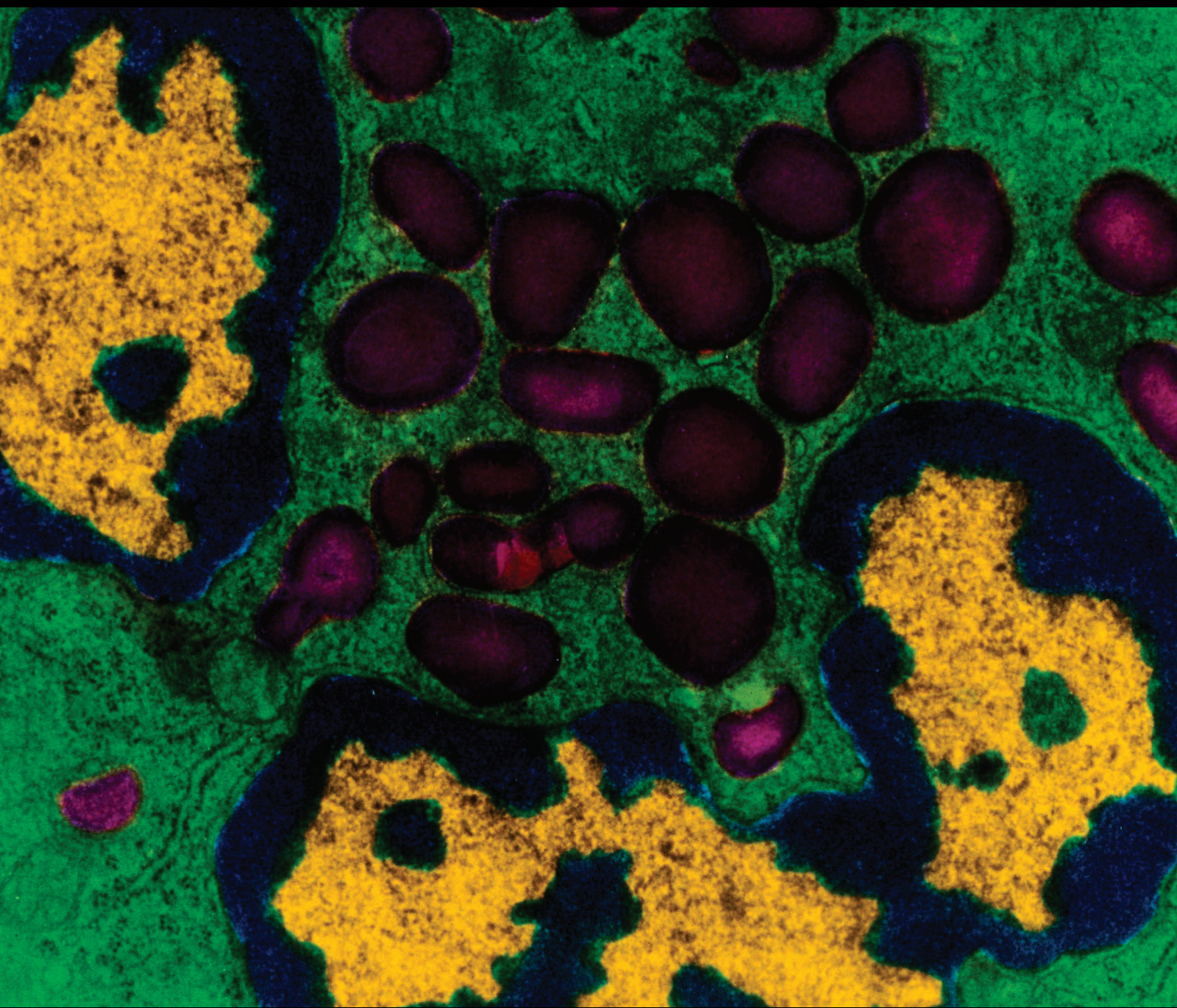
Mediators of Inflammation

Basic and Clinical Advances in Chronic Liver Inflammation

Guest Editors: Hirayuki Enomoto, Akihiro Tamori, Hitoshi Yoshiji, and Ekihiro Seki



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Editorial Board

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Editorial

Basic and Clinical Advances in Chronic Liver Inflammation

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Continuous liver inflammation causes fibrotic changes and leads to the development of liver cirrhosis and liver cancer. Recent biological and medical advances have clarified the mechanisms of chronic liver inflammation and succeeded in providing new therapies for various liver diseases. We proposed this special issue to provide recent basic and clinical findings in chronic liver inflammation and its complications.

Regarding the diagnosis and treatment of liver inflammation, A. Tsutsui et al. showed the clinical utility of the Digestive Disease Week Japan 2004 (DDW-J) scale, which has been used as an objective diagnostic tool for drug-induced liver injury in Japan. A. Tamori et al. reviewed remarkable progression in antiviral treatments for hepatitis C virus (HCV), including DAAs (direct-acting antivirals or direct antiviral agents).

We also introduce papers regarding the complications of progressed liver diseases in this special issue. The prognoses of cirrhotic patients are highly dependent on their liver function, and A. Hassan et al. showed that L-carnitine administration helps maintain and improve liver functions after transarterial chemoembolization. Additionally, malnutrition is a frequently observed complication which is known to be associated with a poor prognosis. Y. Osaki and H. Nishikawa reviewed the nutritional problems of cirrhosis, focusing on a recent hot topic "sarcopenia." Portal hypertension is a major problem along with the progression of chronic liver disease. The paper by K. Kotani et al. suggested the association of the immune system with the development of portal hypertension. Hepatocellular carcinoma is also a prognosis-determining complication of patients with chronic liver inflammation. K. Shindo et al. showed the clinical utility of a semiannual imaging surveillance program in patients without hepatitis viral infection.

With regard to the basic mechanisms of chronic liver inflammation, H. Tsutsui et al. reviewed the roles of IL-1 family cytokines in the development of various liver diseases, including IL-1 family cytokine-mediated molecular and cellular networks. Coinfection of HCV and human immunodeficiency virus (HIV) cooperatively leads to the progression of liver disease. Along this viewpoint, the paper by M. C. Liberto et al., which described the specific cross talk among HIV and HCV proteins in coinfected patients, is interesting and clinically relevant.

We hope that these papers will provide new findings for basic and clinical researchers and help promote their studies for the development of better management for patients with chronic liver inflammation.

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Review Article

Recent Advances in Antiviral Therapy for Chronic Hepatitis C

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Hepatitis C virus (HCV) infection is a major worldwide health problem. Chronic infection induces continuous inflammation in the liver, progression of hepatic fibrosis, eventual cirrhosis, and possible hepatocellular carcinoma. Eradication of the virus is one of the most important treatment aims. A number of promising new direct-acting antivirals (DAAs) have been developed over the past 10 years. Due to their increased efficacy, safety, and tolerability, interferon-free oral therapies with DAAs have been approved for patients with HCV, including those with cirrhosis. This review introduces the characteristics and results of recent clinical trials of several DAAs: NS3/4A protease inhibitors, NS5A inhibitors, and NS5B inhibitors. DAA treatment failure and prognosis after DAA therapy are also discussed.

1. Introduction

Chronic hepatitis C (CHC) due to infection with hepatitis C virus (HCV) affects approximately 170 million people worldwide and is the most common cause of chronic liver disease [1]. Of HCV-infected individuals, 20% to 30% eventually develop liver cirrhosis or hepatocellular carcinoma (HCC). The primary aims of anti-HCV therapy for patients with CHC are prevention of progression to cirrhosis and development of HCC. A combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) completely eradicates HCV in up to 40% to 50% of treatment-naïve patients with high viral loads of HCV genotype 1b [2, 3]. Elongation of the treatment period or retreatment improves the rate of sustained virological response (SVR) in some patients with CHC [4–7]. In patients achieving SVR, IFN-based therapy has improved hepatic fibrosis and prevented the development of HCC. However, only limited numbers of patients show beneficial antiviral effects of IFN-based therapy. The effect depends on the patient's genetic background, presence of hepatic fibrosis, age, HIV coinfection, and other factors. In addition, IFN-based therapy has some adverse effects that may lead to poor drug adherence or treatment discontinuation.

Recently, direct-acting antiviral (DAA) regimens were approved for anti-HCV therapy and have been evaluated. The first-generation protease inhibitors telaprevir (TVR) and boceprevir (BOC) were approved as DAA combination therapy with PEG-IFN and RBV [8–11]. Although triple therapy achieves a higher SVR rate than does conventional IFN-based therapy, treatment is associated with severe adverse effects. Neither the American Association for the Study of Liver Diseases (AASLD) Practice Guidelines nor the European Association for the Study of the Liver (EASL) Clinical Practice Guidelines for CHC recommend TVR or BOC triple therapy [12, 13]. Due to the development of new DAAs with better safety and stronger antiviral effects, it is expected that almost all patients with HCV infection will be able to achieve SVR in the near future (Figure 1). Therefore, it is necessary to consider the long-term prognosis of patients with CHC after eradication of HCV.

Here, we review recent developments in DAA therapy and discuss the management of patients with SVR.

2. Characteristics of DAAs

The development of an *in vitro* culture system for HCV has facilitated the search for agents with anti-HCV effects, and many such agents have undergone clinical trials for use as DAAs [14, 15]. DAAs are classified into three groups: NS3/4A serine protease inhibitors, NS5A inhibitors, and

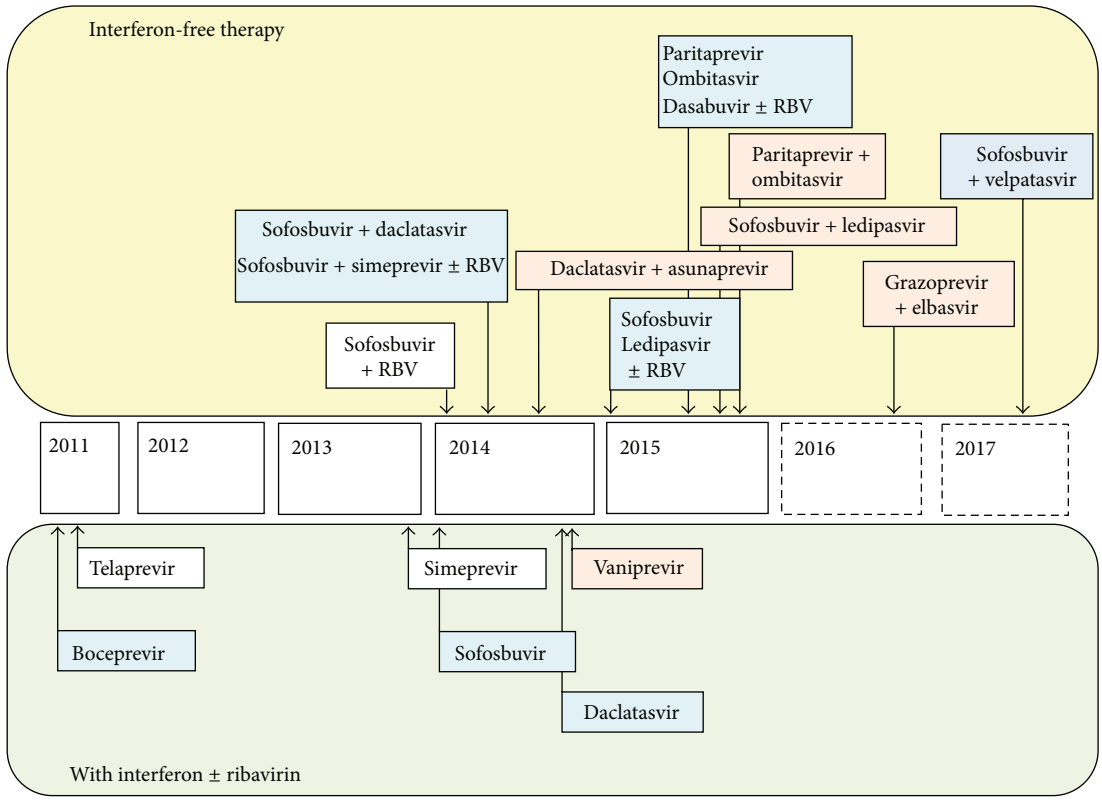

Figure 1: Treatment of HCV with DAAs that are approved or are going to be approved.

NS5B polymerase inhibitors (Tables 1–3). NS3/4A serine protease is required for self-cleavage during HCV replication, the NS5A region plays an important role in viral replication and assembly, and the NS5B region encodes RNA polymerase, which is necessary for HCV replication.

NS3/4A serine protease inhibitors consist of linear first-wave inhibitors and macrocyclic second-wave inhibitors. The NS5B polymerase inhibitors include nucleos(/t)ide types and nonnucleos(/t)ide types [16].

In vitro analysis showed that the antiviral effects of DAAs are dependent on the HCV genotype. In addition, some variants of HCV showed a high EC_{50} for each DAA. Some resistance-associated variants (RAVs) were detected in patients during the natural course of the disease. HCV variants with R155K or A156T in the NS3/4A protease show high resistance to the linear type inhibitors TVR and BOC. The resistance profiles to simeprevir (SMV), a macrocyclic inhibitor of NS3/4A protease, showed overlap with those to TVR and BOC. However, there are specific mutations that confer resistance to SMV [17]. RAV with Q80K was the most commonly observed variant at baseline in particular patients with genotype 1a. D168 mutation is a rarely observed variant associated with virological failure in patients with genotype 1 treated with SMV [18]. In addition, other macrocyclic protease inhibitors, including asunaprevir (ASV) [19], danoprevir, vaniprevir, and paritaprevir (ABT-450), show resistance profiles similar to those of SMV. RAVs with combinations of the mutations in NS3/4A have greater resistance to protease inhibitors than do single mutants. Fortunately, it is rare for such RAVs to emerge at baseline. The second-generation protease inhibitors grazoprevir (MK-5172) and sovaprevir (ACH-1625) [20, 21] have pan-genotypic activities that reduce viral escape through common resistance-associated mutations, such as R155K or Q80K variants.

The NS5A inhibitor daclatasvir (DCV) is a genotype-specific DAA that shows an extremely high antiviral effect against HCV genotype 1, particularly genotype 1b, compared with genotype 1a. In one study, variant viruses with L31M and/or Y93H, which are resistant to DCV, were detected in 4.2% and 14.5% of 214 DAA-naïve patients with HCV genotype 1b, respectively [22]. In another study, ledipasvir (LDV, GS-5885), ombitasvir (ABT-267), and elbasvir (MK-8742), all of which are also NS5A inhibitors, showed resistance profiles identical to that of DCV [23]. ACH-3102 and velpatasvir (GS-5816) are second-generation HCV NS5A inhibitors with potent broad genotype antiviral coverage and broad coverage of first-generation NS5A inhibitor RAVs.

Finally, compounds that inhibit NS5B are classified into two subclasses: nucleos(/t)ide competitive polymerase inhibitors and allosteric inhibitors of RNA polymerase (non-nucleos(/t)ide polymerase inhibitors; NNPIs). NNPIs have a high barrier to resistance [24] and appear to be effective across a broad range of viral genotypes. NNPIs induce conformational changes in the NS5B polymerase enzyme by binding to its various allosteric sites. These agents have a lower barrier of resistance and appear to be genotype specific. As a result of differences in sites of action on the polymerase,

Table 1: Profile of NS3/4A protease inhibitors.

Generation			Active against HCV genotype	Genetic barriers	Resistant association variants					
1st, 1st-wave	VX-950	Telaprevir	1a/1b, 2	Low	V36A/M		R155K/T/Q	A156S/D/T/V		
1st, 1st-wave		Boceprevir	1a/1b, 2	Low	V36A/M		R155K/T/Q	A156S/D/T/V		
1st, 2nd-wave	BI 201335	Faldaprevir	1a/1b, 2	Moderate			R155K/T/Q		D168A/V/T/H	V170A/T
1st, 2nd-wave	TMC-435	Simeprevir	1, 2, and 4–6	Moderate		Q80R/K			D168A/V/T/H	
1st, 2nd-wave	MK-7009	Vaniprevir	1a/1b	Moderate			R155K/T/Q	A156S/D/T/V	D168A/V/T/H	
1st, 2nd-wave	BMS-650032	Asunaprevir	1, 4	Moderate		Q80R/K	R155K/T/Q		D168A/V/T/H	
1st, 2nd-wave	ABT-450	Paritaprevir	1	Moderate			R155K/T/Q		D168A/V/T/H	
2nd	GS-9857		1–4				R155K/T/Q	A156S/D/T/V	D168A/V/T/H	
2nd	ACH-1625	Sovaprevir								
2nd	MK-5172	Grazoprevir	1a/1b, 2, and 4–6	High			R155K/T/Q	A156S/D/T/V	D168A/V/T/H	

Reference [16] Vermehren and Sarrazin 2012.

Table 2: Profile of NS5A inhibitors.

Generation			Active against HCV genotype	Genetic barriers	Resistant association variants			
1st	BMS-790052	Daclatasvir	1b > 2a > 1a	Moderate			L31F/M/V	Y93C/H/N
1st	GS-5885	Ledipasvir	1a, 1b	Moderate			L31F/M/V	Y93C/H/N
1st	ABT-267	Ombitasvir	1 > 2–6	Moderate	M28T	Q30E/R		Y93C/H/N
Broad activity	MK-8742	Elbasvir	1–4	Unavailable	M28T	Q30L/R	L31<	Y93H/N
2nd	GS-5816	Velpatasvir	1–6	Unavailable				
2nd	ACH-3102		1–5	High				Y93H

Reference [23] Kohler et al. 2014.

these two inhibitors have different mechanisms and potencies [16].

Sofosbuvir (SOF) is a uridine nucleotide prodrug NS5B inhibitor [25]. Following absorption, SOF is metabolized in hepatocytes, where it is converted to the active nucleoside triphosphate form. No dose adjustment of SOF is warranted in cases of mild, moderate, or severe hepatic fibrosis, although viral suppression may be slower among patients with Child-Pugh Class B and C liver disease. The safety and efficacy of SOF have not been established in patients with renal impairment (estimated glomerular filtration rate < 30 mL/min) or end-stage renal disease, including patients requiring hemodialysis [26]. S282T is a cross-resistance mutation within the NS5B polymerase to nucleos(/t)ide polymerase inhibitors, including SOF. However, this RAV is extremely rare in patients with a natural clinical course.

The structure of the NS5B polymerase resembles a characteristic "right hand motif" comprising finger, palm, and thumb domains, and at least five different allosteric binding sites at the thumb (sites 1 and 2) and palm (sites 3, 4, and 5) have been identified as targets for NNPIs. The NNPIs beclabuvir (BMS-791325), dasabuvir (ABT-333), and GS-9669 bind to sites 1, 3, and 2, respectively. Compared with NPIs, NNPIs have limitations in their antiviral effectiveness.

3. IFN-Based Therapy with DAAs

3.1. Genotypes 1/4. In clinical trials, addition of first-generation NS3/4A protease inhibitors, such as TVR [9, 10] or BOC [27, 28], substantially increased the rate of SVR to PEG-IFN and RBV. However, treatment is sometimes accompanied by severe adverse events, such as rash, pruritus, and anemia with TVR treatment and anemia and dysgeusia with BOC treatment, and use of first-generation protease inhibitors is currently not recommended.

Second-generation macrocyclic NS3/4A protease inhibitors, such SMV and vaniprevir, are generally well tolerated. The phase III QUEST-1/2 trials showed that SMV plus PEG-IFN and RBV for 12 weeks followed by PEG-IFN and RBV for 12 weeks or 36 weeks according to criteria for response-guided therapy resulted in SVR in 80% to 81% of treatment-naïve patients with genotype 1 infection [29, 30]. However, in patients with genotype 1a with Q80K polymorphism at baseline, the rates of SVR were reduced to 52% to 75%. In Japan, where genotype 1b is predominant and baseline Q80K is rarely observed, the phase III CONCERTO-1 trial showed that SMV-containing triple therapy increased the SVR rate to 89% in previously untreated patients [31]. In addition, the rate of SVR with triple therapy including SMV was 83% in previously untreated patients with genotype 4, in whom the baseline Q80K substitution is rarely detectable [32]. Although approved only in Japan, vaniprevir can produce similar SVR rates [33].

SOF is a nucleotide analog HCV NS5B polymerase inhibitor with pan-genotypic antiviral potency. In the phase III NEUTRINO trial, a 12-week regimen of SOF plus PEG-IFN and RBV resulted in SVR in 92% of previously untreated patients with genotype 1a, 82% of those with genotype 1b, and 96% of those with genotype 4 [34].

DCV is an NS5A replication complex inhibitor. The phase IIb COMMAND-1 study indicated that 60 mg of DCV plus PEG-IFN and RBV for 12 weeks followed by PEG-IFN and RBV with or without DCV for 12 weeks or PEG-IFN and RBV alone for 36 weeks according to protocol-defined response yielded SVR in 55% of previously untreated patients with genotype 1a, 77% of those with genotype 1b, and 100% of those with genotype 4 [35]. The rate of SVR to 60 mg of DCV-containing triple therapy was 90% to 100% in previously untreated Japanese patients with genotype 1b [36, 37].

However, the IFN-based regimens are no longer recommended in the AASLD and EASL guidelines, at least as first-line therapy for treatment-naïve patients, because they are inferior to IFN-free oral DAA combinations in terms of both their safety and tolerability profiles.

3.2. Genotypes 2/3. As described below, an IFN-free SOF and RBV combination is the current standard of care for patients infected with genotypes 2 and 3. However, previously treated patients (especially those with genotype 3) show suboptimal SVR rates. The phase IIb LONESTAR-2 study indicated that SVR was achieved in 96% of previously treated patients with genotype 2 and in 83% of those with genotype 3 by SOF plus PEG-IFN and RBV for 12 weeks [38].

3.3. Genotypes 5/6. In the phase III NEUTRINO trial of a 12-week regimen of SOF plus PEG-IFN and RBV, one patient with genotype 5 and all six patients with genotype 6 showed SVR [34]. However, evidence with which to recommend any regimen for patients with genotype 5 or 6 is still lacking.

4. IFN-Free DAA Therapy

4.1. Genotypes 1/4. An IFN-free combination of SOF and RBV is not recommended for patients with genotype 1

Table 3: Profile of NS5B inhibitors.

		Binding site	Active against HCV genotype	Genetic barriers	Resistant association variants						
Nucleotide											
GS-7977	Sofosbuvir		1a, 1b, and 2–6	High	S282T						
Nonnucleoside											
BMS-791325	Beclabuvir	Site I		Moderate	A421V	P495S/Q/L/A/T					
ABT-333	Dasabuvir	Site III		Moderate	C316Y/N	S368T	M414T/I/V/L	Y448C/H	G554D/S	S556G	D559G
GS9669		Site II		Moderate	L419S	R422K	M423T/I/V/T	I482L/V/T	A486/V/I/T/M	V494A	
MK-3682				Moderate							

Reference [16] Vermehren and Sarrazin 2012.

because the efficacy of the regimen was suboptimal in earlier arms of the phase II ELECTRON trial [39]. Some regimens involving combinations of other DAAs with SOF have since been tested in clinical trials.

The phase IIb COSMOS study showed that the NS5B inhibitor SOF and NS3/4A inhibitor SMV with or without RBV for 12 to 24 weeks resulted in SVR in 90% of previously treated patients with mild fibrosis and 94% of previously treated or untreated patients with advanced fibrosis [40]. RBV, treatment duration, and SMV-resistant baseline Q80K polymorphism had little impact on SVR in this trial.

The phase II AI444040 study indicated that the NS5B inhibitor SOF and NS5A inhibitor DCV with or without RBV for 12 to 24 weeks produced SVR in 98% of previously untreated patients with genotype 1 [41]. An SVR was also obtained with this regimen for 24 weeks in 98% of patients who had previous virological failure with NS3/4A inhibitor TVR or BOC.

In the phase III ION-1 trial, 97% to 99% of previously untreated patients with genotype 1 achieved SVR with a once-daily, fixed-dose combination of the NS5B inhibitor SOF and NS5A inhibitor LDV with or without RBV for 12 to 24 weeks, regardless of the addition of RBV or treatment duration [42]. The results of the phase III ION-3 trial suggested that the duration of treatment with SOF and LDV may be shortened to 8 weeks in treatment-naïve patients with genotype 1 without cirrhosis [43]. The phase III ION-2 trial showed that among patients with genotype 1 previously treated with PEG-IFN and RBV with or without NS3/4A inhibitor the SVR rates with a combination of SOF and LDV with or without RBV were 94% to 96% when given for 12 weeks and 99% when given for 24 weeks [44]. In the Japanese phase III trial, SOF and LDV with or without RBV for 12 weeks yielded SVR in 98% of treatment-naïve patients and 100% of previously treated patients with genotype 1 [45]. The fixed-dose SOF and LDV combination with or without RBV was also efficacious in cirrhotic patients with genotype 1 that had been unresponsive to previous NS3/4A protease inhibitor therapy in the phase II SIRIUS trial [46]. A proof-of-concept phase IIa study suggested that addition of a third potent DAA, such as the NS3/4A inhibitor GS-9451 or the nonnucleoside NS5B inhibitor GS-9669, to this fixed-dose combination can shorten the treatment duration to 6 weeks in noncirrhotic patients with genotype 1 [47]. The SOF and LDV combination appears to be effective for patients with genotype 4 [48]. ASTRAL, clinical trials of SOF plus the next-generation NS5A inhibitor, velpatasvir (GS-5816), have just been published [49]. In detail, SOF and velpatasvir combination therapy for 12 weeks produced SVR in 98% and 100% of patients with genotype 1 and genotype 4, respectively.

In the preliminary AI447-011 study to assess the efficacy of the IFN-free combination of the NS5A inhibitor DCV and the NS3/4A inhibitor ASV, both patients with genotype 1b achieved SVR, compared with only two of nine (22%) patients with genotype 1a [50]. The Japanese phase III AI447-026 study of a 24-week DCV and ASV combination thus included only patients with genotype 1b; 89% of patients who were intolerant to or ineligible for IFN and 80% of patients with a null response to IFN achieved SVR [51]. The regimen has already been approved in Japan, and the results of the Japanese study were reproduced in the multinational phase III multicohort HALLMARK-DUAL trial [52]. However, the efficacy of the DCV and ASV regimen is markedly affected by baseline NS5A RAV. In this study, baseline L31M/V substitutions were detected in 5% of patients, only 41% of whom achieved SVR, and baseline Y93H substitutions were detected in 8% of patients, only 38% of whom achieved SVR. In the phase III UNITY-1 study, addition of the nonnucleoside NS5B inhibitor beclabuvir to DCV and ASV for 12 weeks provided SVR in 92% and 89% of treatment-naïve and previously treated noncirrhotic patients with genotype 1, respectively, irrespective of baseline NS5A RAV [53].

In the phase III SAPPHIRE-I study, among previously untreated patients without cirrhosis, the NS3/4A inhibitor paritaprevir boosted with ritonavir, the NS5A inhibitor ombitasvir, and the nonnucleoside NS5B inhibitor dasabuvir with RBV for 12 weeks produced SVR in 95% and 98% of patients with genotype 1a and genotype 1b, respectively [54]. In the phase III PEARL-IV study, among previously untreated patients with genotype 1a without cirrhosis who were treated with the all-oral three-DAA regimen for 12 weeks, the rate of SVR was 97% with RBV and 90% without RBV, suggesting that RBV is necessary for patients with genotype 1a [55]. In the phase III TURQUOISE-II study, among previously treated or untreated patients with genotype 1a with compensated cirrhosis who were treated with the all-oral three-DAA regimen with RBV, the rate of SVR was 89% in the 12-week arm and 95% in the 24-week arm, suggesting that a 24-week treatment period is preferable for cirrhotic patients [56]. In the phase IIb PEARL-I study, a two-DAA regimen consisting of paritaprevir (with ritonavir) and ombitasvir with or without RBV was sufficient for treatment-naïve or previously treated patients with genotype 4 without cirrhosis [57]. The Japanese phase IIb study suggested that the two-DAA regimen without RBV was effective for previously treated patients with genotype 1b without cirrhosis [58].

The phase III C-EDGE treatment-naive study showed that among cirrhotic and noncirrhotic treatment-naïve patients once-daily fixed-dose treatment with a combination of the second-generation NS3/4A inhibitor grazoprevir and NS5A inhibitor elbasvir for 12 weeks resulted in SVR in 95% of those with genotype 1a and 99% of those with genotype 1b [59].

4.2. Genotypes 2/3. Among previously untreated patients with or without cirrhosis who received SOF and RBV for 12 weeks in the phase III FISSION study, the rate of SVR was 97% in those with genotype 2 and 56% in those with genotype 3 [34]. In the phase III FUSION study of SOF and RBV in previously treated patients with or without cirrhosis, the rates of SVR in genotype 2 were 86% in the 12-week group and 94% in the 16-week group, and the corresponding rates of SVR in genotype 3 were 30% and 62%, respectively [60]. These results suggested that extended treatment is beneficial for previously treated patients, but a 16-week treatment period is still insufficient for patients with genotype 3. Among previously treated or untreated patients who received SOF and RBV in the phase III VALENCE study, SVR was achieved

in 93% of those with genotype 2 who were treated for 12 weeks and 85% of those with genotype 3 who were treated for 24 weeks (91% and 68% of those without and with cirrhosis, resp.) [61]. In a Japanese phase III trial, SOF and RBV for 12 weeks resulted in SVR in 98% of treatment-naïve and 95% of previously treated patients with genotype 2 [62].

The phase III ALLY-3 study to evaluate the 12-week regimen of SOF plus DCV in treatment-naïve and previously treated patients with genotype 3 showed SVR rates of 96% in patients without cirrhosis and 63% in those with cirrhosis [63]. Additional evaluation to optimize efficacy in patients with genotype 3 with cirrhosis is currently underway.

4.3. Genotypes 5/6. Little information is available regarding the efficacy of IFN-free DAA regimens, particularly in patients with genotype 5. In a preliminary report, 96% of treatment-naïve and previously treated patients with genotype 6 showed SVR with a 12-week combination of SOF and LDV [64].

5. Appearance of HCV in Patients Who Previously Relapsed or Showed Breakthrough after DAA Treatment

Patients treated with an NS3 protease inhibitor, an NS5A inhibitor, or a nonnucleoside inhibitor of NS5B who failed to achieve SVR were found to have viruses with amino acid substitutions that confer drug resistance in the NS3 protease, NS5A, and NS5B regions, respectively. For example, resistance-associated variants (RAVs) with NS3 positions V36A/G/I/L/M, T54A/S, I132V (genotype 1a only), R155G/K/T/M, A156F/N/S/T/V, and D168N were identified after failure of treatment with TVR and combined PEG-IFN/RBV therapy [65]. In addition, for patients in whom SMV/PEG-IFN/RBV therapy failed, RAVs were identified with NS3 positions 80, 122, 155, and 168 (mainly R155K in genotype 1a with and without Q80K, and D168V in genotype 1b) at the time of failure [66]. RAVs with both NS5A and NS3 amino acid substitutions emerged in patients in whom IFN-free DAA therapy with DCV and ASV failed. Of note, NS5A variants with both L31V/M and Y93C/N show strong drug resistance [67, 68]. In comparison, few reports have described the detection of NS5B-polymerase-inhibitor-resistant HCV (S282T in NS5B) after failure of SOF combined antiviral therapy. Currently, SOF has the highest barrier to drug resistance.

Another interesting feature of RAVs after DAA failure is the change in the prevalence of RAVs over time. NS5A resistance variants (Q30E/R, L31V/M, and Y93C/N) persisted, while NS3 resistance variants (V36M, R155K, and D168A/E/V/Y) generally decreased [65, 68].

6. Strategy of DAA Retreatment for Patients with Previous DAA Therapy

Although DAA combination therapy can achieve a high SVR rate (>90%), approximately 5% of patients treated with DAAs will fall into relapse or viral breakthrough. It is necessary to consider retreatment for patients with failure of DAA therapy. It was speculated that previously used DAAs (with the same resistance profile) may lose their anti-HCV effect in patients following DAA failure. *In vitro* analysis of combinations with DCV, ASV plus beclabuvir, and DCV, ASV, and beclabuvir plus SOF efficiently cleared resistant replicons to both DCV and ASV. On the other hand, SOF plus SMV and SOF plus LDV did not inhibit the growth of DCV- plus ASV-resistant replicons [69]. Another unique trial has just been reported. It showed that an NS5A inhibitor analogue (Syn-395) induced conformational change in the resistant NS5A protein and that RAVs became resensitized to DCV in *in vitro* and *in vivo* analyses [70].

The phase II C-SALVAGE study evaluated a combination of grazoprevir (NS3/4A protease inhibitor) and elbasvir (NS5A inhibitor) with RBV for patients with chronic HCV genotype 1 infection in whom licensed DAA-containing therapy had failed. The patients had failed to achieve SVR by PEG-IFN and RBV plus either BOC, TVR, or SMV. Grazoprevir (100 mg)/elbasvir (50 mg) QD with weight-based RBV BID for 12 weeks achieved a 96.2% (76/79) overall SVR12 rate, including 43 of 43 (100.0%) patients without baseline RAV, 31 of 34 (91.2%) patients with baseline NS3 RAV, 6 of 8 (75.0%) patients with baseline NS5A RAV, and 4 of 6 (66.7%) patients with both baseline NS3 and RAV [71]. In a phase IIa open-label study, 14 patients with HCV GT-1 who relapsed after treatment with SOF plus RBV for 24 weeks were retreated with SOF plus LDV for 12 weeks. All 14 patients achieved SVR, including one patient with NS5B S282T mutation after previous SOF plus RBV therapy [72]. Taken together, it is speculated that retreatment with DAAs of the same class plus additional DAAs with a different mechanism of action and/or new DAAs could achieve SVR in patients that had previously fallen into relapse or breakthrough after DAA treatment.

7. Prognosis after Achieving SVR by DAAs

IFN-based therapy is well known to prevent the development of hepatocellular carcinoma (HCC) if HCV can be completely eradicated from patients with CHC. In addition, IFN-based therapy could improve the hepatic fibrosis stage in patients who achieved SVR [73–75]. In general, overall survival is prolonged in patients who achieve SVR. On the other hand, patients with advanced hepatic fibrosis or cirrhosis continue to be at risk for the development of HCC after eradication of HCV. The cumulative rate of HCC development is reportedly 1% to 3% in SVR patients in Japan [76–78]. Elderly patients and those with advanced fibrosis prior to IFN-based treatment are at risk for the development of HCC after achieving SVR [79]. Compared with IFN-based therapy, DAAs may be more suitable for elderly patients and patients with hepatic cirrhosis. In addition, IFN-free DAA therapy achieved a higher SVR rate. However, which DAA therapy will reduce the incidence of HCC is questionable. It is necessary to evaluate the long-term prognosis of patients treated with DAAs.

In addition to eradication of HCV, DAA therapy can improve lipid metabolism in patients with SVR. That is, during SOF and RBV therapy for patients with HCV genotype

1, serum lipid concentrations were altered and intrahepatic expression of fatty acid metabolism and lipid transport genes were also changed [80]. With regard to other outcomes in patients treated with ledipasvir and SOF combination therapy, four questionnaires—CLDQ-HCV, SF36, FACIT-F, and WPAI:SHR—were used to evaluate health-related quality of life (HRQL) and work productivity in patients treated with IFN-free therapy. The results indicated that viral eradication with IFN-free therapy led to improvement in HRQL regardless of the hepatic fibrosis stage at baseline [81].

8. Conclusions

DAAs can eradicate HCV in almost all patients with chronic liver disease, including advanced fibrosis, and can inhibit continuous inflammation in the liver. However, this is the treatment goal for HCV infection, but not for liver disease. It has not been confirmed that DAA can improve hepatic fibrosis and prohibit hepatocarcinogenesis. Therefore, it is necessary to evaluate patients' hepatic pathogenesis after achievement of SVR by DAA therapy.

Another problem associated with DAA treatment is that compared with interferon-based therapy new DAA regimens are too expensive to be accessible to all patients with HCV. Therefore, it is necessary to consider not only efficacy, but also cost in selection of anti-HCV therapy.

Disclosure

The English language in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see http://www.textcheck.com/certificate/0A79qH.

Conflict of Interests

Akihiro Tamori has received research grant from Bristol-Myers Squib and Chugai Pharmaceutical Co., Ltd. Norifumi Kawada has received research grant from Bristol-Myers Squib, Chugai Pharmaceutical Co., Ltd., and MSD.

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Clinical Study

Effects of Oral L-Carnitine on Liver Functions after Transarterial Chemoembolization in Intermediate-Stage HCC Patients

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Transarterial chemoembolization (TACE) is usually followed by hepatic dysfunction. We evaluated the effects of L-carnitine on post-TACE impaired liver functions. *Methods*. 53 cirrhotic hepatocellular carcinoma patients at Osaka Medical College were enrolled in this study and assigned into either L-carnitine group receiving 600 mg oral L-carnitine daily or control group. Liver functions were evaluated at pre-TACE and 1, 4, and 12 weeks after TACE. *Results*. The L-carnitine group maintained Child-Pugh (CP) score at 1 week after TACE and exhibited significant improvement at 4 weeks after TACE ($P < 0.01$). Conversely, the control group reported a significant CP score deterioration at 1 week ($P < 0.05$) and 12 weeks after TACE ($P < 0.05$). L-carnitine suppressed serum albumin deterioration at 1 week after TACE. There were significant differences between L-carnitine and control groups regarding mean serum albumin changes from baseline to 1 week ($P < 0.05$) and 4 weeks after TACE ($P < 0.05$). L-carnitine caused prothrombin time improvement from baseline to 1, 4 ($P < 0.05$), and 12 weeks after TACE. Total bilirubin mean changes from baseline to 1 week after TACE exhibited significant differences between L-carnitine and control groups ($P < 0.05$). The hepatoprotective effects of L-carnitine were enhanced by branched chain amino acids combination. *Conclusion*. L-carnitine maintained and improved liver functions after TACE.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer-related deaths [1]. Furthermore, HCC is currently the leading cause of death among patients with liver cirrhosis [2].

Intermediate-stage HCC is defined as an extensive multifocal disease without vascular invasion in patients with preserved liver functions and absence of cancer-related symptoms. Transarterial chemoembolization (TACE) is considered the standard treatment for intermediate-stage HCC [3].

Hepatic failure after TACE is expected even in patients with relatively good hepatic reserve, significantly impairing the outcome of TACE, including patient survival [4, 5]. The maintenance of hepatic functional reserve is a major concern in patients with HCC who, in general, are treated repeatedly with TACE [6].

L-carnitine (4-*N*-trimethyl ammonium 3-hydroxybutyric acid) is a conditionally essential amino acid synthesized from essential amino acids methionine and lysine in human liver, kidneys, and brain but principally obtained from diet [7]. L-carnitine functions by transferring long-chain fatty acids across the mitochondrial membrane, enabling the oxidative release of energy [8]. L-carnitine deficiency is associated with liver cirrhosis because of limited dietary intake, absorption, and endogenous hepatic synthesis [9].

The protective role of L-carnitine against hepatotoxicity has been proposed in many studies [10–12]. Oral L-carnitine supplementation improved liver functions and

histological patterns in patients with nonalcoholic steatohepatitis (NASH) [13]. Cirrhotic patients with minimal hepatic encephalopathy achieved improved quality of life with L-carnitine supplementation [14]. L-carnitine was an effective adjuvant to interferon and ribavirin in patients with chronic hepatitis C viral infection (HCV) [15].

Branched-chain amino acids (BCAA) are essential amino acids and include L-valine, L-leucine, and L-isoleucine. Several studies demonstrated that BCAA nutritional therapy decreased the risk of hepatic failure and improved general outcome in HCC patients undergoing variable treatment options [16].

In this study, we examined the protective effects of L-carnitine regarding liver dysfunction after TACE in intermediate-stage HCC patients when administered alone or in combination with BCAA.

2. Patients and Methods

2.1. Patients. The study included 53 HCC patients who underwent TACE between December 2012 and November 2013 at Osaka Medical College Hospital. All patients provided written informed consent to participate in the study. Study protocol conformed to the ethical guidelines of the Declaration of Helsinki (1975) and was approved by the Osaka Medical College ethical review committee. All patients were Japanese and had liver cirrhosis diagnosed by abdominal ultrasound and liver functions tests. HCC diagnosis was based on data obtained by contrast-enhanced computed tomography and hepatic-artery angiography. No patients had vessel invasion at the time of study enrollment, and none had been prescribed L-carnitine supplements before enrollment to the study.

2.2. Study Design. The study was prospective, and the patients were randomly assigned into two groups; the L-carnitine group included twenty-seven consecutive HCC patients who received a 300 mg tablet of L-carnitine twice daily starting from 2 weeks before TACE to week 12 after TACE. The control group included 26 consecutive HCC patients who did not receive L-carnitine supplementation. Thirty-one of the study patients had already been supplemented by late evening snacks of BCAA granules (LIVACT granules containing L-isoleucine 952 mg, L-leucine 1,904 mg, and L-valine 1,144 mg) prior to enrolment in this study and continued throughout study duration (we identified these patients as BCAA+ patients) while 22 patients did not receive BCAA granules (identified as BCAA−).

All patients were followed up in Osaka Medical College Hospital including clinical follow-up and laboratory measurements. The primary end point was improvement in Child-Pugh (CP) score and serum albumin. The secondary end point included improvement in other liver functions.

2.3. TACE Protocol. TACE for HCC was performed in conformity with Japanese guidelines by catheterization via femoral artery with superselective cannulation to the HCC hepatic feeding artery [17]. The infused agent was an emulsion of 50 mg of cisplatin (IA-call, Nihon-Kayaku) or 50 mg of farmorubicin (epirubicin hydrochloride, Pfizer) or other anticancer agents and 5 mL of Lipiodol (iodine addition products of ethyl esters of fatty acids obtained from poppy seed oil; Mitsui, Japan). The amount of emulsion was determined by the operator.

2.4. Laboratory Measurements. Laboratory measurements were performed 2 weeks before and 1, 4, and 12 weeks after TACE. Ascites was diagnosed by computed tomography or ultrasound. Laboratory measurements included serum albumin, total bilirubin, prothrombin time (PT), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGTP), and aspartate aminotransferase (AST) in addition to calculation of CP scores.

2.5. Statistical Analysis. Data were analyzed using SPSS version 22. Differences in laboratory data between study groups and differences within groups were analyzed using the Mann-Whitney U test and the t-test. Chi-square test was used for categorical data analysis. Probability (P) values < 0.05 indicate statistical significance.

3. Results

3.1. Patients Characteristics. A total of 53 patients with HCC were enrolled in the current study. Three patients were withdrawn from the study because of non-liver-related deaths. There were no statistically significant differences between the two groups regarding demographic characteristics, tumor staging, and anticancer drugs used during TACE and baseline laboratory tests (Table 1). Similarly, no such differences were observed between L-carnitine and control groups in BCAA+ patients and BCAA− patients.

3.2. Effects of L-Carnitine on CP Score. In the L-carnitine group in this study, CP scores showed a nonsignificant deterioration at 1 week after TACE. CP scores showed significant improvement at 4 weeks after TACE ($P < 0.01$ compared with 1 week after TACE scores); CP scores at 4 and 12 weeks after TACE were better than baseline scores (Table 2). In contrast, the control group experienced a significant deterioration in CP scores at 1 week after TACE ($P < 0.05$ compared to baseline), reaching CP scores at 4 weeks after TACE worse than at baseline; CP scores at 12 weeks after TACE were significantly worse than baseline scores ($P < 0.05$) (Table 2). There were significant differences between the L-carnitine and control groups in CP score mean changes from baseline to 4 and 12 weeks after TACE ($P < 0.05$) (Figure 1(a)).

To investigate the combined effects of BCAA granules and L-carnitine, we evaluated BCAA+ and BCAA− patients. L-carnitine group in BCAA+ patients achieved significant CP score improvement from week 1 to week 4 after TACE ($P < 0.05$) (Table 3). There were significant differences between the L-carnitine and control groups in mean CP score changes from baseline to 4 weeks after TACE ($P < 0.05$) and 12 weeks after TACE ($P < 0.05$) (Figure 1(b)). L-carnitine

Table 1: Baseline clinical and laboratory data.

Parameter (mean ± SD)	L-carnitine group ($N = 24$)	Control group ($N = 26$)	P
Sex (male/female)	17/7	21/5	0.5
Age	71.6 ± 7.6	72.3 ± 6.8	0.7
CP score	6.04 ± 1.04	5.88 ± 0.99	0.4
Stage (2/3/4)	7/15/2	9/15/2	0.9
Drug (cisplatin/others)	18/6	21/5	0.3
BCAA (yes/no)	16/8	12/14	0.1
S. albumin (g/dL)	3.22 ± 0.57	3.35 ± 0.56	0.2
PT (%)	86.7 ± 19.4	89.2 ± 17	0.5
T. bilirubin (mg/dL)	1.02 ± 0.57	0.93 ± 0.46	0.4
ALT (U/L)	32.6 ± 17.6	41.8 ± 28.8	0.3
AST (U/L)	51.5 ± 25.9	57 ± 37.8	0.7
GGTP (U/L)	70.7 ± 80.9	66.3 ± 67.3	0.8
Ascites (no/moderate/massive)	21/3/0	21/5/0	0.2
Encephalopathy (yes/no)	24/0	26/0	0.6

SD: standard deviation; CP: Child-Pugh; BCAA: branched-chain amino acids; S. albumin: serum albumin; PT: prothrombin time; T. bilirubin: total bilirubin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGTP: gamma-glutamyl transpeptidase.

group of BCAA− patients showed no significant changes in CP scores (Table 4). Comparison between the L-carnitine and the control groups revealed no significant differences in BCAA− patients.

3.3. Effects of L-Carnitine on Synthetic Liver Functions Tests

3.3.1. Effects of L-Carnitine on Serum Albumin. In the L-carnitine group, serum albumin had significantly decreased by the first week after TACE ($P < 0.05$); serum albumin had significantly improved at 4 weeks after TACE ($P < 0.05$ compared to baseline and $P < 0.01$ compared to week 1 after TACE). Moreover, serum albumin at 12 weeks after TACE was higher than baseline and significantly higher than albumin level at 1 week after TACE ($P < 0.05$) (Table 2).

Nevertheless, in the control group, serum albumin had significantly decreased by the first week after TACE ($P < 0.01$); serum albumin was kept at levels lower than baseline at 4 weeks and 12 weeks after TACE (Table 2). Comparison between the L-carnitine and control groups regarding the means of serum albumin changes from baseline to 1 week and 4 weeks after TACE displayed significant differences ($P < 0.05$) (Figure 1(c)).

At 4 and 12 weeks following TACE, the L-carnitine group in BCAA+ patients had serum albumin levels higher than baseline values. Conversely, in the control group, serum albumin levels had significantly declined at 1 week after TACE ($P < 0.01$); serum albumin levels at 12 weeks after TACE were lower than at baseline (Table 3). There were significant differences between the L-carnitine and control groups in mean serum albumin decline from baseline to 1 week after TACE ($P < 0.05$) (Figure 1(d)). In BCAA− patients, serum albumin at 1 week after TACE showed a significant decline from baseline in the control group ($P < 0.01$) (Table 4). However, no significant differences were observed between the L-carnitine and control groups.

3.3.2. Effects of L-Carnitine on PT. In the L-carnitine group, PT was elevated at week 1 after TACE and significantly higher than baseline at week 4 after TACE ($P < 0.05$). L-carnitine maintained better PT values at week 12 after TACE compared with baseline values (Table 2). Conversely, the control group displayed a significant PT decline at week 1 following TACE ($P < 0.05$) (Table 2). There were significant differences between the L-carnitine and control groups in mean PT changes from baseline to 1 week after TACE ($P < 0.05$) and 4 weeks after TACE ($P < 0.05$) (Figure 2(a)).

At 1, 4, and 12 weeks after TACE, L-carnitine group of BCAA+ patients reported higher PT values compared with baseline. Conversely, the control group demonstrated a significant PT decline at 1 week after TACE ($P < 0.05$); PT values at 12 weeks after TACE were lower than baseline values (Table 3). There were also significant differences between the L-carnitine and control groups in PT mean changes from baseline to 1 week after TACE ($P < 0.05$) (Figure 2(b)). In BCAA− patients, L-carnitine maintained PT values higher than at baseline at all follow-up intervals (Table 4), but there were no significant differences between the groups.

3.3.3. Effects of L-Carnitine on Total Bilirubin. The current study demonstrated that L-carnitine prevented total bilirubin elevation at 1 week after TACE; total bilirubin at 12 weeks after TACE showed nonsignificant elevation over baseline values (Table 2). On the contrary, the control group exhibited total bilirubin levels significantly higher than baseline at the first week after TACE ($P < 0.01$). Furthermore, total bilirubin at week 12 after TACE was significantly higher than at baseline ($P < 0.05$) and at 4 weeks after TACE ($P < 0.05$) (Table 2). A comparison between the L-carnitine and control

Table 2: Effects of L-carnitine in overall study patients.

	Parameter (mean ± SD)	Pretreatment	After TACE		
			1 week	4 weeks	12 weeks
Control group ($N = 26$)	CP score	5.88 ± 0.99	6.24 ± 1.05$^{\dagger *}$	6.04 ± 1.07	6.39 ± 1.55$^{\dagger *}$
	S. albumin (g/dL)	3.35 ± 0.56	2.94 ± 0.57$^{\dagger ***}$	3.29 ± 0.58$^{\ddagger ***}$	3.21 ± 0.63$^{\ddagger ***}$
	PT (%)	89.2 ± 17	85.9 ± 16.9$^{\dagger *}$	88.2 ± 17.7	87.1 ± 18.2
	T. bilirubin (mg/dL)	0.93 ± 0.46	1.13 ± 0.51$^{\dagger *}$	0.96 ± 0.55$^{\ddagger *}$	1.21 ± 0.7$^{\dagger *,\S *}$
	ALT (IU/L)	41.8 ± 28.8	56.2 ± 31$^{\dagger *}$	38.7 ± 21.1$^{\ddagger *}$	37.6 ± 18.8
	AST (IU/L)	57 ± 37.8	50.8 ± 26.5	55.3 ± 31.6	60.3 ± 44.3
	GGTP (IU/L)	66.3 ± 67.3	84.5 ± 100.3$^{\dagger *}$	91.8 ± 84.7$^{\dagger ***}$	59.9 ± 74.7
	Ascitis (no/moderate/massive)	21/5/0	20/5/1	19/5/2	17/5/4
	Encephalopathy (no/yes)	26/0	26/0	26/0	26/0
L-carnitine group ($N = 24$)	CP score	6.04 ± 1.04	6.17 ± 0.86	5.75 ± 0.73$^{\ddagger ***}$	5.91 ± 0.92
	S. albumin (g/dL)	3.22 ± 0.57	3.05 ± 0.46$^{\dagger *}$	3.33 ± 0.5$^{\dagger *,\ddagger ***}$	3.26 ± 0.49$^{\ddagger *}$
	PT (%)	86.7 ± 19.4	91.5 ± 18.4	93.3 ± 16.8$^{\dagger *}$	90.7 ± 16.4
	T. bilirubin (mg/dL)	1.02 ± 0.57	0.97 ± 0.55	0.93 ± 0.46	1.05 ± 0.63
	ALT (IU/L)	32.6 ± 17.6	41.7 ± 23.1$^{\dagger *}$	34.7 ± 20.8	33.9 ± 11.6
	AST (IU/L)	51.5 ± 25.9	50.2 ± 32.1	54.5 ± 28	49.4 ± 15.6
	GGTP (IU/L)	70.7 ± 80.9	91.4 ± 124.4	97.1 ± 148.6	61.8 ± 43.4
	Ascitis (no/moderate/massive)	21/3/0	22/2/0	24/0/0	23/0/1
	Encephalopathy (no/yes)	24/0	24/0	24/0	24/0

SD: standard deviation; CP: Child-Pugh; S. albumin: serum albumin; PT: prothrombin time; T. bilirubin: total bilirubin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGTP: gamma-glutamyl transpeptidase.
†Significant difference compared with baseline; ‡significant difference compared with 1 week after TACE; §significant difference compared with 4 weeks after TACE; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 3: Effects of L-carnitine in BCAA+ patients.

	Parameter (mean ± SD)	Pretreatment	After TACE		
			1 week	4 weeks	12 weeks
Control group ($N = 12$)	CP score	6 ± 1.04	6.5 ± 1.16	6.25 ± 1.28	6.64 ± 1.69
	S. albumin (g/dL)	3.25 ± 0.52	2.81 ± 0.55$^{\dagger **}$	3.25 ± 0.51$^{\ddagger ***}$	3.01 ± 0.52
	PT (%)	87.4 ± 17.3	83.4 ± 19.1$^{\dagger *}$	88.4 ± 18.3	83.6 ± 19.8
	T. bilirubin (mg/dL)	1.1 ± 0.58	1.2 ± 0.55	1.05 ± 0.63	1.41 ± 0.63
	ALT (IU/L)	43.5 ± 19.3	56.5 ± 26.6	42.9 ± 19.8	38.4 ± 11.4
	AST (IU/L)	52.4 ± 20.4	45.8 ± 9	50 ± 21	52.7 ± 21.1
	GGTP (IU/L)	80.4 ± 93.9	104.3 ± 140.5	116.3 ± 120$^{\dagger **}$	43.9 ± 32.3
	Ascitis (no/moderate/massive)	8/4/0	8/3/1	6/4/2	6/4/2
	Encephalopathy (no/yes)	12/0	12/0	12/0	12/0
L-carnitine group ($N = 16$)	CP score	6.25 ± 1.6	6.38 ± 0.88	5.88 ± 0.8$^{\ddagger *}$	5.93 ± 0.99
	S. albumin (g/dL)	3.13 ± 0.61	2.97 ± 0.42	3.25 ± 0.56$^{\ddagger ***}$	3.16 ± 0.49
	PT (%)	87.2 ± 20.7	92.3 ± 16.1	94.1 ± 18.2	91.6 ± 14.2
	T. Bilirubin (mg/dL)	1.01 ± 0.62	1.02 ± 0.62	0.95 ± 0.53	1.1 ± 0.78
	ALT (IU/L)	32.2 ± 12.5	42.7 ± 22.6	30.6 ± 8.3	36.1 ± 11
	AST (IU/L)	51.7 ± 25.3	51.2 ± 31.9	51.3 ± 18.7	53.5 ± 16.8
	GGTP (IU/L)	62 ± 53.7	72.8 ± 56.6	70.6 ± 36.8	60.4 ± 38.5
	Ascitis (no/moderate/massive)	14/2/0	14/2/0	16/0/0	15/0/1
	Encephalopathy (no/yes)	16/0	16/0	16/0	16/0

SD: standard deviation; CP: Child-Pugh; S. albumin: serum albumin; PT: prothrombin time; T. bilirubin: total bilirubin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGTP: gamma-glutamyl transpeptidase; TACE: transarterial chemoembolization.
†Significant difference compared with baseline; ‡significant difference compared with 1 week after TACE; $^{*}P < 0.05$; $^{**}P < 0.01$.

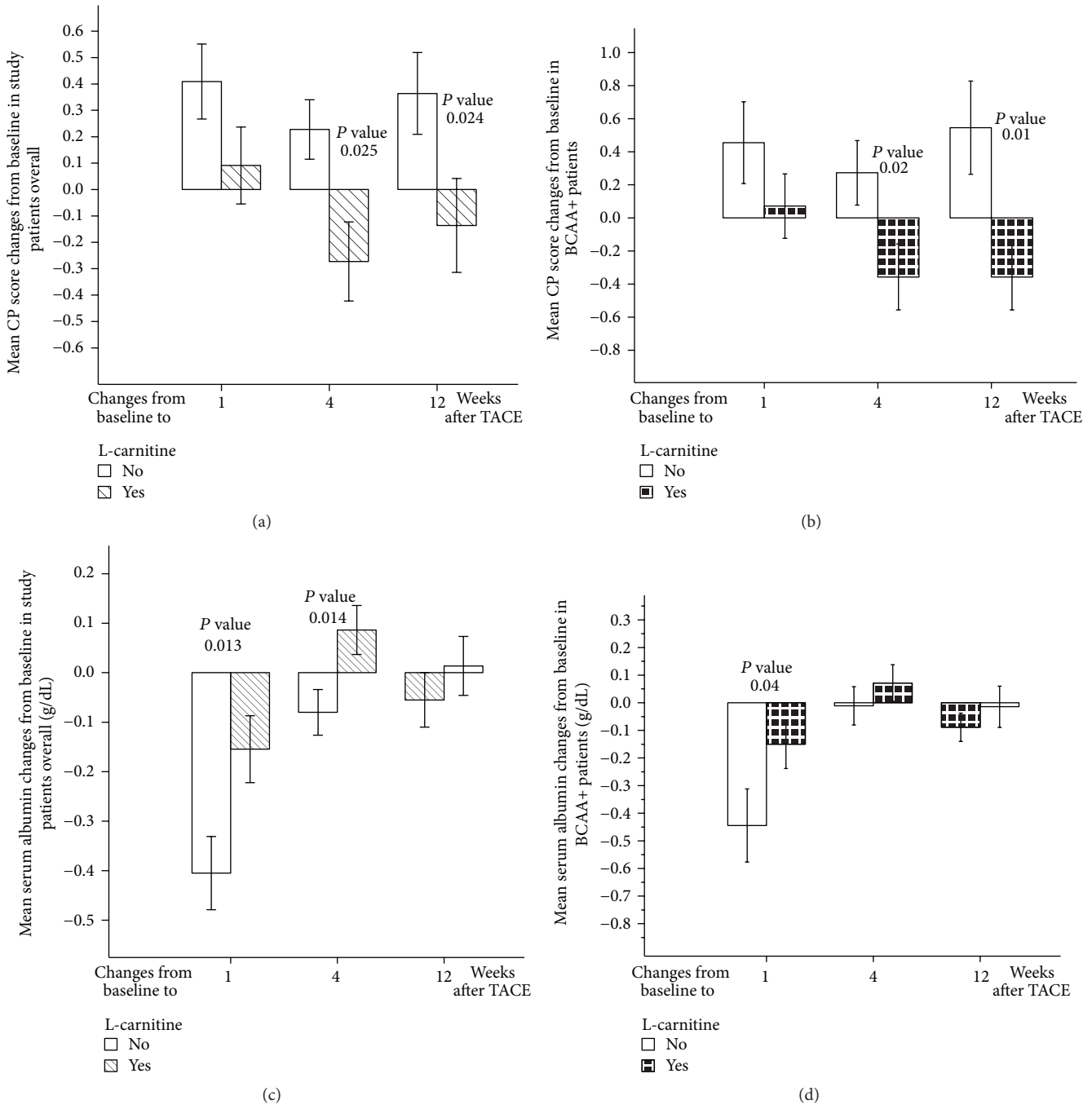

Figure 1: L-carnitine effects on CP score and serum albumin. (a) L-carnitine effects on mean CP score changes from baseline in study patients overall. (b) L-carnitine effects on mean CP score changes from baseline in BCAA+ patients. (c) L-carnitine effects on mean serum albumin changes from baseline in study patients overall. (d) L-carnitine effects on mean serum albumin changes from baseline in BCAA+ patients. CP: Child-Pugh; TACE: transarterial chemoembolization. Error bars represent standard errors.

groups revealed significant differences in mean total bilirubin changes from baseline to 1 week after TACE ($P < 0.05$) (Figure 2(c)).

In BCAA+ patients, we observed no significant differences between L-carnitine and control groups (Table 3). In BCAA− patients, L-carnitine maintained total bilirubin at levels lower than baseline level at 1, 4, and 12 weeks after TACE; on the contrary, total bilirubin levels at week 1 after TACE were significantly higher than baseline level in the control group ($P < 0.05$) (Table 4). Comparison between the L-carnitine and control groups revealed significant differences in mean total bilirubin changes from baseline to 1 week after TACE ($P < 0.01$) (Figure 2(d)).

3.4. Effect of L-Carnitine on Liver Enzymes. A week after TACE, the control group demonstrated a significant rise in

Table 4: Effects of L-carnitine in BCAA− patients.

	Parameter (mean ± SD)	Pretreatment	After TACE		
			1 week	4 weeks	12 weeks
Control group ($N = 14$)	CP score	5.79 ± 0.97	6 ± 0.91	5.86 ± 0.86	6.17 ± 1.46
	S. albumin (g/dL)	3.42 ± 0.6	$3.06 \pm 0.59^{\dagger***}$	$3.32 \pm 0.66^{\ddagger**}$	$3.37 \pm 0.6^{\ddagger***}$
	PT (%)	90.8 ± 17.3	88.3 ± 15.1	88 ± 17.9	90.4 ± 16.9
	T. bilirubin (mg/dL)	0.78 ± 0.29	$1.06 \pm 0.49^{\dagger*}$	0.89 ± 0.48	1.05 ± 0.74
	ALT (IU/L)	40.3 ± 35	55.9 ± 35	35.1 ± 22	36.9 ± 24
	AST (IU/L)	60.9 ± 48	55.3 ± 35	59.7 ± 38	67.3 ± 58
	GGTP (IU/L)	54.2 ± 30	66.3 ± 35	$72.6 \pm 35^{\dagger**}$	74.5 ± 98
	Ascitis (no/moderate/massive)	13/1/0	12/2/0	13/1/0	11/1/2
	Encephalopathy (no/yes)	14/0	14/0	14/0	14/0
L-carnitine group ($N = 8$)	CP score	5.63 ± 0.91	5.75 ± 0.7	5.5 ± 0.53	5.88 ± 0.83
	S. albumin (g/dL)	3.38 ± 0.47	3.22 ± 0.52	3.5 ± 0.54	3.45 ± 0.48
	PT (%)	85.6 ± 17.7	89.7 ± 24.3	91.8 ± 14.8	89.2 ± 20.6
	T. bilirubin (mg/dL)	1.05 ± 0.48	0.87 ± 0.42	0.9 ± 0.34	0.97 ± 0.24
	ALT (IU/L)	33.5 ± 26.1	39.8 ± 25.7	43 ± 34.1	30 ± 12.3
	AST (IU/L)	51.1 ± 28.7	48.1 ± 34.6	61.1 ± 41.9	42.1 ± 10.4
	GGPT (IU/L)	88.1 ± 121	128.6 ± 204	150.1 ± 254	64.3 ± 53
	Ascitis (no/moderate/massive)	7/1/0	8/0/0	8/0/0	8/0/0
	Encephalopathy (no/yes)	8/0	8/0	8/0	8/0

SD: standard deviation; CP: Child-Pugh; S. albumin: serum albumin; PT: prothrombin time; T. bilirubin: total bilirubin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGTP: gamma-glutamyl transpeptidase; TACE: transarterial chemoembolization.
†Significant difference compared with baseline value; ‡significant difference compared with 1 week after TACE; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

ALT ($P < 0.05$). However, L-carnitine limited ALT elevation at 1 week following TACE (Table 2). In the control group, GGTP levels were significantly higher than baseline levels at week 1 ($P < 0.05$) and week 4 after TACE ($P < 0.01$) (Table 2). Conversely, in the L-carnitine group, elevated GGTP levels did not differ significantly from baseline levels (Table 2). There were no significant changes in AST patterns in this study (Table 2). In the control group of BCAA+ and BCAA− patients, GGTP levels were significantly higher than baseline levels at week 4 after TACE ($P < 0.01$); L-carnitine did not affect ALT and AST patterns in both BCAA+ and BCAA− patients (Tables 3 and 4).

3.5. Effects of L-Carnitine on Cirrhotic Symptoms. The protective effects of L-carnitine in preventing occurrence and deterioration of ascites were shown at follow-up of study patients. In L-carnitine group, the number of ascites patients was fewer than baseline at 1 week after TACE and no ascites was detected at 4 weeks after TACE. On the contrary, the number of ascites patients in control group elevated at all follow-up periods with increased observation of massive ascites (Table 2).

Similar results were shown in L-carnitine and control groups in both BCAA+ patients and BCAA− patients (Tables 3 and 4).

In this study, none of study patients developed hepatic encephalopathy after TACE.

3.6. Subgroup Analysis of Effects of BCAA on Liver Functions after TACE. The previously mentioned follow-up results of CP score, serum albumin, and PT demonstrated the additive beneficial effects of combining BCAA and L-carnitine. Patients who received combination of L-carnitine and BCAA achieved better post-TACE liver functions compared to patients who received monotherapy of BCAA or L-carnitine (Tables 3 and 4).

Subgroup analysis of data obtained from BCAA+ and BCAA− patients in the current study irrespective of L-carnitine therapy revealed that BCAA+ patients achieved improvement of CP score at 4 weeks after TACE compared to baseline and significant improvement compared to 1 week after TACE ($P < 0.05$), while no significant improvement was observed in BCAA− patients (Table 5).

BCAA+ patients achieved significant improvement of serum albumin at 4 and 12 weeks after TACE ($P < 0.001$ and $P < 0.05$, resp., compared to 1 week after TACE). On the other hand, less significant improvement of serum albumin was shown in BCAA− patients at 4 and 12 weeks after TACE ($P < 0.01$ and $P < 0.05$, resp., compared to 1 week after TACE) (Table 5).

Moreover, patients who did not receive L-carnitine or BCAA showed highly significant deterioration of serum albumin at 1 week after TACE compared to baseline ($P < 0.001$) and showed PT deterioration at 4 weeks after TACE compared to baseline and 1 week after TACE (Table 4). Conversely, patients who received BCAA alone achieved less significant deterioration of serum albumin at 1 week after TACE ($P < 0.01$) and accomplished PT improvement at 4 weeks after TACE compared to baseline and 1 week after TACE (Table 3).

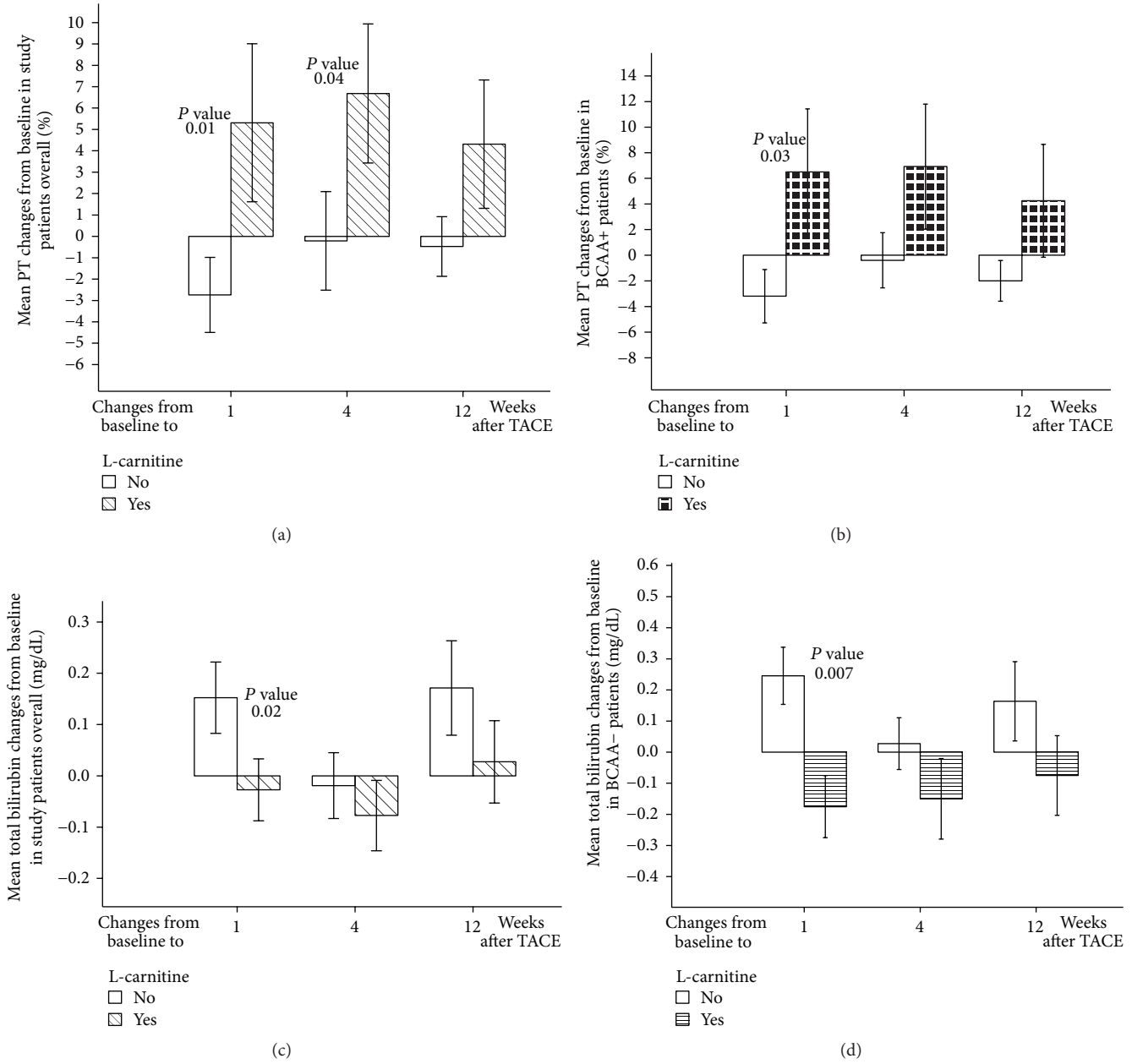

Figure 2: L-carnitine effects on PT and total bilirubin. (a) L-carnitine effects on mean PT changes from baseline in study patients overall. (b) L-carnitine effects on mean PT changes from baseline in BCAA+ patients. (c) L-carnitine effects on mean total bilirubin changes from baseline in study patients overall. (d) L-carnitine effects on mean total bilirubin changes from baseline in BCAA− patients. Footnote: PT: prothrombin time; TACE: transarterial chemoembolization. Error bars represent standard errors.

Total bilirubin showed significant elevation at 1 week after TACE compared to baseline in patients who received neither BCAA nor L-carnitine therapy while no significant elevation was reported in patients who received BCAA (Tables 4 and 3).

4. Discussion

Hepatic failure after TACE is expected and is proportional to pre-TACE synthetic liver functions status [18]. In this study, L-carnitine exhibited hepatoprotective effects following TACE evinced by accomplishing improved CP scores and preventing deterioration of serum albumin, total bilirubin, and PT. To the best of our knowledge, this is the first study evaluating the effects of L-carnitine on liver functions following TACE. The current study clarified early and late post-TACE improvement in liver functions by L-carnitine therapy.

The present study reported very beneficial effects of L-carnitine on CP score showing a significant improvement

Table 5: Subgroup analysis in BCAA+ and BCAA− patients.

	Parameter (mean ± SD)	Pretreatment	After TACE		
			1 week	4 weeks	12 weeks
BCAA+ ($N = 28$)	CP score	6.14 ± 1.04	6.43 ± 0.99	6.04 ± 1.03‡*	6.24 ± 1.36
	S. albumin (g/dL)	3.18 ± 0.56	2.90 ± 0.48†***	3.25 ± 0.53‡***	3.10 ± 0.49‡*
	PT (%)	87.32 ± 18.99	88.50 ± 17.69	91.59 ± 18.19	88.13 ± 16.96
	T. bilirubin (mg/dL)	1.05 ± 0.59	1.10 ± 0.58	1.00 ± 0.56	1.22 ± 0.72
	ALT (IU/L)	37.07 ± 16.50	48.68 ± 24.95†*	35.89 ± 15.43‡**	37.16 ± 11.05‡*
	AST (IU/L)	52.04 ± 22.97	48.93 ± 24.65	50.79 ± 19.39	53.20 ± 18.43
	GGTP (IU/L)	69.89 ± 72.74	86.36 ± 100.41†*	89.26 ± 82.77	53.16 ± 36.19§*
BCAA− group ($N = 22$)	CP score	5.73 ± 0.93	5.90 ± 0.83	5.73 ± 0.76	6.05 ± 1.23
	S. albumin (g/dL)	3.41 ± 0.55	3.12 ± 0.56†***	3.38 ± 0.61‡**	3.40 ± 0.60‡*
	PT (%)	88.9 ± 17.2	88.8 ± 18.2	89.5 ± 16.5	89.9 ± 18.0
	T. bilirubin (mg/dL)	0.88 ± 0.38	0.99 ± 0.46	0.89 ± 0.43	1.02 ± 0.58
	ALT (IU/L)	37.86 ± 32.04	49.81 ± 32.60	38.00 ± 26.65	34.15 ± 20.24
	AST (IU/L)	57.36 ± 42.03	52.62 ± 34.73	60.27 ± 38.91	57.25 ± 46.66
	GGTP (IU/L)	66.59 ± 76.06	90.05 ± 127.71	100.82 ± 154.50	70.50 ± 82.04

SD: standard deviation; CP: Child-Pugh; S. albumin: serum albumin; PT: prothrombin time; T. bilirubin: total bilirubin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGTP: gamma-glutamyl transpeptidase; TACE: transarterial chemoembolization.
†Significant difference compared with baseline value; ‡significant difference compared with 1 week after TACE; §significant difference compared with 4 weeks after TACE; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

at 4 and 12 weeks following TACE. These effects may be attributed to the combination of the favorable L-carnitine effects on serum albumin, PT, total bilirubin, and ascites. As regards ascites, better results were observed in L-carnitine group compared to control group that may be justified by less deteriorated serum albumin profile after TACE with L-carnitine intake.

L-carnitine plays an important role in energy production by transporting long-chain fatty acids across mitochondrial membranes in skeletal muscles [19]. Cirrhotic patients are invariably deficient in L-carnitine [9]. This L-carnitine deficiency inhibits fatty acid mobilization and hence oxidation for energy in skeletal muscles; skeletal muscles are presumed to utilize BCAA as a substitute for fatty acids to produce energy. Cirrhotic patients already suffer from shortage in BCAA that are required for albumin synthesis [20]. BCAA usage as energy source in skeletal muscles adds an additional burden to cirrhotic patients, rendering them more albumin deficient. L-carnitine supplementations correct L-carnitine deficiency; thus, it may preserve BCAA for albumin synthesis and improve serum albumin profiles. In the current study, L-carnitine suppressed early deterioration of serum albumin and maintained its levels better than at baseline.

In a similar fashion, Malaguarnera and his colleagues, in a study evaluating L-carnitine as an adjuvant therapy for interferon plus ribavirin-treated HCV patients, demonstrated that L-carnitine inhibited serum albumin decline at the end of treatment course [15]. Conversely, there was no significant effect of L-carnitine supplementation for 6 months on serum albumin levels in NASH patients [13]. Mean baseline serum albumin levels in L-carnitine-supplemented NASH patients (4.7 ± 0.5 g/dL) were optimal and much better than mean baseline levels in L-carnitine-supplemented cirrhotic patients in our study (3.2 ± 0.5 g/dL); that difference may explain why L-carnitine had no effects on serum albumin levels in patients with NASH.

L-carnitine in this study had improved PT at all follow-up intervals. These finding are in concordance with the effects of L-carnitine on PT reported in children with acute lymphoblastic leukemia receiving chemotherapy [12]. In addition, L-carnitine improved total bilirubin profiles, resembling the observed effect of L-carnitine on total bilirubin in hepatic encephalopathy patients [14]. Conversely, L-carnitine showed no effects on total bilirubin in interferon plus ribavirin-treated patients with HCV [15]. This controversy may be due to long duration of follow-up for HCV patients as total bilirubin was evaluated at 12 months after treatment initiation, while the adverse effect of ribavirin elevating total bilirubin because of hemolysis was usually observed within 3 months from treatment initiation [21].

The favorable effects of L-carnitine on PT and total bilirubin may be due to its ability to relieve hepatic oxidative stress [22]. L-carnitine significantly improved liver antioxidant capacity in cisplatin-treated rats by increasing the Glutathione pool, blockage of the free radical production [23], and regulation of peroxisome proliferator-activated receptor alpha [22].

HCC patients undergoing variable treatment options earned many benefits from BCAA oral therapy [16]. BCAA granules therapy for decompensated cirrhotic patients revealed improvement in event-free survival, quality of life, and serum albumin concentrations [20]. BCAA oral intake improved functions of hepatic parenchymal cells in cirrhotic patients [24]. Moreover, Nishikawa et al. reported that BCAA

granules significantly suppressed the deterioration in hepatic functional reserve (serum albumin and CP score) at 3 and 6 months after TACE [25].

In our study, L-carnitine combination with BCAA revealed more pronounced effects on CP scores, serum albumin, and PT than observed effects in BCAA− patients. We found no available data of previous studies evaluating BCAA and L-carnitine combination therapy. This study is also the first report of the combination effect of L-carnitine and BCAA on liver functions.

On the other hand, comparison between BCAA+ and BCAA− patients regardless of L-carnitine therapy revealed lesser post-TACE deterioration of CP score, serum albumin, and liver enzymes in BCAA+ patients.

The beneficial effects of BCAA therapy without L-carnitine intake were more cleared up in the present study by analyzing data obtained from patients who received neither BCAA nor L-carnitine compared to patients who received BCAA alone. These data demonstrated favorable effects of BCAA on serum albumin, PT, and total bilirubin.

There were no adverse effects by L-carnitine intake in any of study patients.

This study has the following limitations: (1) The relatively small number of patients may inflate the beneficial effects of L-carnitine on liver functions after TACE. (2) Oral administration may decrease the L-carnitine efficacy as it has low oral bioavailability and poor absorption in cirrhotic patients. Parenteral administration is more effective but decreases patient tolerability.

In conclusion, L-carnitine maintained and improved liver functions following TACE. The hepatoprotective effects of L-carnitine in this study were enhanced by BCAA granules combination. L-carnitine and BCAA combination therapy may be offered as a new liver support tool in patients with HCC.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Comparison of Liver Biopsy Findings with the Digestive Disease Week Japan 2004 Scale for Diagnosis of Drug-Induced Liver Injury

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The liver biopsy remains a valuable tool in the diagnosis of drug-induced liver injury (DILI). The Digestive Disease Week Japan 2004 (DDW-J) scale proposed as an objective tool for the diagnosis of DILI has been widely used in Japan. So far, the histological features have not been compared with DDW-J scale in detail. Herein, we examined the correlation between liver biopsy findings and clinical features, particularly DDW-J scales. A total of 80 patients with liver injuries of unknown cause were enrolled. Based on the histological findings, these cases were categorized into 3 groups: A (DILI was strongly suspected), B (DILI was suspected), and C (DILI should be considered in the differential diagnosis). Histological groups and DDW-J scale were moderately correlated ($\kappa = 0.60$). The mean total DDW-J scale scores were as follows: 4.89 for A, 3.26 for B, and 0.75 for C ($p < 0.05$). While hepatocellular type was coincided in a majority of cases by histological and DDW-J scale evaluation, cholestatic type was not well coincided. In conclusion, biopsy findings and DDW-J scale were well correlated, and the hepatocellular type of liver injuries was well coincided by both evaluations, though there were several discrepant cases, particularly in cholestatic type.

1. Introduction

Drug-induced liver injury (DILI) is an important cause of liver injuries with significant morbidity and mortality [1, 2]. Accurate and early diagnosis is important but the diagnosis of DILI is complicated and nonstandardized because of the difficulty in identification drug(s) causing liver injuries and lack of reliable markers to facilitate and establish a diagnosis of DILI [3, 4]. The most important factors to be considered in the diagnosis of drug-induced liver injury (DILI) are the time relationship between the drug administration and appearance/disappearance of liver injury, and the exclusion of other potential causes. Recently, clinical scales or scores have developed to facilitate a diagnosis of DILI. The Council for International Organizations of Medical Sciences/the Roussel Uclaf Causality Assessment Method (CIOMS/RUCAM) scale was proposed [5] and has been generally used as a standardized diagnostic tool. In Japan, the Digestive Disease Week Japan 2004 (DDW-J) scale, which is highly sensitive and specific, was developed by modifying the CIOMS/RUCAM scale [6–8]. This DDW-J scale was proposed as an objective tool for the diagnosis of DILI and has been widely used in Japan [9].

Despite the associated limitations and aggressive diagnostic tool, the liver biopsy remains still a valuable tool in the evaluation of patients suspected to have DILI and can provide valuable information for the diagnosis and management of DILI. That is, when the cause of the liver injury is not

apparent, liver biopsy can indicate the possible presence of DILI and can enable an assessment of the histological patterns by referring to known histological patterns related to potential drugs. Second, liver biopsy can contribute to the management of DILI by assessing the severity and histological features of liver injury.

However, the histologic findings of liver biopsy alone are often insufficient for confirming a diagnosis of DILI, as the diverse histological patterns can mimic any primary liver disease [5, 10–13]. Therefore, clinical investigations are also essential to make a definite diagnosis of DILI. While the comparison of the histological features of liver biopsies and DDW-J scale in the patients with a suspected diagnosis of DILI seems important, detailed correlational studies have not been performed, so far.

In this study, we investigated the relationship between pathological findings of liver biopsies and clinical features, particularly DDW-J scale for the diagnosis of DILI, by using 80 patients with liver biopsy findings showing a suspicion of DILI.

2. Materials and Methods

2.1. Selection of Patients and Tissue Preparation. Subjects were selected from 2115 patients who underwent liver biopsy, when the liver biopsy examination indicated a potential presence of DILI, in two hospitals (Kanazawa University Hospital and Kagawa Prefectural Central Hospital) from April 2007 to December 2014. A total of 80 patients (35 men and 45 women), with a mean age of 55.0 years (range: 15 to 83 years), were enrolled in this study. Clinical and laboratory data were obtained from medical records in these hospitals.

Liver biopsy specimens obtained from these patients were fixed in 10% formalin and embedded in paraffin and were processed routinely for histological diagnosis.

2.2. The Digestive Disease Week Japan 2004 (DDW-J) Scale. DDW-J scale was developed by modifying the CIOMS/RUCAM scale [6–8]. In particular, the factor of comedication was excluded, and the factors of drug lymphocyte stimulation test and eosinophilia were included according to Japan's clinical environment. Details regarding these two scales are summarized in Table 1. Each case was assessed according to DDW-J scale, Japanese clinical diagnostic criteria for DILI [9]. First, the cases were scored for 8 items indicated: time to onset, course, risk factors, other causes, previous information on hepatotoxicity, eosinophilia, drug-lymphocyte stimulation test, and response to readministration. Based on the total scores, individual cases were classified to 3 grades with respect to a diagnosis of DILI; 5 or more, probable; 3 to 4, possible; 2 or less, unlikely (Table 1(a)). Then, the cases were classified to the hepatocellular, cholestatic, and mixed hepatocellular and cholestatic type according to the serum levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP). Hepatocellular type was defined as ALT $> 2 \times$ ULN (upper limit of the normal range) and ALP $\leqq$ ULN, or $R \geqq 5$, where the R value was calculated as (ALT/ULN)/(ALP/ULN). Cholestatic type was defined as ALT $\leqq$ ULN and ALP $> 2\times$ULN, or $R \leqq 2$. Mixed type was defined as ALT $> 2\times$ULN, ALP $>$ ULN, and $R > 2$ and <5.

Causality assessment of drug-induced liver injury (hepatocellular type) is as follows:

(1) time to onset (Table 1(b));

(2) course of the reaction, after cessation of the administration of the drug: score +3: the course is very suggestive if there is decrease of ALT $\geqq$ 50% of the excess over the upper limit of normal within 8 days. Score +2: the course is suggestive if there is decrease of ALT $\geqq$ 50% of the excess over the upper limit of normal within 30 days. Score 0: the course is inconclusive if there is decrease of ALT $<$ 50% of the excess over the upper limit of normal within 30 days or no information regarding liver tests. Score −2: the course is not suggestive if there is decrease of ALT $<$ 50% of the excess over the upper limit of normal after 30 days or increase of ALT again;

(3) in case of readministration of the drug, Score +3: the response is positive if there is at least a doubling of ALT irrespective of date, duration without another drug. Score +1: the response is compatible if there is at least a doubling of ALT irrespective of date, duration with another uninterrupted drug. Score 0: the response is uninterpretable under other conditions. Score −2: the response is negative if the increase is less than normal range, provided that the drug has been given in the same dose, for the same duration and with the same combined drugs as for the first administration.

Causality assessment of drug-induced liver injury (cholestatic type) is as follows:

(1) time to onset (Table 1(c));

(2) course of the reaction, after cessation of the administration of the drug: Score +2: the course is suggestive if there is decrease of ALP $\geqq$ 50% of the excess over the upper limit of normal within 180 days. Score +1: the course is intermediate if there is decrease of ALP $<$ 50% of the excess over the upper limit of normal within 180 days. Score 0: the course is inconclusive if the levels are stable, and there is increase of ALP or no information regarding liver tests. If the drug is continued, the course is always inconclusive as regards causality assessment. Score −2: the course is not suggestive if there is decrease of ALP $<$ 50% of the excess over the upper limit of normal after 30 days or increase of ALP again;

(3) in case of readministration of the drug, Score +3: the response is positive if there is at least a doubling of ALP irrespective of date, duration without other drug. Score +1: the response is compatible if there is at least a doubling of ALP irrespective of date, duration with other uninterrupted drug. Score 0: the response is uninterpretable under other conditions. Score −2: the response is negative if the increase is less than normal range, provided that the drug has been given in the

Table 1: (a) Comparison between DDW-J and CIOMS/RUCAM scales for drug-induced liver injury (DILI). (b) Time to onset: hepatocellular type. (c) Time to onset: cholestatic type.

(a)

Scales	DDW-J	CIOMS/RUCAM
	Score	
Time to onset	0 to +2	0 to +2
Course of the reaction	−2 to +3	−2 to +3
Risk factor		
Age		0 to +1
Alcohol, pregnancy	0 to +1	0 to +1
Comedication		−3 to 0
Other causes*	−3 to +2	−3 to +2
Previous information on hepatotoxicity	0 to +1	0 to +2
Eosinophilia	0 to +1	
Drug-lymphocyte stimulation test	0 to +2	
Response to readministration	−2 to +3	−2 to +3
	Thresholds	
Highly probable		>8
Probable	≧5	6 to 8
Possible	3 to 4	3 to 5
Unlikely	≦2	1 to 2
Excluded		≦0

*Score +2: all causes in groups I and II are ruled out. Score +1: all causes in group I are ruled out. Score 0: 5 or 4 causes in group I are ruled out. Score −1: less than 4 causes in group I are ruled out. Score −2: less than 3 causes in group I are ruled out. Score −3: nondrug cause highly probable.
Group I: HAV, HBV, HCV, biliary obstruction, alcoholism, and acute recent hypotension history. Group II: cytomegalovirus and Epstein-Barr virus.

(b)

	Suggestive from onset of drug administration (score +2)	Compatible (score +1)		Incompatible (score 0)	
		From onset of drug administration	From cessation of drug administration	From onset of drug administration	From cessation of drug administration
Initial treatment	5–90 days	<5 or >90 days	≦15 days	—	>15 days
Subsequent treatment	1–15 days	>15 days	≦15 days	—	>15 days

(c)

	Suggestive from onset of drug administration (score +2)	Compatible (score +1)		Incompatible (score 0)	
		From onset of drug administration	From cessation of drug administration	From onset of drug administration	From cessation of drug administration
Initial treatment	5–90 days	<5 or >90 days	≦30 days	—	>30 days
Subsequent treatment	1–90 days	>90 days	≦30 days	—	>30 days

same dose, for the same duration and with the same combined drugs as for the first administration.

2.3. Histological Examination. Histological examination was performed by three pathologists (Yasuni Nakanuma, Satoko Nakamura, and Hiroko Ikeda) with 20 years of experience of diagnostic liver pathology. DILI was suspected when at least one of the pathological features suggesting DILI, as shown in Box 1, was observed. Based on the histological findings, other potential causes of liver injuries were ruled out as far as possible. When a patient had a history of drug(s) administration with possibility to cause liver injury, the histological pattern(s) were examined by referring to the histopathologic patterns reported for individual drug(s) in the literatures. In this study, we retrospectively analyzed the pathological findings examined as described above.

Based on the comprehensive evaluation of histologic findings of liver, individual cases were categorized into three groups: group A (DILI is strongly suspected, when at least one of the pathological features suggesting DILI, as shown in Box 1, was observed and all pathologists suspected DILI), group B (DILI is suspected, when at least one of the pathological features suggesting DILI was observed), and group C (DILI should be considered in the differential diagnosis, when pathological features suggesting DILI were not observed) (Table 2). Patients who were finally diagnosed

(1) The inflammatory changes in the portal areas are relatively mild compared with those in the liver parenchyma
(2) Demarcated perivenular (acinar zone 3) necrosis
(3) Confluent necrosis with little inflammation
(4) Minimal hepatitis with canalicular cholestasis
(5) Fat deposits in hepatocytes (≥30%)
(6) Abundant neutrophil infiltration
(7) Abundant eosinophil infiltration
(8) Presence of epithelioid-cell granulomas
(9) Biliary damage and inflammation
(10) Severe cholestasis

Box 1: Pathological features suggestive of DILI.

Table 2: Category of groups.

	Category	Histological findings
Group A	DILI is strongly suspected	At least one of the pathological features suggesting DILI* was observed and all pathologists suspected DILI
Group B	DILI is suspected	At least one of the pathological features suggesting DILI* was observed
Group C	DILI should be considered in the differential diagnosis	Pathological features suggesting DILI* were not observed

*Pathological features suggesting DILI were shown in Box 1.
DILI: drug-induced liver injury.

clinically with DILI (see below) were further histologically classified into three types: the hepatocellular type, cholestatic type, and mixed hepatocellular and cholestatic type [5]. The hepatocellular type was characterized by predominant necrotic and/or inflammatory changes of hepatic parenchyma including periportal regions, the cholestatic type by predominant canalicular cholestasis with or without other features of cholestasis, and the mixed type by considerable hepatocellular and cholestatic changes (Figure 1).

2.4. Final Clinical Diagnosis and Comparative Analyses between Histologic Groups and DDW-J Scale. The final clinical diagnosis of DILI was comprehensively made based on clinical course including laboratory and clinical findings with a help of consideration of histologic findings and also DDW-J scale for a diagnosis of DILI. Then, the final clinical diagnosis of DILI and the histological grouping were compared. The DDW-J scale scores and the histological groups were also compared. Furthermore, the histological patterns (hepatocellular type, cholestatic type, and mixed type) and clinical types according to the DDW-J scale (hepatocellular type, cholestatic type, and mixed type) were compared for the patients who were finally diagnosed as DILI.

2.5. Statistical Analysis. Statistical analysis was carried out using Student's t-test and weighted κ test. Differences were considered significant when the p value was less than 0.05.

3. Results

3.1. Histological Grouping and Final Clinical Diagnosis of DILI. By histological examinations, 36 patients were assigned to group A, 19 patients to group B, and 25 patients to group C. By comprehensive clinical and laboratory analyses of these 80 patients, a total of 41 patients were finally diagnosed with DILI. It was found that 33 of 36 (91.7%) patients in group A, 7 of 19 patients (36.8%) in group B, and 1 of 25 patients (4%) in group C were diagnosed finally with DILI (Figure 2). The incidence of final diagnosis of DILI was higher in group A compared to groups B ($p < 0.05$) and C ($p < 0.01$), and the incidence was also higher in group B than in group C ($p < 0.05$), suggesting a good correlation between the histological groups and the final clinical diagnosis of DILI. In 3 of 36 patients of group A, a diagnosis of DILI was eventually excluded: two patients had autoimmune hepatitis (AIH) (Figure 3) and one had graft versus host disease (GVHD). Among the 19 patients in group B, one patient was finally diagnosed with hepatitis E (Figure 4).

3.2. Correlation between Histologic Grouping and DDW-J Scale. Table 3 shows distribution of 80 cases with respect to histologic groups (A, B, and C) and DDW-J scale (probable, possible, and unlikely). Among the 41 patients with a final clinical diagnosis of DILI, 28 of 28 patients (100%) were classified as probable by DDW-J scale, suggesting a good correlation between final clinical diagnosis and DDW-J scale assessment. A majority of group A cases were diagnosed finally as probable DILI, while only 4 of 25 cases of group C were diagnosed as possible DILI and a majority of group C cases were diagnosed as unlikely DILI category. Group B cases were rather evenly distributed to probable, possible, and unlikely DILI. These two distributions were moderately correlated ($\kappa = 0.60$). The mean total DDW-J scale scores for the diagnosis of DILI in these three groups were as follows: 4.89 ± 1.96 for group A, 3.26 ± 1.95 for group B, and 0.75 ± 1.79 for group C (Figure 5): the scores were high in group

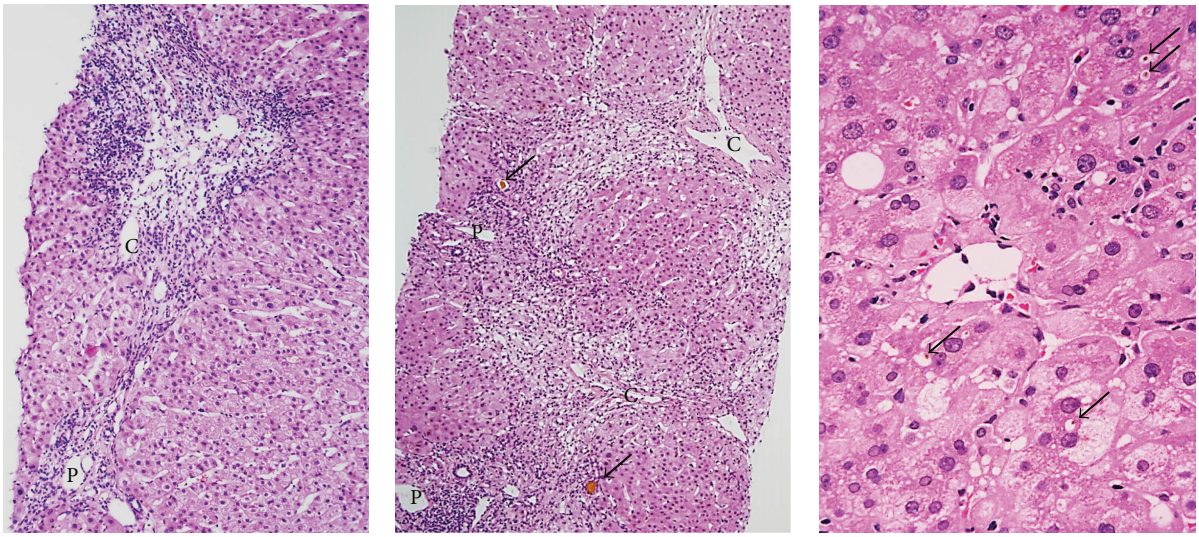

Figure 1: Typical histological images of DILI. (a) Hepatocellular type. Histological examination showed centrilobular hepatic necrosis with punched out lesions. The inflammatory changes were relatively mild in the portal areas, compared with those in the liver parenchyma. (b) mixed type. There is dropout of liver cells in the perivenular zone and bridges of necrosis ("central to portal"). Note periportal mild to moderate inflammatory response. There is dropout of liver cells in the perivenular zone. Additionally cholestasis is observed. (c) Cholestatic type. Ballooning degeneration of liver cells and marked cholestasis (needle biopsy, H&E). C: central vein, P: portal vein, and black arrows: bile thrombus.

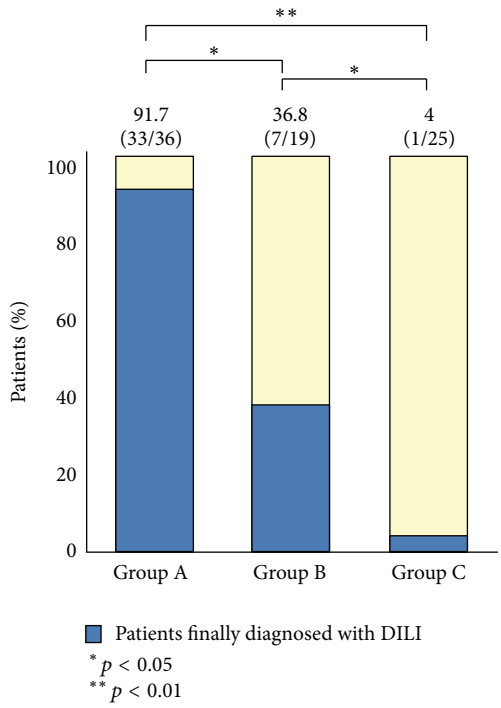

Figure 2: The histological examination results and the final diagnosis of DILI.

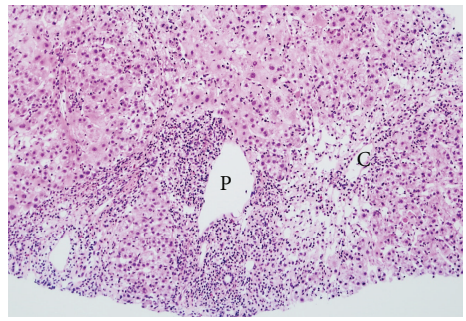

Figure 3: Autoimmune hepatitis. Histological examination showed centrilobular hepatic necrosis with punched out lesions, acidophilic body formation, enlargement of Kupffer cells, and sinusoidal lymphocyte infiltration. Moderate lymphocyte infiltration was observed in the portal areas. Eosinophilic infiltration was also observed. The histological findings were consistent with a diagnosis of acute severe hepatitis with confluent necrosis (needle biopsy, H&E). C: central vein; P: portal vein.

A and lower in group C, and group B was between them, and there was statistical difference between groups A and C ($p < 0.05$) and also between groups B and C ($p < 0.05$), suggesting a good correlation between histological grouping and the scores of the DDW-J scale. There were no statistical differences between group A and group B.

3.3. Three Types of Liver Injuries of DILI by Histological Evaluation and by DDW-J Scales. Among the 41 patients with a final clinical diagnosis of DILI, 30 of 34 patients (73.2%) were classified as the hepatocellular type by histological evaluation and also DDW-J scale, suggesting a good correlation as for the hepatocellular type between histological and DDW-J scale assessment (Table 4).

Among these 41 patients, three patients were classified histologically as the cholestatic type. Of these three patients,

Table 3: Correlation between histologic groups and DDW-J scale for a diagnosis of DILI.

	Histologic groups for a diagnosis of DILI		
	A	B	C
DDW-J scale for a diagnosis of DILI			
Probable	●●●●●●● ●●●●●● ●●●●●●	●●●●● ●	
Possible	●●●◇◇○	●◇○○○	●◇◇○
Unlikely	●●●●	◇◇○○○ ○○○	◇◇○○○○ ○○○○○○ ○○○○○○

Final clinical diagnosis of individual case: DILI ●, not DILI ○, and AIH ◇.
DILI: drug-induced liver injury and AIH: autoimmune hepatitis.

Table 4: The histological patterns and clinical types according to DDW-J scale.

	The histological patterns			Total (cases)
	Hepatocellular type	Cholestatic type	Mixed type	
Clinical types according to the DDW-J scale				
Hepatocellular type	30	2	2	34
Cholestatic type	3	1	0	4
Mixed type	1	0	2	3
Total (cases)	34	3	4	41

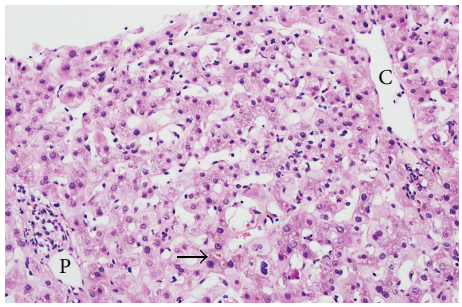

Figure 4: Hepatitis E. Mild lymphocyte infiltration was observed in the portal areas, but interface hepatitis was not present. Cholestasis and bile thrombus (black arrow) were clearly observed in the hepatocytes and predominantly in the periphery of the central vein (needle biopsy, H&E). C: central vein; P: portal vein.

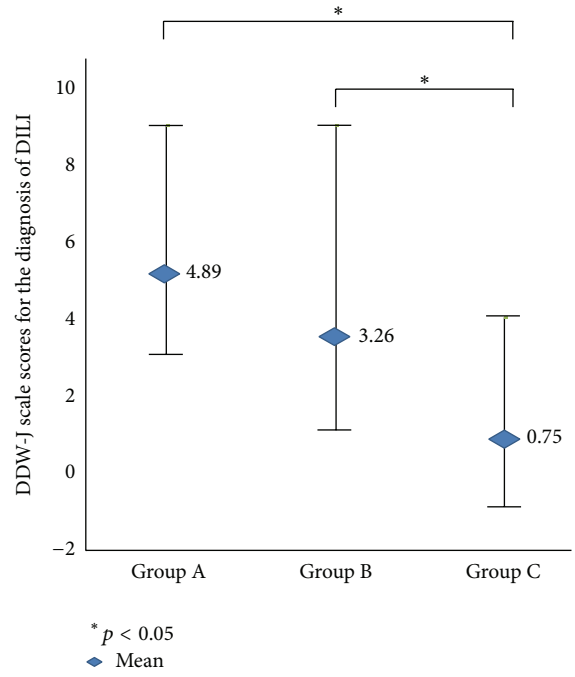

Figure 5: The histological examination results and DDW-J scale scores for the diagnosis of DILI.

one was classified as the cholestatic type and two were clinically classified as the hepatocellular type by DDW-J scale assessment. The latter two patients had higher ALT levels of ≥500, along with a high ALT/AST ratio. Although these two patients had elevated levels of ALP and T-bil, they were clinically classified as the hepatocellular type according to the diagnostic criteria (Table 5).

Among these 41 patients, four patients were clinically classified as the cholestatic type by DDW-J scale. Of the four patients, one was histologically classified as the cholestatic type and three were histologically classified as the hepatocellular type. Among the latter three patients, two had rheumatoid arthritis as an underlying disease; thus, an increase in the ALP level might have been bone-derived rather than hepatogenic, suggesting that ALP did not reflect the degree of cholestasis among these patients (Table 6).

4. Discussion

The data obtained in this study were summarized as follows. (i) Liver biopsy cases were classified into three groups according to a likelihood of DILI. (ii) Good correlation between histological groups and three grades of DILI based on the DDW-J scale was obtained. (iii) Among three patterns of

Table 5: Two patients histologically classified as the cholestatic type but clinically classified as the hepatocellular type.

Gender	Age	The histological examination	DDW-J scale scores	The histological patterns	Clinical types according to DDW-J scale	Medicines which have been taken	DLST	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	T-bil (mg/dL)
M	62	A	4	Cholestatic type	Hepatocellular type	Prednisolone, tranilast, teprenone	Negative	591	189	1003	7.4
F	37	A	6	Cholestatic type	Hepatocellular type	Phenytoin, famotidine, valaciclovir, lorazepam, and etizolam	Negative	718	331	616	1.9

DLST: drug-lymphocyte stimulation test.

liver injuries, the hepatocellular type was well coincided in both assessments, but cholestatic type was not well coincided. Taken together, liver biopsy findings and DDW-J scale were well correlated for diagnosis of DILI, and the hepatocellular type was well coincided, though there were several discrepant cases, particularly in cholestatic type. There might be some limits to classification of cholestatic type in DDW-J scale, because drug-induced cholestatic injury consisted mainly of bile accumulation in the cytoplasm of liver cells (hepatocellular cholestasis) and in canaliculi (canalicular cholestasis). We considered that liver biopsy was useful for diagnosis of DILI in cholestatic type.

In this study, we first categorized liver biopsy findings into three groups: group A (DILI is strongly suspected), group B (DILI is suspected), and group C (DILI should be considered in the differential diagnosis), and it was found that a good correlation was obtained between three groups of liver biopsy diagnosis and the final clinical diagnosis of DILI, suggesting that this grouping of liver biopsies seems useful in clinical practice. This grouping was comprehensively done by combination of histologic findings suggesting DILI (Box 1). However, the combination of pathological findings was different in individual cases and could not be formalized or subjected at the moment. Furthermore, the histological examinations are based on experiences of individual pathologists. More formalized categorization of liver biopsy evaluation for a diagnosis of DILI is necessary.

Recently, the Drug-Induced Liver Injury Network (DILIN) is an ongoing, multicenter observational study of consecutive cases of DILI enrolled at eight geographically distributed academic medical centers in the United States [14]. The central aims of the DILIN Network are to more fully characterize the clinical syndromes of liver injury caused by medications, herbals, and dietary supplements (HDS), to standardize terminology and grading systems, and to provide resources for mechanistic studies of DILI. Kleiner et al. have classified the pathological pattern of liver injury and systematically evaluated histological changes in liver biopsies obtained from 249 patients with suspected DILI enrolled in the prospective, observational study conducted by DILIN [15]. They described that adoption of a standardized and systematic approach to describe the histology of DILI will also allow for comparison of findings across studies and will help in standardizing management and providing insights into pathogenesis as well as approaches to therapy.

The Digestive Disease Week Japan 2004 (DDW-J) scale, which is highly sensitive and specific, was developed by modifying the CIOMS/RUCAM scale [6–8], was proposed as an objective tool for the diagnosis of DILI, and has been widely used in Japan [9]. Based on the total scores, individual cases were classified to 3 grades with respect to a diagnosis of DILI: probable, possible, and unlikely (Table 1(a)). It was found in this study that three groups of liver biopsy findings and three grades of DDW-J scales were well correlated. That is, the distribution of cases with respect to three grades of DDW-J scales and three groups of liver biopsies were moderately correlated, and the scores of DDW-J scales were higher in group A and lower in group C, and scores of group B were between them. However, there were also discrepant cases. In the manual of DDW-J scale, it is described that differential diagnoses of idiopathic autoimmune hepatitis (AIH) and DILI are difficult. In our study, among the 4 patients who were strongly suspected initially to have DILI based on the histological examination results (group A) but were not eventually diagnosed with DILI, two patients had AIH. These conditions might have been mediated by immunological reactions and thus show considerable resemblance in clinical and histopathologic features [16]. Suzuki et al. performed a standardized histologic evaluation to explore potential hallmarks to differentiate AIH versus DILI. The study showed that no single feature was indicative of AIH or DILI, but rather the combination of distinct findings, such as the types of inflammatory cells in different areas, severity of injury/inflammation, and presence of cholestasis, was very helpful in differentiating DILI versus AIH [17]. We considered that liver biopsy was useful in cases where the differential diagnoses were difficult.

HEV infection contributes to a small but important proportion of cases of acute liver injury that are suspected to be drug induced. In our study, among the 19 patients who were possibly suspected to have DILI based on the histological examination results (group B), one patient was finally diagnosed with hepatitis E. Also in Europe and the United States, there has been an increase in the prevalence of hepatitis E in individuals who had no history of overseas travel. Davern et al. examined the prevalence of hepatitis E virus in 318 patients who were previously diagnosed with DILI and reported that nine out of the 318 individuals were positive for the IgM hepatitis E virus antibody [18]. They said that hepatitis E should be considered in the differential

Table 6: Three patients histologically classified as the hepatocellular type but clinically classified as the cholestatic type.

Gender	Age	The histological examination	DDW-J scale scores	The histological patterns	Clinical types according to DDW-J scale	Medicines which have been taken	Underlying disease	DLST	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	T-bil (mg/dL)
M	83	A	5	Hepatocellular type	Cholestatic type	Meloxicam, bucillamine	RA	Negative	161	231	1268	1.4
F	49	B	5	Hepatocellular type	Cholestatic type	Alendronate sodium hydrate, methotrexate, and sodium ferrous citrate	RA	Not available	97	102	766	0.6
F	55	B	3	Hepatocellular type	Cholestatic type	Candesartan, trichlormethiazide, miglitol, and rabeprazole sodium	DM HT	Negative	168	152	1350	1.0

RA: rheumatoid arthritis, DM: Diabetes Mellitus, and HT: hypertension.

diagnosis of patients with acute hepatitis of unknown cause. Although the patient in the present study showed changes suggestive of acute viral hepatitis, we suspected DILI because the changes in the portal areas were relatively mild compared with those in the liver parenchyma.

DILI is generally classified histologically into three types: hepatocellular, cholestatic, and mixed. DDW-J scale also proposed such classification into the hepatocellular, cholestatic, and mixed hepatocellular and cholestatic type according to the serum levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP). It was found in this study that a majority of hepatocellular type cases were coincided in both histological and laboratory assessment by DDW-J scale. However, the cholestatic cases were not coincided. As we discussed in Section 3, this discrepancy may be some limitations in DDW-J scale and more comprehensive clinical analysis of DILI cases is necessary in classification of liver injuries of DILI.

In conclusion, our study suggested a good correspondence between the histological groups with likelihood to a diagnosis of DILI and three grades of DDW-J scales. Hepatocellular type was well coincided by both liver biopsy and DDW-J scale, though cholestatic type was not well classified by DDW-scale. More objective and formalized grouping of liver biopsy findings and the exact classification of cholestatic type in DDW-J scale seem mandatory in a diagnosis of DILI.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Interleukin-1 Family Cytokines in Liver Diseases

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The gene encoding IL-1 was sequenced more than 30 years ago, and many related cytokines, such as IL-18, IL-33, IL-36, IL-37, IL-38, IL-1 receptor antagonist (IL-1Ra), and IL-36Ra, have since been identified. IL-1 is a potent proinflammatory cytokine and is involved in various inflammatory diseases. Other IL-1 family ligands are critical for the development of diverse diseases, including inflammatory and allergic diseases. Only IL-1Ra possesses the leader peptide required for secretion from cells, and many ligands require posttranslational processing for activation. Some require inflammasome-mediated processing for activation and release, whereas others serve as alarmins and are released following cell membrane rupture, for example, by pyroptosis or necroptosis. Thus, each ligand has the proper molecular process to exert its own biological functions. In this review, we will give a brief introduction to the IL-1 family cytokines and discuss their pivotal roles in the development of various liver diseases in association with immune responses. For example, an excess of IL-33 causes liver fibrosis in mice via activation and expansion of group 2 innate lymphoid cells to produce type 2 cytokines, resulting in cell conversion into pro-fibrotic M2 macrophages. Finally, we will discuss the importance of IL-1 family cytokine-mediated molecular and cellular networks in the development of acute and chronic liver diseases.

1. Introduction

The supernatants of activated leukocytes were shown to have the capacity to activate lymphocytes in the 1940s [1]. In 1979, four decades later, the active molecule was designated as interleukin- (IL-) 1. In 1984, the cDNA for murine IL-1 was identified. IL-1 is composed of two different molecules, IL-1α and IL-1β, which are recognized by the same receptor (R), known as the IL-1 receptor, which consists of IL-1R1 and IL-1RAcP [1]. Both IL-1α and IL-1β play roles in innate immunity. Subsequently, a third molecule, IL-1R antagonist (IL-1Ra), was discovered [2]. IL-1Ra competitively binds to IL-1R1 but does not have the capacity to activate IL-1 signaling. In 1995, another member of the IL-1 family, interferon-γ inducing factor (IGIF), was identified [3]. Due to its homology with IL-1α and IL-1β and to the similarity of its mode of production to that of IL-1β, IGIF was temporarily called "IL-1γ" [4]. Indeed, like IL-1β, IGIF lacks a leader peptide and is produced as a biologically inactive precursor (pro). Pro-IGIF, like pro-IL-1β, requires posttranslational processing for activation and secretion [5]. However, IGIF has been shown to require IL-18R but not IL-1R for signaling transduction; neither IL-1α nor IL-1β requires IL-18R [6]. This indicates that IGIF is a member of the IL-1 family but not of IL-1 itself. Thus, IGIF was designated as IL-18. IL-18 is a unique cytokine that activates both innate and acquired immunity, including both Th1 and Th2 immune responses, and has important pathophysiological functions. In 2005, another member of the IL-1 family, IL-33, was identified [7]. Unlike IL-1 or IL-18, IL-33 is important for the development of the type 2 immune response and several diseases. Subsequently, several proteins that show homology with IL-1 family members but have different biological functions have been discovered, such as IL-36α, IL-36β, IL-36γ, IL-36Ra, IL-37, and IL-38 [8]. In this review, we will focus on the roles of IL-1, IL-18, and IL-33 in various liver diseases, following a brief introduction to the IL-1 family, with the aim of clarifying the molecular and cellular networks involving each cytokine that are activated in liver diseases. We will focus on the unique mechanism of liberation of each cytokine and on the liver resident cells that may release and respond to each cytokine. Many excellent reviews have been published that can provide the reader with recent, detailed histological findings on the members of the IL-1 family [9–11].

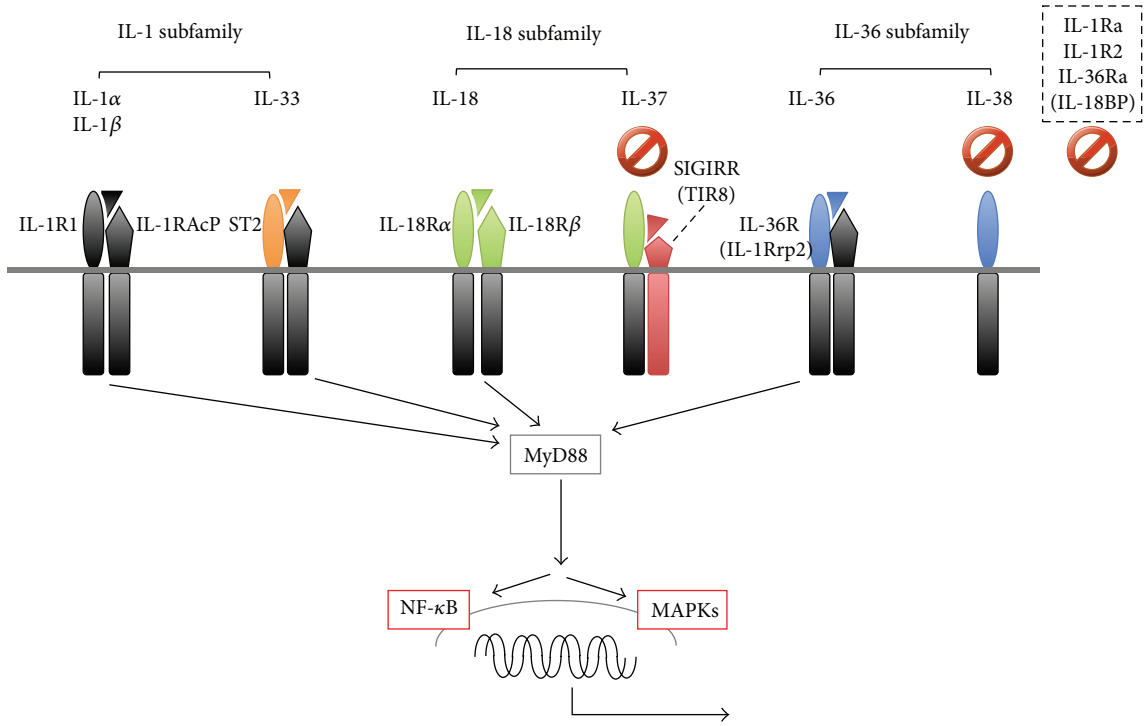

Figure 1: IL-1 family agonists and antagonists. The IL-1 family is divided into three subfamilies based on the length of the N-terminal pro-pieces. The IL-1 subfamily consists of IL-1α, IL-β, IL-1 receptor antagonist (IL-1Ra), and IL-33. The IL-18 subfamily is composed of IL-18 and IL-37. IL-36α, IL-36β, IL-36γ, and IL-38 belong to the IL-36 subfamily. The receptor for each IL-1 family cytokine is a heterodimer of the proper or common subunits. IL-1R1, ST2, IL-18Rα, and IL-36R are ligand-binding subunits, whereas IL-1R accessory protein (IL-1LRAcP), IL-18Rβ, and SIGIRR (TIR8) are signaling subunits. Upon engagement of the binding subunits with the corresponding ligands, they recruit the corresponding signaling receptor subunit, which, except in IL-37 signaling, ultimately translocates nuclear factor- (NF-) κB to the nucleus and activates MAPKs such as p38 and JNK. IL-38, IL-1Ra, IL-18-binding protein (IL-18BP), and IL-36Ra inhibit IL-36, IL-1, IL-18, and IL-36 signaling, respectively. IL-37 inhibits the signal pathways of the innate and acquired immune responses via mechanisms that are poorly identified. IL-1R2 acts as a decoy receptor for IL-1α and IL-1β. Stop signs indicate proteins that inhibit corresponding ligand signaling or those that negatively regulate other signal pathways as well. JNK: c-jun-N-terminal kinase; MAPK: mitogen-activated protein kinase; SIGIRR: single immunoglobulin IL-1-related receptor; ST2: suppression of tumorigenicity 2.

2. The IL-1 and IL-1R Families

The IL-1 family ligands and the corresponding receptor subunits are shown in Figure 1. All of the receptor subunits are members of the IL-1R family and are characterized by possession of a Toll-like receptor (TLR)/IL-1R (TIR) domain within their cytoplasmic tails. TIR is required by IL-1 family members and TLR ligands for signaling. IL-1 family ligands comprise seven members with agonistic functions (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ) and four members with antagonistic activities, such as IL-1Ra, IL-36Ra, IL-37, and IL-38. IL-1R family members include four ligand-binding subunits, IL-1R1, ST2, IL-18Rα, and IL-36R, and three signaling subunits, IL-1RAcP, IL-18Rβ, and single immunoglobulin IL-1-related receptor (SIGIRR), alternatively named TIR8. SIGIRR/TIR8 is regarded as an orphan receptor with regulatory functions [12].

IL-1 family ligands are classified into three groups, based on the size of the N-terminal pro-pieces that remain after cleavage of the full-length ligands [9–11]. Members of the IL-1 subfamily, such as IL-1α, IL-1β, and IL-33, possess the longest pro-pieces, composed of approximately 270 amino acids. Both IL-1α and IL-1β transduce signals via IL-1R, a heterodimer of IL-1R1 and IL-1RAcP. IL-33 uses ST2 and IL-1RAcP. IL-18 subfamily ligands, including IL-18 and IL-37, also possess long pro-pieces composed of approximately 190 amino acids. The receptor for IL-18 consists of a binding subunit, IL-18Rα, and a signaling chain, IL-18Rβ. IL-37 binds to IL-18Rα, and SIGIRR (TIR8) is required as the signaling subunit. Notably, the mouse homologue of IL-37 remains unknown. IL-36 subfamily ligands such as IL-36α, IL-36β, IL-36γ, and IL-38 possess small pro-pieces of approximately 150 amino acids. All except IL-38 bind to IL-36R, followed by signaling via the coreceptor, IL-1RAcP.

The biological functions of IL-1 (IL-1α and IL-1β) and IL-36 (IL-36α, IL-36β, and IL-36γ) are negatively regulated by IL-1Ra and IL-36Ra, respectively. IL-1Ra, in competition with IL-1α and IL-1β, binds to IL-1R1, but unlike the IL-1α/IL-1R1 or IL-1β/IL-R1 complexes, the IL-1Ra/IL-1R1 complex cannot recruit the signaling chain, IL-1RAcP, eventually resulting in inhibition of IL-1 signaling [13]. Similarly, IL-36Ra binds to IL-36R and inhibits recruitment of the IL-36 signaling receptor chain, IL-1RAcP, onto IL-36R [14]. IL-1R2 is a "decoy receptor" that competitively inhibits IL-1α/β binding to

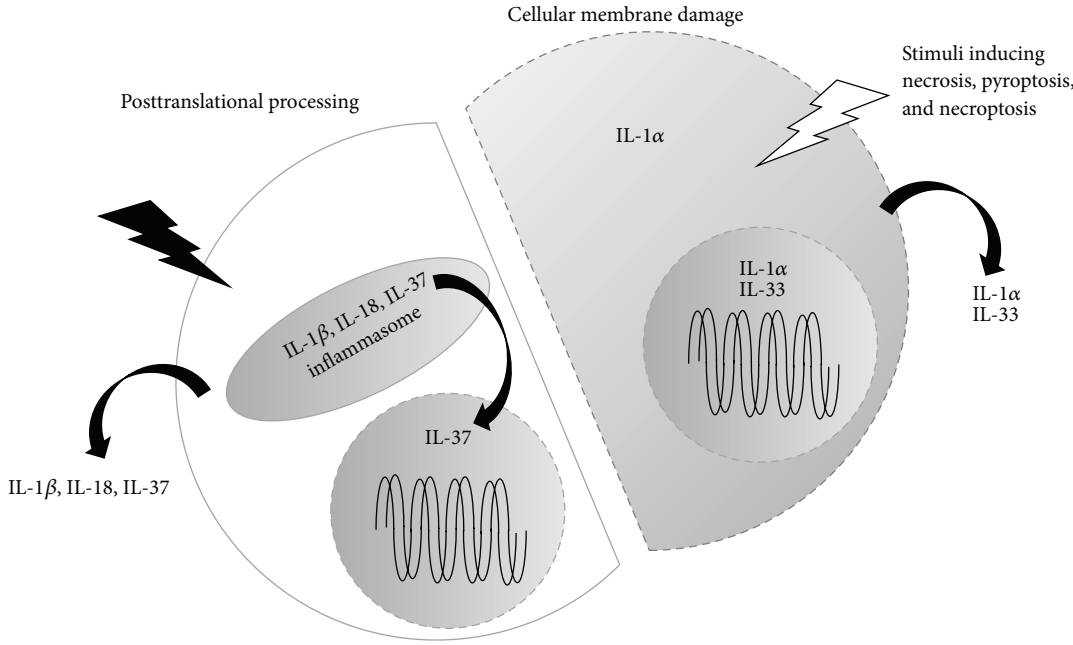

Figure 2: Modes of action of IL-1 family cytokines. All cytokines belonging to the IL-1 family, except IL-1 receptor antagonist (IL-1Ra), lack the leading peptides that are required for cell secretion. Furthermore, all full-length cytokines, except IL-1α and IL-33, are biologically inactive unless they receive appropriate posttranslational processing. Even biologically active full-length IL-1α and IL-33 need the appropriate cellular stimuli to be secreted from cells. IL-33 is localized in cellular nuclei, whereas IL-1α is localized in lysosomes and perhaps in nuclei. After receiving stimuli that induce cell death via destruction of the cellular membranes (i.e., pyroptosis and necroptosis), IL-1α and IL-33 are extracellularly liberated. In contrast, precursor- (pro-) IL-1β, pro-IL-18, and pro-IL-37 require cleavage by caspase-1 in the inflammasome, a large multiple-protein complex (shown in Figure 3). Following appropriate stimuli, the inflammasomes are activated. Consequently, biologically active IL-1β, IL-18, and perhaps IL-37 fragments are secreted. Bioactive IL-37 can also be translocated into nuclei. IL-36 subfamily members also require posttranslational processing for activation and excretion. However, they cannot be processed by caspase-1; their processing enzymes remain to be elucidated. pro: precursor.

IL-1R1. IL-18-binding protein (IL-18BP), which does not belong to the IL-1 family, is a natural inhibitor of IL-18 [15].

Following binding of a ligand to its corresponding binding receptor subunit, the ligand/binding receptor subunit complex recruits the corresponding signaling subunit. In this setting, the cytoplasmic tails of the binding and signaling subunits can recruit and interact with the signaling adaptor molecule MyD88 via TIR-TIR interaction, which eventually results in the nuclear translocation of nuclear factor- (NF-) κB and the activation of mitogen-activated protein kinases (MAPKs) such as p38 and c-jun-N-terminal kinase (JNK) [14, 16, 17] (Figure 1).

3. Modes of Liberation of Biologically Active Ligands

Because they lack leader peptides, none of the members of the IL-1 family, except IL-1Ra, are extracellularly released immediately after translation. The full-length cytokines remain inside the cell, unless the cell is appropriately stimulated or damaged. Some full-length ligands, such as IL-33 and IL-1α, are biologically active but require cellular damage for release [18–20] (Figure 2 right). Full-length IL-1α is biologically active, but processed IL-1α has been reported to exert higher activity than full-length IL-1α [21, 22]. Full-length IL-1α and IL-33 are not released unless the cellular membrane is destroyed. Thus, IL-1α and IL-33 are designated as alarmins.

Some of the IL-1 family members are produced as biologically inactive precursor proteins and require appropriate posttranslational processing for activation and release from cells, such as IL-1β, IL-18, IL-37, IL-36α, IL-36β, IL-36γ, and IL-36Ra. IL-1β, IL-18, and IL-37 are processed by caspase-1, which was initially termed IL-1β-converting enzyme (ICE) [5, 18, 23–25] (Figure 2 left). In contrast, IL-36 subfamily members commonly lack caspase-1 cleavage sites. Their processing enzyme remains to be identified [14].

Caspase-1 is a member of the caspase family [26]. Caspase family members are produced as enzymatically inactive zymogens. Based on function, caspases have been classified into two groups, cell death-associated caspases (caspase-8, caspase-9, caspase-6, caspase-7, and caspase-3) and inflammatory caspases (caspase-1, caspase-4 caspase-5, and caspase-11). The activation cascade of the cell death-associated caspases was identified in the 1990s. However, the molecular mechanism for the activation of inflammatory caspases remained enigmatic until 2002. The late Dr. J. Tschopp proposed that a large protein complex, named the inflammasome, was the cytoplasmic machinery that activates caspase-1 for release of resultant mature IL-1β and IL-18 [27]. Several types of inflammasomes, with different cytoplasmic sensors, such as NACHT, leucine-rich repeat (LRR), and pyrin

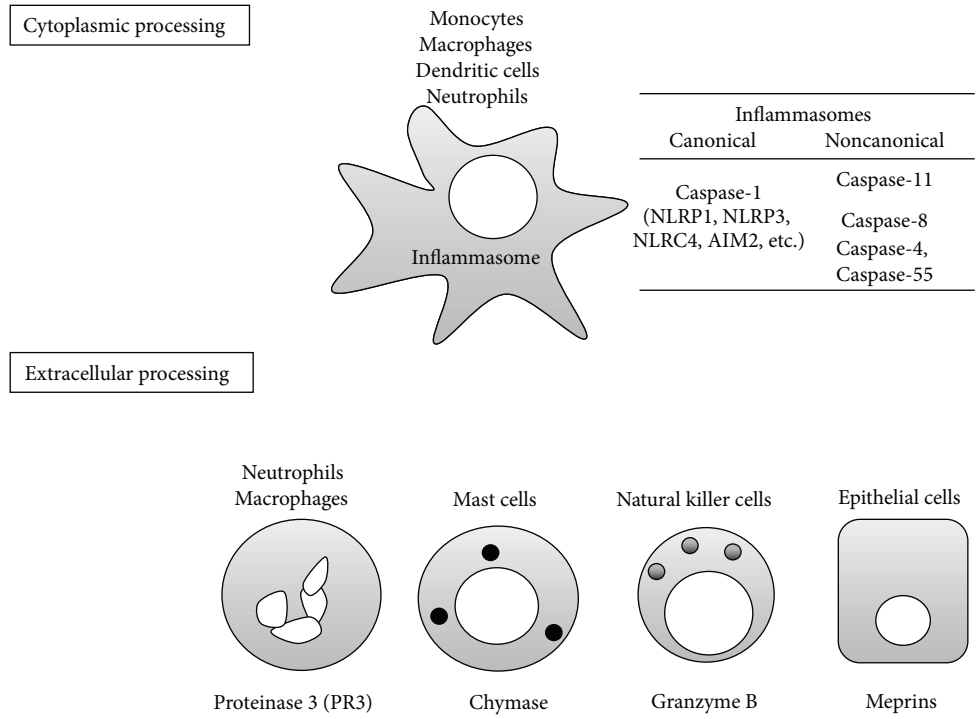

Figure 3: Processing enzymes dependent on and independent of the inflammasome. Caspase-1 is an enzyme that putatively converts IL-1β and IL-18. Caspase-1 is produced as zymogen and requires cleavage. Inflammasomes are the machinery in which caspase-1, IL-1β, and IL-18 are processed. Inflammasomes are classified as canonical and noncanonical. Canonical inflammasomes can be divided into 4 main types based on differences in cytoplasmic PRRs, such as NLRP1, NLRP3, NLRC4, and AIM. Noncanonical inflammasomes activate caspase-11, caspase-8, caspase-4, and caspase-5 and often collaborate with the canonical inflammasomes. Monocyte-macrophage lineage cells, dendritic cells, and neutrophils can harbor inflammasomes. Various enzymes have the capacity to process IL-1β and IL-18 extracellularly. Neutrophil-derived proteinase 3, mast cell chymase, granzyme B produced by NK cells, and meprins produced by epithelial cells can convert pro-IL-1β and pro-IL-18 into mature ligands. AIM: absent in melanoma 2; NK: natural killer; NLRC: NACHT, leucine-rich repeat, and caspase recruitment domain-containing protein; NLRP: NACHT, leucine-rich repeat, and pyrin domain-containing protein; PRR: pattern recognition receptor.

domain- (PYD-) containing protein- (NLRP-) 1 (NLRP1), NLRP3, caspase recruitment domain- (CARD-) containing protein- (NLRC-) 4 (NLRC4), and absent in melanoma 2 (AIM2) have since been identified (Figure 3 upper). Those proteins are cytoplasmic pattern recognition receptors (PRRs) that sense extrinsic pathogen-associated molecular patterns (PAMPs) and intrinsic damage-associated molecular patterns (DAMPs). Three of the proteins, NLRP1, NLRP3, and NLRC4, belong to the Nod-like receptor (NLR) family. AIM2 is a cytoplasmic DNA sensor that does not belong to the NLR family. When cells such as neutrophils, macrophages, or dendritic cells are appropriately stimulated [28], one of these sensors, a caspase-1 adaptor protein named apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC) and procaspase-1, begins to assemble to form the inflammasome [29–33]. Structural analysis has revealed that, in some inflammasomes, such as NLRP3 and AIM2 inflammasomes, many procaspase-1 proteins likely polymerize to form a filamentous structure that permits autocleavage of procaspase-1 [34]. We found that the NLRP3 inflammasome is essential for the elevated serum levels of IL-18 observed in heat-killed *Propionibacterium acnes*-primed mice after challenge with lipopolysaccharide (LPS) [35]. Intriguingly, deletion of the genes encoding either component of the NLRP3 inflammasome or the resulting IL-18 prevented LPS-induced liver injury in *P. acnes*-primed mice. Indeed, $Nlrp3^{-/-}$, $Asc^{-/-}$, $Caspase1^{-/-}$, and $Il18^{-/-}$ mice are resistant to liver injury induced by sequential treatment with *P. acnes* and LPS [3, 35, 36]. Recently, it was shown that IL-37 requires the NLRP3 inflammasome for activation and nuclear translocation [24]. In addition, several different types of noncanonical inflammasomes have been demonstrated to be required for activation of the canonical inflammasomes. For example, the caspase-11 noncanonical inflammasome was demonstrated to be essential for the activation of the canonical NLRP3 inflammasome in macrophages after Gram-negative bacterial infection [37–39]. Notably, ES cells derived from mouse strain 129 express little caspase-11, and the $Caspase1^{-/-}$ mice currently used worldwide were generated from ES cells expressing $Caspase11^{129/129}$. Even after heavy backcrossing with C57BL/6 (B6) mice, $Caspase1^{-/-}$ B6 mice still possess $Caspase11^{129/129}$, because of the close proximity of *Caspase1* and *Caspase11*. Therefore, data obtained using these $Caspase1^{-/-}Caspase11^{129/129}$ mice are equivalent to data for $Caspase1^{-/-}Caspase11^{-/-}$ mice. Before the function of endogenous caspase-1 could be determined, B6 ES cell-derived $Caspase1^{-/-}$ mice had to be developed. Previous to

the development of *Caspase1*$^{-/-}$ B6 mice, we believed that caspase-1 was responsible for both release of IL-1β/IL-18 and pyroptosis, a type of regulated cell death accompanied by cell membrane rapture and resulting amplifying inflammatory responses, based on the observation that *Caspase1*$^{-/-}$*Caspase11*$^{129/129}$ B6 macrophages (Mø) impaired both phenotypes. Finally, using *Caspase11*$^{-/-}$ B6 mice, Kayagaki et al. demonstrated that caspase-11 participates in activation of the canonical inflammasome and is also responsible for pyroptosis. In contrast, *Caspase1*$^{-/-}$ B6 mice did not show IL-1β and IL-18 release but did show pyroptosis comparable to wild-type (WT) B6 mice [37–40]. We now know that both noncanonical inflammasomes, involving caspase-11, and canonical inflammasomes, involving caspase-1, are required for IL-1β/IL-18 release, but that the canonical inflammasome is not required for pyroptosis upon infection with intracellular-facultative Gram-negative bacterial infection or LPS challenge. Kayagaki et al. clearly demonstrated that caspase-11-dependent pyroptotic cell death, but not caspase-1-mediated IL-1β/IL-18 release, is responsible for the morbidity and mortality of LPS-induced septic shock. Very recently, two human caspase-11 orthologues, caspase-4 and caspase-5, as well as caspase-11, were verified to directly recognize cytoplasmic LPS to activate the canonical inflammasome [41]. Furthermore, a noncanonical inflammasome involving caspase-8, an apoptosis-associated caspase, was also verified to be essential for processing IL-1β and IL-18 after microbial infection [42–46]. Thus, activation of canonical and noncanonical inflammasomes might result not only in release of IL-1β and IL-18 but also in activation of regulated cell death processes such as pyroptosis and apoptosis, which are characterized by the presence and absence of cell membrane rupture, respectively [47–50]. Thus, caspase family members are important for host defense via development of inflammatory responses and activation of regulated cell death in infected cells. Recent comprehensive reviews, including a description of the mechanisms for negative regulation of the inflammasome, provide detailed information on the inflammasome [47, 48, 51–57].

4. Extracellular Processing of Precursor Cytokines

Precursor cytokines can be activated by machinery other than inflammasomes. Many enzymes other than caspases can cleave pro-IL-1β and pro-IL-18 into biologically active fragments (Figure 3 lower).

Serine proteases can process IL-1β and IL-18. Neutrophils possess several types of serine proteases in their azurophilic granules. Neutrophil elastase, proteinase 3 (PR3), and cathepsin G are representative of neutrophil serine proteases [58, 59]. Purified PR3 activates the human Mø cell line THP-1 to release IL-1β independent of caspase-1 [60]. The importance of PR3-mediated IL-1β processing has also been demonstrated in vivo. WT mice, but not *Il1β*$^{-/-}$ mice, developed chronic arthritis after multiple intra-articular challenges with streptococcal cell wall. *Dppi*$^{-/-}$ mice, which lack the ability to activate PR3, are partly resistant to this chronic arthritis and show reduced induction of biologically active IL-1β in synovial explants, whereas *Caspase1*$^{-/-}$ mice are similarly susceptible to WT mice [61]. The importance of PR3-mediated IL-1β processing has also been demonstrated in peritonitis induced by intraperitoneal challenge with monosodium urate crystals, a self-derived molecule relevant to gout [62]. Thus, multiple IL-1β-converting enzymes, including PR3, are required for inflammatory arthritis in mice [63]. PR3 is also involved in the processing of IL-18 and perhaps IL-33 [64, 65].

Human mast cell chymase was demonstrated to convert pro-IL-18 into biologically active IL-18 fragments [66]. Cutaneous mastocytosis is a common feature of atopic dermatitis in humans and also in murine models, such as skin-specific caspase-1 transgenic (tg) mice and IL-33 tg mice [67, 68]. In these mouse models, chronic dermatitis spontaneously develops with severe mastocytosis in the skin lesions. As IL-18 and IL-33 are potent activators of mast cells [69, 70], it is plausible that mast cells in the lesion release chymase. Chymase might, in turn, cleave pro-IL-18, which is liberated from the damaged keratinocytes, into active fragments. This might serve as a positive feedback circuit for exacerbation of chronic dermatitis in either mouse model.

Granzyme B is a cysteine protease, produced by natural killer cells and cytotoxic T lymphocytes, that is involved in apoptosis of target cells [71]. Recently, granzyme B was shown to exert processing activity on pro-IL-18 [72], although the biological settings involving granzyme B-mediated IL-18 processing are unclear.

As described below, many IL-1 family cytokines are produced as full-length proteins in nonhematopoietic cells, including epithelial cells, fibroblasts, and endothelial cells (Table 1). Membrane-bound and secreted astacin metalloproteinases, meprins, were verified to have the potential to cleave pro-IL-1β and pro-IL-18 into bioactive fragments [73, 74]. Meprin α is reported to be expressed in the stratum basale of human skin, whereas meprin β is observed in the cells of the stratum granulosum. Intriguingly, in the lesions of patients with psoriasis vulgaris, meprin α expression is predominant in the uppermost layers rather than the basal layer [75, 76]. This indicates that meprins may be involved in processing of IL-36 subfamily cytokines, which are preferentially expressed in the skin (as described above). Meprin β, but not meprin α, has been shown to be essential for IL-18 processing in a murine model of colitis induced by dextran sulfate sodium (DSS). After challenge with DSS, meprin β-deficient mice, but not meprin α-deficient mice, display a significant reduction in the elevation of serum levels of IL-18 in comparison with WT-mice [74]. Thus, in nonhematopoietic cells, meprins are IL-1β- and IL-18-converting enzymes.

Previously, inflammasome-independent enzymes were believed to be involved in the processing of IL-1β in autoinflammatory disease of *Pstpip2*^{cmo} mice [77–81]. *Pstpip2*^{cmo} mice express a Leu98Pro missense mutation in the Pombe Cdc15 homology family protein proline serine-threonine phosphatase interacting protein 2 (PSTPIP2) and develop osteomyelitis when they are fed a normal, low-fat diet. *Pstpip2*^{cmo}*Il1β*$^{-/-}$ and *Pstpip2*^{cmo}*Il1r1*$^{-/-}$ mice are free of osteomyelitis, whereas *Pstpip2*^{cmo}*Nlrp3*$^{-/-}$ and

Table 1: Pathophysiological roles of IL-1 family members. The cellular source, target cells, and major functions of IL-1 family cytokines are shown. Representative diseases involving each cytokine are also shown. The precise disease relevancy is described in the text.

Cytokine	Cellular sources	Targets	Major functions	Liver diseases	Other diseases
IL-1α	Many cell types	Many cell types	Inflammation	Sterile inflammation	Sterile inflammation
IL-1β	Many cell types	Many cell types	Inflammation, Th17 induction	Sterile inflammation NAFLD	Sterile inflammation Autoinflammatory diseases
IL-33	Many cell types	ICL2, basophils, eosinophils, mast cells	Wound healing Type 2 response	Liver fibrosis	Nematode-associated disease, allergic diseases, fibrosis
IL-18	Many cell types	NK cells, Th1 cells, mast cells, basophils	Inflammation, Th1 response, allergic response	Innate immunity-mediated liver injury	Chronic dermatitis, intrinsic allergy
IL-37	Unknown murine orthologue	Many cell types	Inhibition of innate and acquired immunity	Unknown	Protection of sepsis
IL-36	Many cell types	Skin	Recruitment of neutrophils	Unknown	Psoriasis

COPD: chronic obstructive pulmonary disease; ICL2: group 2 innate lymphoid cells; NAFLD: nonalcoholic fatty liver disease.

Pstpip2^{cmo}*Casp1*$^{-/-}$ mice suffer similarly to *Pstpip2*^{cmo} mice [77, 78]. Very recently, osteomyelitis was reported to be absent in *Pstpip2*^{cmo} mice fed a high-fat diet [82]. Intriguingly, in contrast to control normal mice, the microbiota of *Pstpip2*^{cmo} mice fed a low-fat diet was found to show a predominance of inflammation-associated commensals. In contrast, when these mice are fed a high-fat diet (HFD), their microbiota is restored. Furthermore, *Pstpip2*^{cmo} mice can evade osteomyelitis even when fed a low-chow diet, if the genes encoding caspase-1 and caspase-8 are both knocked out. However, the deletion of either *Caspase1* or *Caspase8* alone does not prevent osteomyelitis. Thus, aberrant activation of either caspase-1 or caspase-8 seems to determine the severity of this autoimmune disease. The precise molecular mechanisms underlying this disease, in particular how caspase-1 and caspase-8 inflammasomes are involved, remain unclear.

Recently, a comprehensive review of inflammasome-independent regulation of IL-1 family cytokines was published [83]. It provides a detailed discussion of inflammasome-independent activation of IL-1 family cytokines.

5. Biological Functions

IL-1 family agonists signal via different corresponding receptor complexes, but commonly activate NF-κB and MAPKs via recruitment of the signal adaptor MyD88 (Figure 1), resulting in the ability to perform their biological functions (Table 1). Recent findings on the function of each member will be briefly introduced.

5.1. IL-1. Innate immunity is required for the rapid eradication of invading microbes. However, the same innate immune response can be evoked upon exposure to stimuli from host-derived substances, such as PAMPs translocated from the gut microbiome or self-derived alarmins and DAMPs. This triggers or exacerbates inflammatory diseases. In contrast to inflammation induced by extrinsic microbial infection, this is known as sterile inflammation. Sterile inflammation underlies many types of local inflammatory diseases [84]. IL-1α has been demonstrated to play a major role in the development of sterile inflammatory diseases including atherosclerosis and myocardial infarction [85, 86]. Indeed, intraperitoneal administration of dead endothelial cell-derived particles can induce peritonitis in mice, dependent on IL-1α but not IL-1β [87]. Furthermore, it has been demonstrated that the mode of cell death largely determines whether cells release IL-1α, IL-1β, or neither [88]. Apoptosis and pyroptosis are two types of regulated cell death; necroptosis is a third. In contrast to apoptosis, pyroptosis and necroptosis are characterized by destruction of the cellular membrane. Apoptotic cells do not release their cellular contents, whereas both necroptotic and pyroptotic cells liberate their cellular contents, including IL-1α [48]. Pyroptosis, a type of inflammatory cell death induced by inflammasome activation as described above, is accompanied by IL-1β release. Necroptosis is mediated by receptor interacting protein kinase-3 (RIP3) and by its substrate, mixed lineage kinase like (MLKL), which is located in the cytosol [49, 89]. RIP3-phosphorylated MLKL is oligomerized and translocated to the plasma membrane, where it destroys the cell membrane by disruption of ion channels and/or induction of pore formation [90–93]. Necroptosis develops when cells are stimulated via cell death receptors, such as TNFR1, Fas, and receptors of TNF-related apoptosis-inducing ligand (TRAIL), under limited, apoptosis-inhibiting conditions. Other stimuli, such as TLR ligands, viral infection, and type 1 IFN, can induce necroptosis as well [49]. Unfortunately, no method has been established for selectively distinguishing necroptotic cell death from other types of cell death. Necroptosis can liberate IL-1α. In addition to IL-1α, IL-1β is responsible for sterile inflammation, because DAMPs in the liberated cellular contents can activate innate immune responses including inflammasome-mediated IL-1β release. Therefore, it is plausible that both IL-1α and IL-1β are involved in sterile inflammation [84].

Recent progress in antibody technology and tools for identification of disease-responsible genes has enabled cures for many patients with recurrent or chronic inflammatory diseases. In particular, many inflammatory diseases can be efficiently treated by IL-1 blockade. For example, patients with hereditary systemic autoinflammatory disease due to impaired regulation of inflammasome activation or impaired regulation of IL-1 signaling can be treated with an IL-1Ra named Anakinra and/or a soluble IL-1R named Rilonacept [13, 94–97]. Type 2 diabetes, to which the aberrantly activated inflammasome has been shown to be relevant [98–100], can also be treated with IL-1Ra [101].

5.2. IL-33. Analogous to IL-1α, IL-33 is liberated following cell death accompanied by cell membrane rupture (i.e., necroptosis but not apoptosis) [19].

Endogenous IL-33 is important for the type 2 immune response against helminth infection. Several cell types involved in innate immunity, such as group 2 innate lymphoid cells (ILC2), basophils, eosinophils, and mast cells, express IL-33R and play a role in triggering and/or enhancement of type 2 immunity. In particular, in response to IL-33, ILC2 promptly produces large amounts of IL-5 and IL-13, which eventually leads to eosinophilia and mucin accumulation around the helminth. IL-33-mediated ILC2 activation is important for the rapid development of Löffler syndrome, which is characterized by pulmonary eosinophilia after cutaneous infection of mice with intestinal nematode larva [102]. This type of ILC2-mediated host response is critical for the expulsion of intestinal nematodes via induction of parasite-specific type 2 immunity [103]. Thus, IL-33 activation of ILC2 is required for host defense against helminths via activation of both innate and acquired immune responses [104, 105].

Cerebral malaria, induced by infection with the protozoan *Plasmodium* spp., is a lethal disease in children. Th1 immunity against the protozoan is important for host defense but simultaneously activates collateral severe inflammation in the brain. Very recently, extrinsic IL-33 was demonstrated to protect against lethal cerebral malaria by activating ILC2, inducing regulatory T cells, and causing cellular conversion into M2 Mø that produce anti-inflammatory and regulatory cytokines [106].

IL-33 is pivotal for allergic diseases as well. ILC2 plays a role in the development of allergic airway inflammation [107–111]. For example, peripheral blood mononuclear cells (PBMCs), including ILC2, prepared from patients with allergic asthma produce larger quantities of IL-13 and IL-5 in response to IL-33 than those from control subjects [112]. In addition, the IL-33/IL-33R axis in mast cells and basophils participates in allergic respiratory diseases [113]. After intranasal infection of mice with a mouse parainfluenza virus, Sendai virus, chronic obstructive pulmonary disease (COPD) develops. The virus-infected WT mice show elevated airway resistance, whereas IL-33- and ST2-deficient mice do not [114]. The IL-33-IL-33R axis is also important for airway inflammation and for induction of IL-33 downstream cytokines and mucin. Notably, lung samples from patients with COPD exhibit higher levels of IL-33 in their lung epithelial cells than those from non-COPD patients [114]. IL-33 is essential for the development of allergic rhinitis induced by intranasal treatment with ragweed in mice [115]. This is also true for food allergies induced by topical application of peanut extracts [116]. Furthermore, *Il33* tg mice, which specifically overexpress IL-33 in their keratinocytes, spontaneously develop atopic dermatitis-like chronic cutaneous alterations [68].

Accumulating lines of evidence strongly suggest that IL-33 is involved in fibrotic diseases, an important cause of morbidity and mortality in humans. It is well established that type 2 immune responses involving cell conversion into M2 Mø are important for wound healing and fibrosis [117–120]. Because IL-33 is associated with type 2 immunity, as described above, it is plausible that IL-33 is causative of fibrotic diseases [121]. Serum levels of IL-33 are elevated in patients with systemic sclerosis, a connective tissue disease characterized by fibrosis of the skin and other organs, such as the lungs [122]. There is a positive relationship between serum IL-33 levels and disease severity [123]. In addition, daily subcutaneous injection of IL-33 induces cutaneous fibrosis in WT mice and mast cell-deficient mice ($cKit^{w/v}$) but not in $St2^{-/-}$, $Il33^{-/-}$, $Rag2^{-/-}$, or eosinophil-deficient mice (ΔdblGATA mice), indicating the importance of aberrant accumulation of IL-33, its downstream cytokine IL-13, acquired immunity, and eosinophils [124]. Large numbers of cells expressing abundant IL-33 are observed in the lungs of patients with idiopathic pulmonary fibrosis, as well as systemic sclerosis [125]. Furthermore, gene-delivery of *Il33* exacerbates bleomycin-induced pulmonary fibrosis [125, 126]. The roles of IL-33 in liver cirrhosis will be described in a subsequent section.

5.3. IL-18. IL-18 is a pleiotropic cytokine, similar to other IL-1 agonists, and its major biological functions have been described in several reviews [69, 127–130]. For example, IL-18 contributes to both type 1- and type 2-mediated host defense and inflammatory diseases. In collaboration with IL-12, IL-18 activates NK cells to produce a large quantity of interferon-(IFN-) γ. Convincingly, $Il18^{-/-}$ mice are highly susceptible to infection with the intracellular bacterium *Listeria monocytogenes*, particularly in the early infectious phase [131]. IL-18 is also involved in the expulsion of the intracellular fungus *Cryptococcus neoformans*. In addition, together with IL-2, IL-18 activates NKT cells and T helper (Th) cells to produce the Th2-related cytokines, IL-4 and IL-13 [132]. IL-18 is known to be beneficial for the expulsion of the intestinal nematode, *Strongyloides venezuelensis* [133]. Unexpectedly, IL-18 activates Th1 cells to produce IL-13 and chemokines as well as Th1-related cytokines [134]. Based on this biological function, IL-18 can trigger the development of intrinsic atopic dermatitis and bronchial asthma, in which both IFN-γ and IL-13 play a critical role [67, 135, 136].

IL-18 has also been reported to be involved in metabolic syndrome [137]. In contrast to IL-1 [101], however, IL-18 protects against metabolic syndrome, although patients with the metabolic syndrome show increased serum levels of IL-18 [138, 139].

5.4. IL-37. In 2001, database analyses identified a new, IL-18Rα-binding human protein named IL-37 [140]. To date, its mouse orthologue has not been identified. Various cell types have been reported to produce IL-37. IL-37 is a potent inhibitor of both innate and acquired immune responses. *IL37*tg mice are resistant to LPS-induced shock and DSS colitis compared to WT mice [141, 142]. IL-37 is associated with Behçet's disease, an intractable, chronic inflammatory disease [143]. IL-37 also dampens type 2 immune responses. Indeed, intranasal administration of human IL-37 ameliorates ovalbumin-induced type 2 allergic airway inflammation [144]. Upon IL-37 engagement, IL-18Rα recruits SIGIRR/TIR8, which negatively regulates many signaling pathways, including phosphatase and tensin homolog deleted from chromosome 10 (PTEN) and signal transducers and activators of transcription- (STAT-) 3, to inhibit transforming growth factor β activated kinase- (TAK-) 1 and the transcription factors NF-κB and MAPKs and to interact with Smad3, an anti-inflammatory signaling molecule [141, 145]. The full network that is activated by IL-37-mediated signaling remains to be elucidated.

5.5. IL-36. The IL-36 subfamily includes IL-36α, IL-36β, IL-36γ, IL-36Ra, and IL-38. All were discovered by database searches on the basis of their homology with IL-1α and IL-1β [8, 146]. IL-36 cytokines are expressed in the skin under normal conditions [147]. Skin-specific *Il36α* tg mice develop psoriasis-like skin alterations; they spontaneously suffer from flaky skin within 1 week of birth, but by week 3 their skin phenotypes have disappeared [148]. Thereafter, they exhibit recurrence of skin lesions. Treatment with anti-IL-23, anti-TNF, or anti-IL-36R antibodies rescues *Il36α* tg mice from the skin alterations [149]. Furthermore, following topical application of the ligand of TLR7/TLR8, imiquimod, $Il36ra^{-/-}$ mice develop more severe psoriasis-like skin alterations than WT mice. In contrast, $Il23^{-/-}$ and $Il17a^{-/-}$ mice are resistant to imiquimod-induced psoriasis [150]. Several reports identified a homozygous missense mutation in *IL36RN* that encodes IL36Ra in patients with familial generalized pustular psoriasis and found that the mutations in *IL36RN* cause poor affinity or labile binding of IL-36Ra to IL-36R [151–153]. Thus, impaired IL-36Ra function might be responsible for the development of generalized pustular psoriasis. In addition, IL-36 agonists might be involved in sporadic psoriasis [154].

IL-38 is a recently identified IL-1 family antagonist that is reported to function similarly to IL-36Ra [155]. However, the biological roles of IL-38 remain to be elucidated.

6. The Roles of IL-1 Family Members in Liver Diseases

As mentioned, IL-1 family agonists and antagonists are involved in health and disease, and it is plausible that these molecules are involved in liver diseases. This section highlights recent advancements in the understanding of the molecular and cellular mechanisms of liver diseases associated with IL-1 family cytokines, particularly focusing on IL-1, IL-33, and IL-18.

6.1. IL-1 and IL-18 in Viral Hepatitis (Figure 4). Because the viruses that cause liver diseases in humans are species-specific, it is difficult to demonstrate the mechanism of viral hepatitis in animal models other than primates, limiting advancement in the study of viral hepatitis. Study of immunodeficient mice with transplanted human liver cells and receiving human autologous hematopoietic stem cells may shed light on this issue. Mice with human liver and immune cells developed by induced pluripotent stem cells (iPS cells) show promise of becoming a useful tool in the near future.

6.1.1. Hepatitis B Virus. It is well established that types I, II, and III IFNs directly inhibit the replication of hepatitis B virus (HBV) [156]. Recently, Watashi et al. demonstrated that IL-1 can protect against HBV infection [157]. The authors incubated hepatocytes with IL-1β, and after vigorous washing of the cells they added HBV to the cell culture. After 4 days of incubation they measured HBV replication. In this setting, IL-1β protected against HBV infection, whereas IFN-α, an HBV replication inhibitor, did not, suggesting that IL-1β inhibits the early phase of HBV infection, including attachment, entry, and nuclear import. This restriction of HBV infection is mediated by activation-induced cytidine deaminase (AID) induced by IL-1β/NF-κB signaling. Indeed, introduction of AID suppresses the permission of HBV. Thus, IL-1β can restrict HBV infection via induction of AID, although the molecular mechanism underlying the AID-mediated restriction is unknown. Intriguingly, HBeAg, a soluble protein produced by HBV, can inhibit production of IFN-γ from IL-12/IL-18-activated NK cells via downregulation of IL-18/NF-κB signaling [158]. Indeed, serum from HBeAg-positive patients induces lower IFN-γ production by IL-12/IL-18-stimulated NK cells than that from HBeAg-negative patients [158]. The liver contains abundant NK cells, which were initially named "pit cells" on the basis of their unique localization in the hepatic sinusoid [159], as well as Kupffer cells, which have the potential to produce both IL-18 and IL-12 in the same anatomical compartment. Thus, after microbial infection, robust IFN-γ is promptly produced by the intimate interaction between NK cells and Kupffer cells. HBV may evade this IFN-γ production by releasing HBeAg and can easily infect hepatocytes.

6.1.2. Hepatitis C Virus. Type I and type III IFNs are important for eradication of HCV [160]. It is well documented that serum levels of IL-18 are elevated in chronic HCV patients. IL-18 levels reflect the severity and activity of HCV infection [161]. THP-1 cells or monocyte-derived Mø produce IL-1β and IL-18 after infection with HCV [162]. This HCV-induced IL-1β/IL-18 production requires activation of the TLR7/MyD88 pathway, but not TLR3/TRIF, and of NLRP3 inflammasomes. In contrast, IFN-α production requires mitochondrial antiviral-signaling protein (MAVS), which is an essential signal adaptor for the signaling of RIG-1-like receptors (RLRs), which are sensors of viral products. Thus, upon HCV infection, distinct modes of RRP signaling participate in production of IL-1β/IL-18 and IFN-α [163, 164].

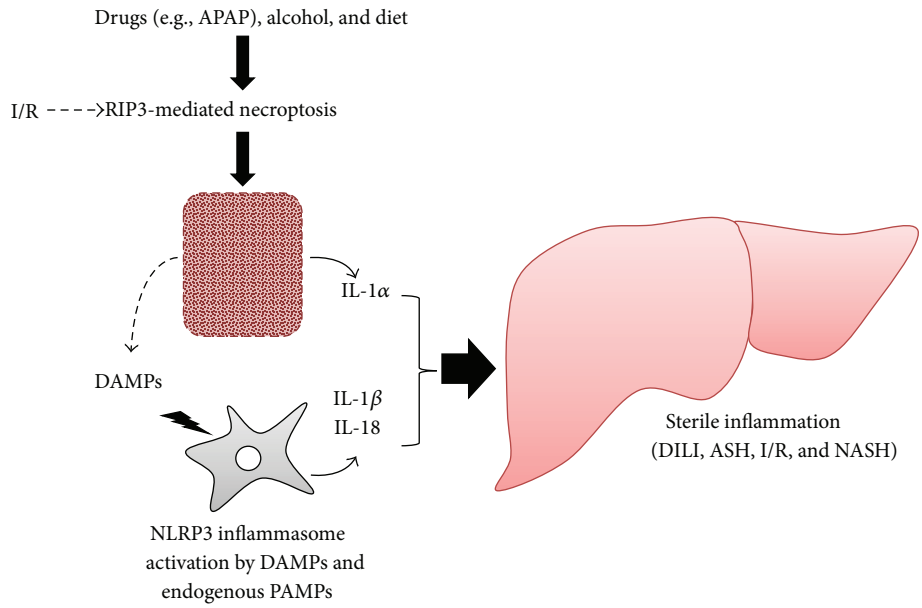

Figure 4: Mechanisms for IL-1-mediated liver diseases. Intake of acetaminophen, alcohol, or perhaps diet induces necroptosis of hepatocytes via activation of RIP3. Ischemia-reperfusion also causes RIP3-mediated hepatocyte necroptosis. Necroptotic hepatocytes release their cellular contents, including IL-1α and DAMPs. DAMPs such as saturated FFAs, which are metabolites of high-calorie diets, and endogenous PAMPs, such as LPS translocated from the gut microbiome, can activate NLRP3 inflammasomes to release IL-1β. In turn, IL-1β and IL-1α exacerbate the liver inflammation and injury. This inflammation-amplifying mechanism is known as "sterile inflammation," given the absence of exogenous microbes or their PAMP products. In contrast, IL-18 regulates NASH via maintenance of healthy intestinal microbiota. APAP: acetaminophen; ASH: alcoholic induced steatohepatitis; DAMPs: damage-associated molecular patterns; DILI: drug-induced liver injury; I/R: ischemia-reperfusion injury; NASH: nonalcoholic steatohepatitis; NLRP3: NACHT, leucine-rich repeat, and pyrin domain-containing protein-3; PAMPs: pathogen-associated molecular patterns; RIP3: receptor interacting protein kinase-3.

6.2. The Importance of Necroptosis in IL-1α-Mediated Liver Diseases (Figure 4). As described above, necroptosis is a form of regulated cell death that involves cellular membrane rupture. Necroptosis-driven liberation of the cell contents, including IL-1α, is important for the activation of sterile inflammation, which is a powerful mechanism for amplifying inflammation (described below). Hepatocyte necroptosis is involved in various liver diseases [165]. Acetaminophen (APAP) overdose is a predominant cause of acute, hepatotoxic liver failure in the USA and UK. An APAP-derived metabolite has been shown to have direct hepatocytotoxic action. Recent studies suggested that necroptotic hepatocyte death is a direct pharmaceutical effect of the APAP metabolite [166]. The liver of APAP-treated mice showed induction of RIP3, a key molecule that induces necroptosis, and RIP3 morpholin treatment protected against APAP-induced liver injury. In addition, $Rip3^{-/-}$ hepatocytes are somewhat resistant to APAP. Very recently, Li et al. demonstrated that APAP-induced liver injury is induced by necroptosis of hepatocytes [167]. They verified that the B-Raf^{v600E} inhibitor dabrafenib, which is an anticancer drug, is a potent RIP3 inhibitor as well. Dabrafenib can inhibit necroptosis of human hepatocytes incubated with APAP. Importantly, inhibition of cell death by the RIP1 inhibitor necrostatin-1 had been regarded as a hallmark of necroptosis, but this inference is made dubious by the evidence of RIP1-independent necroptosis [49]. Thus, the necrostatin-1 test is not always useful for diagnosis of necroptosis. Similarly, necrostatin-1 treatment does not improve APAP-induced hepatocyte cell death [167]. Notably, positive staining for phosphorylated MLKL, a hallmark of necroptosis, has been observed in the hepatocytes of patients with APAP-induced liver failure [92]. Necroptosis has also been demonstrated to be relevant to ethanol-induced liver injury in mice [168]. This is also the case in liver biopsy of patients with alcoholic liver disease (ALD). Thus, some drug-induced liver injuries appear to develop in a manner dependent on RIP3-mediated necroptosis.

Ischemia-reperfusion injury of the kidney or retina was also demonstrated to be caused by necroptosis [169, 170]. Similarly, ischemia-reperfusion-induced liver injury might also involve the necroptotic death of hepatocytes. A precise analysis of whether and how necroptosis is involved in ischemia-reperfusion-induced liver injury would be useful.

RIP3 is overexpressed in the livers of patients with NASH [171]. In this paper, the authors showed that WT mice were susceptible to diet-induced NASH, whereas $Rip3^{-/-}$ mice, which lack the capacity for necroptosis, were resistant. Therefore, necroptosis might underlie hepatocytic cell death during NASH. However, the endogenous factor(s) that are responsible for the initiation of RIP3-mediated necroptosis remain to be elucidated.

6.3. Sterile Inflammation Mediated by IL-1α and IL-1β (Figure 4). Necroptotic hepatocytes in NASH, drug-induced liver injury, and possibly ischemia-reperfusion-induced liver injury can trigger sterile inflammation by activation of

the NLRP3 inflammasome with liberated DAMPs and/or by IL-1α. It has been clearly demonstrated that saturated, but not unsaturated, free fatty acid (FFA), which is a host-derived metabolite, is capable of activating the NLRP3 inflammasome to produce IL-1β and that this IL-1β might lead to progression to NASH in mice with simple hepatic steatosis [172, 173]. The following scenario has been proposed. Saturated FFA can inhibit autophagy, especially mitophagy, in cells. Under normal conditions, impaired mitochondria are rapidly eliminated via mitophagy. However, saturated FFA-induced impairment of mitophagy might allow long-term retention of impaired mitochondria. Subsequently, aberrant production of reactive oxygen species (ROS) by the impaired mitochondria might continuously and robustly activate ROS-sensitive inflammasomes [172]. Miura et al. reported that the induction and exacerbation of NASH were explained by IL-1β, which is produced by the activation of TLR9/MyD88 signaling, and the NLRP3 inflammasome by DAMPs, possibly derived from necroptotic hepatocytes [174]. In fact, *Tlr9*$^{-/-}$ mice are resistant to diet-induced NASH and show accompanying reduced induction of serum elevation of IL-1β. Consistently, *Myd88*$^{-/-}$ and *Il1r1*$^{-/-}$ mice show resistance to NASH similar to *Tlr9*$^{-/-}$ mice. The authors reported that Kupffer cells were a major cellular source of IL-1β, which accelerated cytocidal lipid deposits in hepatocytes, induced cell death, and simultaneously activated hepatic stellate cells to produce profibrotic proteins. *Tlr2*$^{-/-}$ mice evade NASH via impaired induction of IL-1α and IL-1β, even when fed a NASH-prone diet [175]. The importance of hematopoietic cells, including Kupffer cells, for the development of NASH via activation of TLR2 has been demonstrated in chimeric mice. Indeed, *Tlr2*$^{-/-}$ Kupffer cells cannot produce IL-1α or IL-1β after stimulation with saturated free fatty acid palmitate together with TLR2 agonists. Sterile inflammation is also activated by hepatocyte-derived IL-1β. Hepatocytes prepared from mice suffering from NASH express enhanced levels of NLRP3 inflammasome components and have the capacity to release IL-1β in response to palmitate, a saturated fatty acid, in combination with LPS [176]. Consistently, treatment with pan-caspase inhibitors such as Emricasan and VX-166 can suppress liver injury, inflammation, and fibrosis in mice fed a NASH-prone, high-fat diet [177]. In contrast to IL-1, IL-18 is reported to be beneficial for preventing NASH. IL-18 secreted following activation of the NLRP3 inflammasome negatively regulates the development of murine NASH through maintenance of a healthy gut microbiota [178, 179]. This may imply that the harmful effects of IL-1β might overcome the beneficial action of endogenous IL-18.

Sterile inflammation is involved in the exacerbation of APAP-induced acute liver failure [180]. Imaeda et al. showed that treatment with a TLR9 antagonist and deletion of *Tlr9* decreased the severity of APAP-induced liver failure with impaired induction of *proIl1β*. NLRP3 inflammasome-deficient mice, such as *Caspase1*$^{-/-}$, *Asc*$^{-/-}$, and *Nlrp3*$^{-/-}$ mice, showed comparably reduced induction of liver failure to *Tlr9*$^{-/-}$ mice. This suggests that DAMPs, including a TLR9 agonist, which are liberated from APAP-induced necroptotic hepatocytes, might activate the TLR9 pathway and the NLRP3 inflammasome.

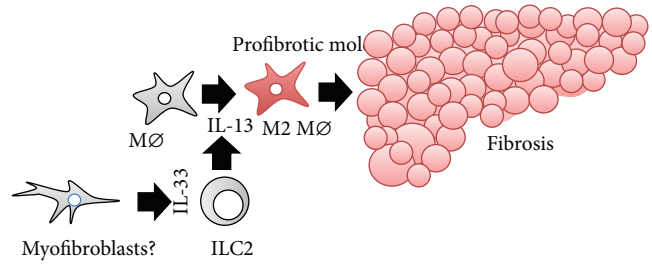

Figure 5: IL-33–ILC2–IL-13 axis-mediated liver fibrosis. The IL-33–ILC2–IL-13 axis is important for physiological wound healing. The same process is involved in the development of liver fibrosis. IL-33 activates ILC2 to release IL-13. In response to IL-13, liver macrophages undergo cell conversion into M2 Mø, which begin to produce profibrotic proteins, eventually leading to the development of liver fibrosis. Myofibroblasts are regarded as a major cellular source of IL-33 in cases of liver fibrosis. ILC2: group 2 innate lymphoid cells; Mø: macrophages.

The TLR9-mediated signal pathway has also been demonstrated to participate in the activation of sterile liver inflammation induced by ischemia and reperfusion [181]. A scenario similar to that of NASH and APAP-induced acute liver failure underlies the progression of this disease as well. An excellent review has been published that is helpful in understanding the other mechanisms underlying sterile inflammation in hepatic ischemia-reperfusion-induced liver injuries [182].

In the above-mentioned liver diseases, sterile inflammation may accelerate disease progress, such as lethal liver failure and fibrosis. However, the pathway that links TLR/MyD88-mediated pro-IL-1β production to activation of the NLRP3 inflammasome remains to be elucidated.

6.4. IL-33 in Liver Fibrosis (Figure 5). Until recently, liver cirrhosis was regarded as the irreversible end stage of chronic liver disease. However, recent studies have revealed that liver fibrosis is reversible. Many cell types, such as Mø, pro-fibrotic myofibroblasts, which are mainly transdifferentiated from stellate cells, and innate and acquired immune lymphocytes, participate in the development of liver fibrosis. The mechanisms underlying the initiation of liver fibrosis have been gradually unraveled by intensive efforts using animal models and clinical data. For example, pro-fibrotic and pro-resolving Mø have been shown to contribute to the progression and regression of liver fibrosis, respectively [183, 184]. A comprehensive discussion of the cellular and molecular mechanisms of liver fibrosis discovered to date is out of the scope of this review. For further information, the reader may consult recent expert reviews [185–188]. Herein, we will focus on the roles of IL-33 in liver fibrosis.

The importance of IL-33 for liver fibrosis was demonstrated by analyses of several mouse models of liver fibrosis. Multiple injections of carbon tetrahydrochloride (CCl_4) or thioacetamide, infection with the helminth *Schistosoma mansoni*, or bile duct ligation induces liver fibrosis in WT mice, accompanied by elevation of serum IL-33 levels [189, 190].

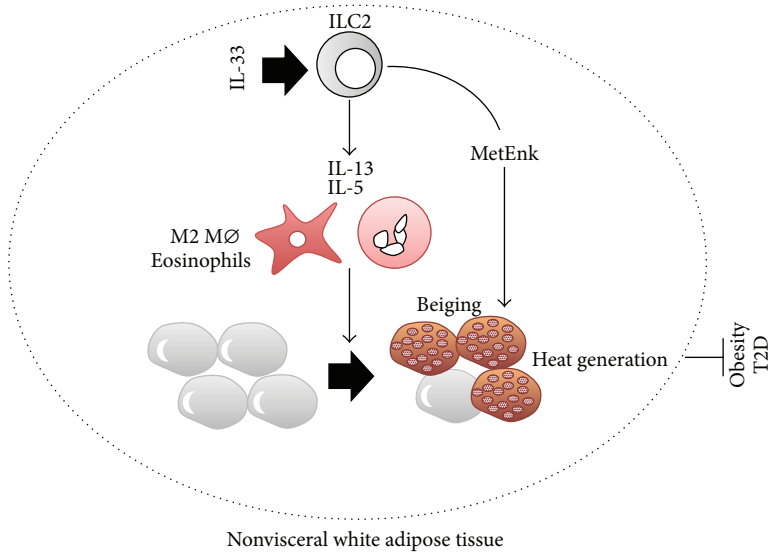

FIGURE 6: IL-33–ILC2 beiges white adipose tissue to protect against obesity via heat generation and energy expenditure. IL-33–ILC2–IL-13/IL-5 is also critical for transformation of white adipocytes into thermogenic beige adipocytes. In response to IL-33, ILC2 in white adipose tissue produces methionine-enkephalin peptide (MetEnk) as well as pro-allergic IL-13 and IL-5. IL-13 and IL-5 induce recruitment and activation of eosinophils and cell conversion of Mø into M2 Mø. MetEnk, combined with M2 Mø and eosinophils, contributes to beiging of white adipocytes, which leads to protection against obesity and the related disease T2D via heat generation and energy consumption. ILC2: group 2 innate lymphoid cells; MetEnk: methionine-enkephalin peptide; Mø: macrophages; T2D: type 2 diabetes mellitus.

IL-33-expressing cells are accumulated in the fibrotic liver, particularly in αSMA$^+$ cells [189, 190]. *Il33*$^{-/-}$ mice are resistant to induction of liver fibrosis by these treatments. Intriguingly, multiple introduction of *Il33* in the liver of mice induces liver fibrosis. *St2*$^{-/-}$, *Il13*$^{-/-}$, and *Il4rα*$^{-/-}$ mice are resistant to this treatment, strongly suggesting that the IL-33–IL-13 axis is important for the development of IL-33-induced liver fibrosis. *Rag1*$^{-/-}$ mice lack both T- and B-lymphocytes but normally possess group 2 ILC (ILC2), which are capable of responding to IL-33 by prompt and robust production of type 2 cytokines including IL-13 [191–193]. Intriguingly, in vivo depletion of ILC2 prevents *Rag1*$^{-/-}$ mice from developing IL-33-induced liver fibrosis. Thus, the IL-33–ICL2–IL-13 axis is critical for development of some types of liver fibrosis. Consistent with data from mice, serum levels of IL-33 are elevated in patients with liver cirrhosis [189]. In addition, *IL33* and *ST2* expression levels in cirrhotic livers positively correlate with disease severity.

The IL-33–ILC2–IL-13 axis has also been demonstrated to be involved in biliary repair and possibly in carcinogenesis in the biliary tract. Biliary atresia is a bile duct disease that is common in childhood. A subset of patients with biliary atresia display an increase in pro-Th2 cytokines. Recently, it was demonstrated that the IL-33–ILC2–IL-13 axis plays a pivotal role in the development of biliary atresia-associated biliary repair and carcinogenesis [194, 195]. Serum IL-33 levels are elevated in patients with biliary atresia. Expression levels of *Il33* mRNA are correlated with numbers of replicating cholangiocytes in an experimentally induced mouse model of biliary atresia. Biliary atresia is induced by infection of neonate mice with rhesus rotavirus type A (RRP). Treatment with anti-ST2 antibodies inhibits cholangiocyte replication in RRP-challenged mice. Conversely, extrinsic IL-33 treatment induces profound replication of extrahepatic cholangiocytes. ILC2-deficient *Rag2*$^{-/-}\gamma c^{-/-}$ mice, as well as *Il13*$^{-/-}$ mice, fail to show cholangiocyte growth upon IL-33 treatment. Thus, the IL-33–ILC2–IL-13 axis is important for bile duct repair. Similarly, it was reported that IL-33 facilitated oncogene-induced cholangiocarcinoma in mice [196].

6.5. IL-33 in Obesity, a Precondition for NASH (Figure 6). IL-33/ST2 signaling protects against obesity-associated inflammation in visceral adipose tissue (VAT) and maintains insulin sensitivity. It is well established that chronic, low-grade inflammation involving Mø in VAT links obesity to metabolic diseases [197, 198]. In parallel, regulatory T cells (Treg) are functionally and numerically reduced in the VAT of obese mice, which might partly explain the dysregulated inflammation observed in the VAT of obese mice [198, 199]. Multiple injections of IL-33 improved insulin sensitivity and glucose tolerance in *ob/ob* mice, concomitant with cellular conversion into anti-inflammatory M2 Mø in their VAT [200]. Consistent with this, *St2*$^{-/-}$ mice were highly susceptible to HFD-induced impairment of glucose tolerance. IL-33 treatment could reverse the deficit of ST2$^+$ Treg in diet-induced obese mice [201]. Furthermore, accumulation of Treg in the VAT of lean, aged mice was reported to be dependent on IL-33 in VAT. Indeed, the proportion of Treg in CD4$^+$ T cells in the VAT of *St2*$^{-/-}$ mice is reduced compared to that of WT mice. Conversely, IL-33 treatment increases the proportion of Treg [202, 203]. In addition, ILC2 is required for the accumulation of eosinophils and cell conversion to M2 Mø in the VAT via production of IL-5 and IL-13 [204].

White adipose tissue (WAT) stores energy. Unlike WAT, brown adipose tissue (BAT) contributes to energy consumption via thermogenesis and is regarded as a possible target organ for treatment of obesity. Although the VAT is lost by adulthood in humans, adult humans harbor beige adipocytes that express a thermogenic protein, uncoupling protein 1 (UCP1), in subcutaneous WAT (SAT) in cold conditions. Thus, beige fat shows promise as a therapeutic target for the treatment of obesity. It has been demonstrated that both IL-4 production by eosinophils and the IL-4 responsiveness of M2 macrophages are equally required for the development of thermogenic beige fat that expresses UCP1 [205]. Brestoff et al. and Lee et al. independently demonstrated that, in this setting, IL-33 activation of ILC2 is important for beiging [206, 207]. In obese humans, the SAT harbors lower numbers of ILC2 than in nonobese humans. This is also true for diet-induced obese WT mice. *Il33*$^{-/-}$ mice harbor small numbers of ILC2 in the SAT and show higher gain of body and fat mass than WT mice, even when fed a normal, low-fat diet. The SAT of *Il33*$^{-/-}$ mice is characterized by the presence of few beige adipocytes and by much lower expression of *Ucp1*, whereas in WT mice the SAT as well as the VAT harbors substantial numbers of beige adipocytes. IL-33 administration induces an increase in ICL2 in the SAT and enhances calorie expenditure in WT mice. Transfer of ILC2 prepared from IL-33-treated WT mouse SAT upregulates *Ucp1* expression in the SAT and increases energy expenditure in WT mice. Transfer of ILC2 induces SAT *Ucp1* expression in ILC2-null *Rag2*$^{-/-}\gamma c^{-/-}$ mice as well. Levels of proprotein convertase subtilisin/kexin type 1 (*Pcsk1*), an endopeptidase involved in processing prohormones into active form, were shown to increase in ILC2 [206]. Following administration of methionine-enkephalin peptide, which is a substrate of PCSK1, WT mice showed increased numbers of UCP1^{+} beige adipocytes and increased energy consumption. This may imply that IL-33 induces beiging in the WAT, at least partly via production of methionine-enkephalin peptide. Taken together, these results show that endogenous IL-33 sustains beige adipocytes in the SAT by activating ILC2, presumably in collaboration with M2 Mø and eosinophils, which eventually contributes to metabolic homeostasis by tuning calorie expenditure. Endogenous opioid-like peptides, including the methionine-enkephalin produced by IL-33-activated ILC2, show promise as a potent therapeutic agent for obesity and obesity-associated diseases.

7. Closing Remarks

Recent studies have revealed the importance of ILC2 in health and disease. ILC2 plays a critical role in the development of liver fibrosis and in protection against obesity. However, these conclusions were drawn from studies using mouse models, and although the definition of mouse ICL2 is well established, definitions of human ILC2 differ among research groups [208, 209]. A uniform definition of ICL2 will be required to make progress in the translation of the findings on murine ICL2 to human diseases.

NASH is characterized by liver fibrosis. Overexpression of IL-33 can induce liver fibrosis in mice. However, it remains to be elucidated whether endogenous IL-33 is involved in diet-induced NASH fibrosis. Many investigators have reported the elevation of IL-33 or IL-33 mRNA levels in the liver or in circulation. However, it remains unclear whether the IL-33 elevation is a result of or relevant to liver fibrosis. To address this, we must examine whether *Il33*$^{-/-}$ and *St2*$^{-/-}$ mice are resistant to liver fibrosis. In addition, the cellular source of IL-33 in these diseases remains unknown. Liver parenchymal cells have been shown to be a major cellular source of IL-33 in the liver under normal conditions. During the progression of NASH, α-SMA-expressing myofibroblasts become the predominant IL-33-producing cell type. However, this does not indicate whether myofibroblast-derived IL-33 initiates and/or exacerbates liver fibrosis, because we do not know whether or how the myofibroblasts undergo the necroptotic or pyroptotic cell death that allows liberation of intracellular IL-33. It is well documented that hepatocytes become necroptotic under NASH conditions (Figure 4), suggesting that hepatocytes might be a major continuous cellular source of IL-33 during NASH.

Obesity is an important problem in both developed and developing countries. Obesity plays a central role in the development of various life-threatening diseases, including cardiovascular diseases and cancers. However, the causes of the rapid, global increase in obesity remain to be elucidated. Recent studies clearly demonstrate that artificial sweeteners and dietary emulsifiers, which are a common part of the daily diet, are responsible for the development of metabolic syndrome in mice [210, 211]. Eating a diet without these agents might be important for health. In addition, several active compounds in a "healthy diet" have been identified. The biological compounds thymoquinone, curcumine, and resveratrol can be purified from *Nigella sativa* (black seed), *Zingiberaceae* spp. (e.g., ginger and turmeric), and grape skins, respectively [212–214]. These compounds are beneficial for human health. Many traditional foods have been shown to promote health. The identification of pro-health compounds will contribute to human health and longevity.

Abbreviations

AID: Activation-induced cytidine deaminase
AIM2: Absent in melanoma 2
ALD: Alcoholic liver disease
APAP: Acetaminophen
ASC: Apoptosis-associated speck-like protein containing a carboxy-terminal CARD
BAT: Brown adipose tissue
B6: C57BL/6
CARD: Caspase recruitment domain
CCl_4: Carbon tetrachloride
COPD: Chronic obstructive pulmonary disease
DAMPs: Damage-associated molecular patterns
DSS: Dextran sulfate sodium
FFA: Free fatty acid
HBV: Hepatitis B virus
HFD: High-fat diet
ICE: IL-1β-converting enzyme
IFN: Interferon
IGIF: Interferon inducing factor

IL: Interleukin
IL-18BP: IL-18-binding protein
IL-1Ra: IL-1R antagonist
ILC: Innate lymphoid cells
ILC2: Group 2 ILC
JNK: c-Jun-N-terminal kinase
MAPK: Mitogen-activated protein kinase
LPS: Lipopolysaccharide
LRR: Leucine-rich repeat
MAVS: Mitochondrial antiviral-signaling protein
MLKL: Mixed lineage kinase like
Mø: Macrophages
NF-κB: Nuclear factor-κB
NLR: Nod-like receptor
NLRC: NACHT, LRR, and CARD-containing protein
NLRP: NACHT, LRR, and PYD-containing protein
Pro: Precursor
PAMPs: Pathogen-associated molecular patterns
PYD: Pyrin domain
PBMC: Peripheral blood mononuclear cells
PCSK1: Proprotein convertase subtilisin/kexin type 1
Penk: Proenkephalin A
PR3: Proteinase 3
PRR: Pattern recognition receptor
PSTPIP2: Proline serine-threonine phosphatase interacting protein 2
R: Receptor
RIP: Receptor interacting protein kinase
RLR: RIGI-like receptor
RPP: Rhesus rotavirus type A
SAT: Subcutaneous WAT
SIGIRR: Single immunoglobulin IL-1-related receptor
Th: T helper
TIR: TLR/IL-1R
TLR: Toll-like receptor
TRIF: TIR-domain-containing adapter-inducing interferon-β
TRAIL: TNF-related apoptosis-inducing ligand
Treg: Regulatory T cells
UCP1: Uncoupling protein 1
VAT: Visceral adipose tissue
WAT: White adipose tissue
WT: Wild-type.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Comprehensive Screening of Gene Function and Networks by DNA Microarray Analysis in Japanese Patients with Idiopathic Portal Hypertension

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The functions of genes involved in idiopathic portal hypertension (IPH) remain unidentified. The present study was undertaken to identify the functions of genes expressed in blood samples from patients with IPH through comprehensive analysis of gene expression using DNA microarrays. The data were compared with data from healthy individuals to explore the functions of genes showing increased or decreased expression in patients with IPH. In cluster analysis, no dominant probe group was shown to differ between patients with IPH and healthy controls. In functional annotation analysis using the Database for Annotation Visualization and Integrated Discovery tool, clusters showing dysfunction in patients with IPH involved gene terms related to the immune system. Analysis using network-based pathways revealed decreased expression of adenosine deaminase, ectonucleoside triphosphate diphosphohydrolase 4, ATP-binding cassette, subfamily C, member 1, transforming growth factor-β, and prostaglandin E receptor 2; increased expression of cytochrome P450, family 4, subfamily F, polypeptide 3, and glutathione peroxidase 3; and abnormalities in the immune system, nucleic acid metabolism, arachidonic acid/leukotriene pathways, and biological processes. These results suggested that IPH involved compromised function of immunocompetent cells and that such dysfunction may be associated with abnormalities in nucleic acid metabolism and arachidonic acid/leukotriene-related synthesis/metabolism.

1. Introduction

Idiopathic portal hypertension (IPH) is characterized by portal hypertension due to obstruction or stenosis of the intrahepatic peripheral portal branches [1, 2]. Patients with IPH present with splenomegaly, anemia, and portal hypertension and are free of obstruction of the extrahepatic portal vein or hepatic vein, hematological disease, parasitic disease, granulomatous liver disease, or congenital hepatic fibrosis. IPH does not usually lead to liver cirrhosis [3–5]. IPH resembles a disease called noncirrhotic portal fibrosis in India or hepatoportal sclerosis/noncirrhotic portal hypertension in Western countries [5, 6]. The etiology of IPH remains unclear. To date, researchers have suggested that IPH may be attributed to intrahepatic peripheral portal vein thrombosis, splenic factors, abnormal autoimmunity, and related factors [7–11].

Our research team has shown that the connective tissue growth factor (CTGF) gene is expressed specifically in patients with IPH. Additionally, we previously reported that patients with IPH exhibit high levels of CTGF in the blood and overexpression of CTGF mRNA in liver tissues [12]. However, in rats with transient overexpression of CTGF induced by recombinant adenovirus, no changes were observed in liver tissues, despite the expression of fibrosis-related genes [13]. Thus, we aimed to examine the specific expression of other genes in patients with IPH.

Recently, comprehensive gene expression analysis with DNA microarrays has been used in a variety of diseases, such as cancer and immunological disease, and has been employed for pathophysiological analysis of these diseases [14–17]. Such analysis enables detection of genes or biological pathways showing significant increases or decreases in expression in the target specimens; this is useful for estimation of the features of the target disease. To the best of our knowledge, no studies have reported comprehensive analysis of gene expression using DNA microarrays in patients with IPH. Thus, in this study, we aimed to perform comprehensive gene expression analysis using DNA microarrays in blood samples from patients with IPH to identify genes showing significant changes in expression. Moreover, we performed biological pathway analysis using the data obtained from DNA microarrays.

2. Materials and Methods

2.1. Patients. Analysis was conducted using blood samples from four patients with IPH satisfying the IPH diagnostic criteria (prepared under the Intractable Hepatobiliary Disease Program of the Ministry of Health, Labour and Welfare, Japan) and four healthy volunteers as controls [18, 19]. The diagnosis of IPH was based on the following criteria: (1) general findings: one or more cytopenia, normal function or mild dysfunction of the liver, or collateral circulation such as upper gastrointestinal varix, (2) imaging analysis: splenomegaly, atrophy of peripheral parenchyma and enlargement of central parenchyma in the liver, irregular hepatic surface, increase in splenic venous flow, abnormality of intrahepatic peripheral portal branches, mutual binding of hepatic veins, or increase of hepatic venous pressure, (3) pathological findings: collapse or stenosis of peripheral branches of intrahepatic portal veins, fibrosis of splenic sinus, or Gamma-Gandy bodies formation in splenic trabeculae, (4) exclusion of other diseases causing portal hypertension such as cirrhosis, extrahepatic occlusion, Budd-Chiari syndrome, blood disease, parasitic disease, granulomatous liver disease, congenital liver fibrosis, chronic viral hepatitis, and primary biliary cirrhosis, among others. These blood samples had been stored without identifying information at the Sample Storage Center of Kyushu University installed by the Portal Hemodynamics Abnormalities Study Group within the framework of the Intractable Hepatobiliary Disease Program of the Ministry of Health, Labour and Welfare, Japan. The samples were randomly chosen for use in this study. This study was conducted in compliance with the ethical principles of the Declaration of Helsinki (1964) and was approved by the ethics committees of Osaka City University and Kyushu University. Informed consent was obtained from all participants.

2.2. Sample Adjustment and Microarray Data. All blood samples were stored at −80°C. Total RNA was extracted by ISOGEN-LS (NIPPON GENE Co., Ltd., Tokyo, Japan). RNA levels were measured with a spectrophotometer SmartSpec 3000 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the quality of each RNA sample was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Three samples from patients with IPH and three samples from healthy volunteers (with RNA integrity numbers of ≥ 6) were subjected to microarray analysis. The WT-Ovation Pico RNA Amplification System (NuGEN Technologies, Inc., San Carlos, CA, USA) was employed for cDNA synthesis, conversion into sense chain, fragmentation, and biotin labeling, and a Human Gene ST 1.0 Array (Affymetrix, Inc., Santa Clara, CA, USA) was then used for hybridization of the sense chain cDNA. Array scanning was conducted with a GeneChip 3000 Scanner (Affymetrix, Inc.) to yield image data. The array image data from each sample were converted into files in a format enabling numerical extraction using GeneChip Operating Software (GCOS), a standard data analysis system included with the GeneChip system. Microarray data in this paper have been submitted to National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible through the GEO series accession number GSE69601.

2.3. Cluster Analysis. The signal intensity values for data from the three patients with IPH and the three healthy controls were divided by the mean signal intensity values for all six samples, and the quotient was converted into a logarithm. The calculated relative signal intensity values were presented on a heat map and subjected to cluster analysis using Cluster 3.0 software with the following parameters: *k*-means; number of clusters, 12; correlation (uncentered) selected for similarity metric; and number of runs, 1000. The results were visualized with Java TreeView software [20].

2.4. Functional Annotation Analysis for Classification of Gene Function. To extract information on the functions of genes identified as differentially expressing using the GeneChip numerical data, the Database for Annotation Visualization and Integrated Discovery (DAVID) tool was used; this database includes the Gene Ontology Database (http://geneontology.org/) and the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) [21–23]. For each gene, the ratio between the mean values of the IPH group and the healthy control group, that is, the expression ratio (fold-change), and p value obtained using t-test were calculated. We used the volcano plot method, which reflects both biological significance and statistical significance. By changing the cut-off value of the expression ratio and p value to include gene function terms that may be important, four gene groups were defined, including three groups showing a significant reduction in expression in the presence of IPH (Group A: healthy control/IPH > 2.0, $p < 0.05$; Group B: healthy control/IPH > 1.5, $p < 0.05$; Group C: healthy control/IPH > 1.5, $p < 0.1$) and one group showing significant elevation of expression in the presence of IPH (Group D: IPH/healthy control > 1.5, $p < 0.1$). For each group, functional annotation chart analysis and functional annotation clustering analysis were performed.

2.5. Biological Interpretation Using Gene Ontology and Network-Based Pathway Analysis. Gene network analysis

Table 1: Number of charts and clusters extracted by functional annotation analysis.

	Gene expression ratio	Number of charts	Number of clusters
Group A	Control/IPH > 2.0, $p < 0.05$	28	17
Group B	Control/IPH > 1.5, $p < 0.05$	124	45
Group C	Control/IPH > 1.5, $p < 0.1$	167	89
Group D	IPH/Control > 1.5, $p < 0.1$	3	14

was performed using Ingenuity iReport (Ingenuity Systems, http://www.ingenuity.com/, Redwood City, CA, USA). This analysis enabled us to identify differentially expressed genes and molecular interactions for the target disease. On the basis of iReport data, differentially expressed genes were focused using t-tests and fold changes, followed by biological interpretation with Ingenuity ontology and canonical pathways. For each pathway, biological process, and disease classification, categories were arranged in the order of involvement rate ranking. Furthermore, relevant genes and networks were also explored by Ingenuity Pathways Analysis (IPA; Ingenuity Systems), with reference to the top 10 significant pathways selected by IPA. Biological interpretation was made from these results.

3. Results

3.1. Cluster Analysis. Figure 1 represents the results of cluster analysis. Samples from all three healthy controls showed similar trends (increases or decreases) in gene expression, while samples from the three patients with IPH showed differing trends. No dominant probe group showing a clear difference between the IPH group and the healthy control group was obtained.

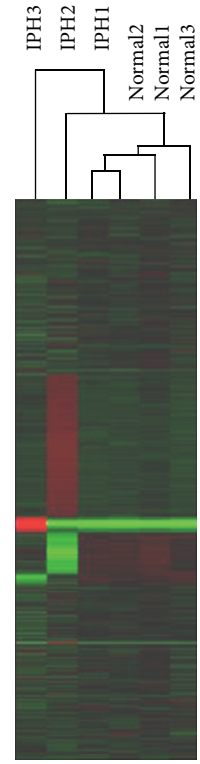


Figure 1: Results of cluster analysis of gene expression data. The dark purple to red regions indicate relative increases in expression, while the dark green to yellow-green regions indicate relative decreases in expression. There were no dominant increases/decreases in gene expression in the IPH group as compared to the healthy control group.

3.2. Functional Annotation Analysis of Differentially Expressed Genes. Table 1 shows the number of charts extracted with the functional annotation chart and the number of clusters extracted by functional annotation clustering. The number of charts was smallest (three) in Group D. Among all groups, clusters for Group C (exhibiting the highest enrichment score (3.37)) contained many gene terms related to the immune system, including "lymphocyte activation" and "leukocyte activation" (Figure 2(a)). This finding suggested that immunological abnormalities were involved in IPH. In Group D, the enrichment score was low (0.94), and no gene function terms were found to be specifically increased in the presence of IPH (Figure 2(b)).

3.3. Biological Interpretation Using Network-Based Pathway Analysis. Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/349215 lists all genes selected on the basis of the iReport. For each pathway, biological process, and disease classification, we extracted categories in the top 25 positions of the involvement rate ranking (Tables 2–4). The highest ranked target in pathway classification was reduced adenosine deaminase (ADA) expression. Additionally, the target at rank 21 in the pathway classification was abnormal purine metabolism. Targets 8 and 15 in pathway classification and targets 1, 2, and 11 in biological process classification were associated with abnormalities in the synthesis/metabolism of arachidonic acid- (AA-) prostaglandin- (PG-) leukotriene (LT). Expression levels of cytochrome P450, family 4, subfamily F, polypeptide 3 (CYP4F3), and glutathione peroxidase 3 (GPX3), which are involved in leukotriene B4 (LTB4) metabolism, were increased, while the expression levels of the PG E receptor 2 (PTGER2) were reduced. Furthermore, target 22 in pathway classification and target 13 in disease classification were associated with abnormal endothelin (ET) signals. The expression of the ET receptor type A (EDNRA) was reduced, while the expression of natriuretic peptide receptor 3 (NPR3) was increased. Additionally, the expression levels of cluster of differentiation 44 (CD44) and transforming growth factor (TGF)-β were reduced. Figure 3(a) shows combination of

89 Cluster(s) Download File

Annotation Cluster 1	Enrichment Score: 3.37	G		Count	p_Value	Benjamini
GOTERM_BP_FAT	lymphocyte activation	RT		19	$2.4E-7$	$4.3E-4$
GOTERM_BP_FAT	leukocyte activation	RT		19	$4.1E-6$	$3.7E-3$
GOTERM_BP_FAT	cell activation	RT		20	$1.2E-5$	$7.1E-3$
GOTERM_BP_FAT	T cell activation	RT		10	$1.5E-3$	$2.5E-1$
GOTERM_BP_FAT	lymphocyte differentiation	RT		9	$1.6E-3$	$2.4E-1$
GOTERM_BP_FAT	leukocyte differentiation	RT		10	$1.9E-3$	$2.5E-1$
GOTERM_BP_FAT	T cell differentiation	RT		7	$2.5E-3$	$2.6E-1$
GOTERM_BP_FAT	hemopoietic or lymphoid organ development	RT		13	$9.7E-3$	$4.5E-1$
GOTERM_BP_FAT	hemopoiesis	RT		12	$1.2E-2$	$4.6E-1$
GOTERM_BP_FAT	immune system development	RT		13	$1.5E-2$	$4.6E-1$

(a)

14 Cluster(s) Download File

Annotation Cluster 1	Enrichment Score: 0.94	G		Count	p_Value	Benjamini
GOTERM_CC_FAT	actin cytoskeleton	RT		4	$5.5E-2$	$1.0E0$
GOTERM_MF_FAT	actin binding	RT		4	$1.1E-1$	$1.0E0$
GOTERM_MF_FAT	cytoskeletal protein binding	RT		4	$2.6E-1$	$1.0E0$
Annotation Cluster 2	**Enrichment Score: 0.78**	**G**		**Count**	**p_Value**	**Benjamini**
GOTERM_ CC_FAT	non-membrane-bounded organelle	RT		13	$1.1E-1$	$1.0E0$
GOTERM_CC_FAT	intracellular non-membrane-bounded organelle	RT		13	$1.1E-1$	$1.0E0$
GOTERM_CC_FAT	cytoskeleton	RT		8	$1.5E-1$	$1.0E0$
GOTERM_MF_FAT	structural molecule activity	RT		5	$1.9E-1$	$1.0E0$
GOTERM_CC_FAT	cytoskeletal part	RT		5	$3.7E-1$	$1.0E0$

(b)

Figure 2: The expression ratio (ratio of the mean in the IPH group to the mean in the healthy control group) was calculated for each gene. Results of functional annotation clustering analysis are given for (a) the group showing significantly reduced expression among patients with IPH (Group C: healthy control/IPH > 1.5, $p < 0.1$) and (b) the group showing significantly increased expression among patients with IPH (Group D: IPH/healthy control > 1.5, $p < 0.1$). (a) Group C had the highest enrichment score (3.37), and its cluster included many gene terms related to the immune system, such as "lymphocyte activation" and "leukocyte activation." (b) In Group D, the enrichment score was low (0.94), and no gene function terms were found to be specifically increased in the presence of IPH.

the first pathway with the second pathway, and Figure 3(b) shows combination of the first pathway with the third and fifth pathways; each of these plots was prepared with reference to the top 10 significant pathways selected by IPA. Reduced expression of the ectonucleoside triphosphate diphosphohydrolase 4 (ENTPD4), ATP-binding cassette, subfamily C, member 1 (ABCC1), ADA, and TGF-β and increased expression of CYP4F3 were noted. Thus, the results from the iReport were also observed within the IPA network.

4. Discussion

IPH is a rare disease. In Japan, IPH was officially added to the list of specific intractable diseases covered by the medical expense subsidy program in January of 2015. IPH has been reported to be associated with autoimmune abnormalities [10, 11, 24]; however, the exact pathophysiology of IPH remains unclear. In this study, we attempted, for the first time, to explore the features of IPH through comprehensive gene analysis with DNA microarrays.

The results of cluster analysis showed that there were no dominant increases/decreases in gene expression in the IPH group as compared to the healthy control group. IPH has been conventionally viewed as a syndrome observed in individuals free of any other disease that can elevate portal pressure. The diagnosis of IPH generally involves ruling out other potential diagnoses, and the pathophysiological features of IPH are not uniform. Thus, these characteristics of IPH may explain the above-mentioned results.

In the analysis of functional annotation charts, many genes showed significantly reduced expression in the IPH group, but only three charts in the IPH group showed significantly increased expression. In functional annotation clustering analysis, the enrichment score of the Group C cluster was the highest (3.37), and this cluster contained many gene terms related to the immune system. Coupled

Table 2: Top 25 canonical pathways associated with IPH in Ingenuity iReport.

Number	Pathways	DEGs	p value	Genes
1	Primary immunodeficiency signaling	4	0.000120085	ADA, CD79A, IGHD, IGLL1/IGLL5
2	Altered T-cell and B-cell signaling in rheumatoid arthritis	4	0.000772903	CD28, SLAMF1, TGFB1, CD79A
3	T-helper cell differentiation	3	0.004440048	CD28, TGFB1, TNFRSF11B
4	Cyclins and cell cycle regulation	3	0.006251755	TGFB1, CDK7, E2F5
5	B-cell development	2	0.010284999	CD79A, IGHD
6	Leukocyte extravasation signaling	4	0.013183985	CXCR4, CD44, MLLT4, MMP12
7	Atherosclerosis signaling	3	0.019146088	TGFB1, CXCR4, PLA2G10
8	Arachidonic acid metabolism	3	0.021798453	GPX3, PLA2G10, CYP4F3
9	Colorectal cancer metastasis signaling	4	0.027547337	RHOQ, TGFB1, PTGER2, MMP12
10	Aryl hydrocarbon receptor signaling	3	0.028201448	TGFB1, NFIA, GSTA5
11	Hepatic fibrosis/hepatic stellate cell activation	3	0.029806364	TGFB1, EDNRA, TNFRSF11B
12	Glioma invasiveness signaling	2	0.030646264	RHOQ, CD44
13	Glutathione metabolism	2	0.031643803	GPX3, GSTA5
14	Cell cycle: G1/S checkpoint regulation	2	0.03367696	TGFB1, E2F5
15	Eicosanoid signaling	2	0.03367696	PLA2G10, PTGER2
16	Induction of apoptosis by HIV1	2	0.03367696	CXCR4, TNFRSF11B
17	Hypoxia signaling in the cardiovascular system	2	0.037891658	UBE2D2, HIF1AN
18	Tight junction signaling	3	0.038540606	TGFB1, MLLT4, TNFRSF11B
19	Germ cell-sertoli cell junction signaling	3	0.039164578	RHOQ, TGFB1, MLLT4
20	Mitotic roles of Polo-like kinase	2	0.040070987	TGFB1, PTTG1
21	Purine metabolism	4	0.043235613	ENTPD4, ABCC1, ADA, SEPT1
22	Endothelin-1 signaling	3	0.046366635	PLA2G10, EDNRA, PTGER2
23	Regulation of IL-2 expression in activated and anergic T Lymphocytes	2	0.05532422	CD28, TGFB1
24	Chronic myeloid leukemia signaling	2	0.073630475	TGFB1, E2F5
25	HMGB1 signaling	2	0.075004378	RHOQ, TNFRSF11B

Underlined genes indicate increases in expression. Not underlined genes indicate decreases in expression.
DEGs: differentially expressed genes.

with the observation that some particular trends are found when the enrichment score rises to 2.0 or higher, our data suggested that immunological abnormalities are involved in the pathophysiology of IPH. We also found that the enrichment score in Group D was relatively low (0.94), with no gene function terms showing increased expression in the presence of IPH.

We attempted further clarification of the IPH pathophysiology through network analysis. First, canonical pathway analysis was conducted using the Ingenuity iReport. In pathway classification, reduced expression of ADA was noted. Severe immunodeficiency has been shown to be induced by accumulation of hazardous adenosine metabolites resulting from ADA defects [25, 26]. Thus, reduced expression of ADA may cause induction of autoimmune disease from immunodeficiency, resulting in attack of specific organs. This theory is consistent with the view that immune system abnormalities, particularly suppressor T-cell dysfunction and accompanying autoimmunity, are candidate factors responsible for IPH [24, 27, 28]. ADA dysfunction has also been shown to affect liver differentiation and function [29–31]. It is likely that reduced ADA activity serves as a core factor, and combination of ADA with autoimmunity-dependent injury and direct liver injury leads to manifestation of IPH. In this analysis, abnormal purine metabolism was selected. Based on this connection, ADA deficiency induces accumulation of intermediate metabolites due to abnormal nucleic acid metabolism, thereby exerting cytotoxic effects and disrupting lymphocyte differentiation, leading to onset of severe immunodeficiency [32]. Similar to the effects of ADA deficiency, patients with IPH also have abnormal systemic nucleic acid metabolism as a primary feature; this may lead to induction of abnormal lymphocyte differentiation and abnormal function.

The results of the current study showed that the expression levels of CYP4F3 and GPX3, which are known to be involved in degradation of LTB4 and the LTB4 precursor 5-HPETE, were increased. In contrast, the expression of PTGER2 was decreased. LTB4 is known to activate neutrophils [33], and PGE2 is known to have vasodilatory effects [34]. These results may be viewed as compensatory reactions, corresponding to the increased production of LTB4 and PGE2; elevation of these components in the blood may compensate for leukocyte dysfunction and increased blood pressure caused by other factors. Furthermore, analysis of ET signals revealed reduced EDNRA expression and increased NPR3 expression. Decreased expression of the ET receptor leads to vascular contraction [35], while increased expression of NPR3 leads to vascular dilation [36]. Taken together, these findings suggest that NPR3 expression increases as

Table 3: Top 25 biological processes associated with IPH in Ingenuity iReport.

Number	Biological process	DEGs	p value	Genes
1	Synthesis of leukotriene B4	4	$6.76164E-06$	TGFB1, ABCC1, ADA, PTGER2
2	Synthesis of leukotriene	5	$8.36612E-06$	TGFB1, PLA2G10, ABCC1, ADA, PTGER2
3	Quantity of pro-B lymphocytes	5	$9.10676E-06$	CD28, CXCR4, CD79A, IGLL1/IGLL5, TNFRSF11B
4	T-cell migration	8	$1.0515E-05$	CD28, TGFB1, CXCR4, GNLY, RASGRP1, PLA2G10, ABCC1, CD44
5	Arrest in cell cycle progression of keratinocyte cancer cell lines	2	$2.27598E-05$	TGFB1, MELK
6	Function of leukocytes	12	$2.82837E-05$	CD28, SLAMF1, TGFB1, CXCR4, RASGRP1, PLA2G10, ABCC1, ADA, CD44, PTGER2, MMP12, LAX1
7	Transcytosis of HIV-1	2	$6.80667E-05$	CXCR4, CD79A
8	Cell movement of hairy leukemia cells	2	$6.80667E-05$	TGFB1, CD44
9	Invasion of keratinocyte cancer cell lines	2	$6.80667E-05$	TGFB1, PTGER2
10	Signaling of T lymphocytes	3	$8.23941E-05$	CD28, SLAMF1, CD44
11	Metabolism of eicosanoid	7	0.000109678	TGFB1, PLA2G10, ABCC1, ADA, CYP4F3, EDNRA, PTGER2
12	Reorganization of membrane rafts	2	0.000135709	CD28, CD44
13	Response of memory T lymphocytes	2	0.000135709	CD28, TGFB1
14	Fusion of leukocytes	3	0.000153367	CXCR4, CD44, TNFRSF11B
15	Lymphopoiesis of cells	3	0.000153367	TGFB1, CXCR4, CD44
16	Migration of mammary tumor cells	3	0.000153367	TGFB1, CXCR4, CD44
17	Cell viability of leukocytes	7	0.000162768	CD28, TGFB1, CXCR4, CD44, LAX1, CD79A, TNFRSF11B
18	Transmigration of mononuclear leukocytes	4	0.000170867	CD28, TGFB1, CXCR4, CD44
19	Lymphopoiesis	4	0.000182632	TGFB1, CXCR4, CD44, IGLL1/IGLL5
20	Adhesion of prostate cancer cell lines	3	0.000200182	TGFB1, CXCR4, CD44
21	Invasion of breast cell lines	3	0.000200182	TGFB1, CXCR4, PTGER2
22	TH1 immune response of naive T lymphocytes	2	0.000225478	CD28, TGFB1
23	Adhesion of hyaluronic acid	2	0.000225478	TGFB1, CD44
24	Morphology of cardiovascular tissue	4	0.000235591	TGFB1, CXCR4, CD44, TNFRSF11B
25	Migration of tumor cell lines	10	0.000252381	TNFAIP8, TGFB1, CXCR4, RASGRP1, CDK7, PTTG1, SERPINA5, CD44, E2F5, MLLT4

Underlined genes indicate increases in expression. Not underlined genes indicate decreases in expression.
DEGs: differentially expressed genes.

a compensatory reaction to vascular contraction, possibly causing dilation of blood vessels.

When viewed as a whole, the expression of CD44 and TGF-β was reduced. CD44 is known to induce T-cell homing and activate T-helper 1 (Th1) cells, T-helper 2 (Th2) cells, cytotoxic T lymphocytes (CTLs), and natural killer (NK) cells and is thus involved in stem cell proliferation and differentiation [37–39]. TGF-β suppresses the proliferation of blood cells and induces the proliferation and activation of mesenchymal cells involved in blood cell differentiation [40, 41]. Thus, in patients with IPH, immune system responses and the differentiation-inducing environment appear to be disturbed.

Furthermore, we explored the network pathway involved in IPH using IPA network analysis. Although the IPA network results did not reveal any additional finding, many genes selected by iReport were also observed in the high-ranked IPA pathways, thus strongly supporting the iReport results.

Taken together, these findings suggest that abnormal systemic nucleic acid metabolism is the first factor involved in the onset of IPH and that this abnormality induces abnormal differentiation and function of immunocompetent

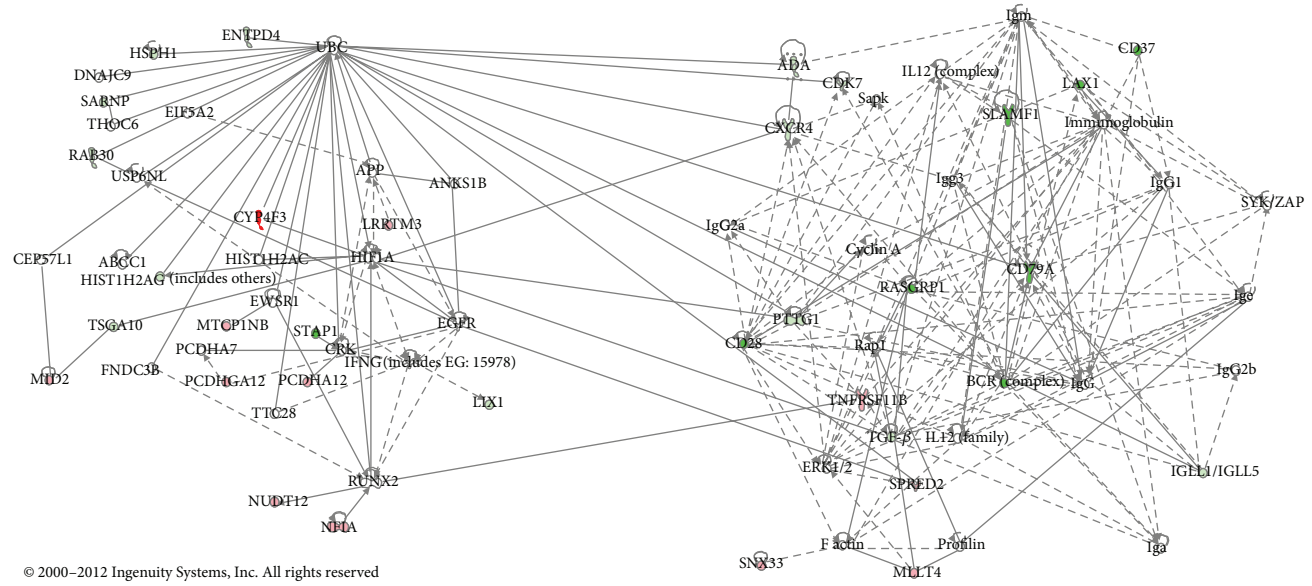
Table 4: Top 25 diseases associated with IPH in Ingenuity iReport.

Number	Disease	DEGs	p value	Genes
1	Immunodeficiency	6	$1.805E-06$	CD28, CXCR4, RASGRP1, ADA, CD79A, IGLL1/IGLL5
2	Leprosy	5	$5.11653E-05$	CD28, SLAMF1, TGFB1, CD79A, IGLL1/IGLL5
3	Transcytosis of HIV-1	2	$6.80667E-05$	CXCR4, CD79A
4	Genital tumor	15	0.000101439	CXCR4, CDK7, FOLH1, STAP1, MKI67, GPX3, ABCA8, TGFB1, ABCC1, SERPINA5, CD44, EDNRA, PTGER2, TNFRSF11B, PEG3
5	Agammaglobulinemia	2	0.000135709	CD79A, IGLL1/IGLL5
6	Metastasis of tumor	5	0.000161747	CD28, TGFB1, CXCR4, CD44, PTGER2
7	Digestive organ tumor	20	0.000177474	ERAP2, HIST1H4A, CXCR4, CDK7, PTTG1, FOLH1, TPD52L1, MELK, MKI67, IGLL1/IGLL5, GPX3, TGFB1, OIT3, ABCC1, C7, E2F5, CD44, MMP12, PEG3, TNFRSF11B
8	Colorectal cancer	14	0.000211188	ERAP2, HIST1H4A, CXCR4, PTTG1, CDK7, FOLH1, TPD52L1, MELK, MKI67, GPX3, TGFB1, CD44, TNFRSF11B, PEG3
9	Tuberculoid leprosy	3	0.000255384	CD28, SLAMF1, CD79A
10	Metastasis	10	0.000432136	CD28, ERAP2, TGFB1, CXCR4, PTTG1, CD44, MLLT4, PTGER2, PEG3, TNFRSF11B
11	Granulocyte colony stimulating factor-induced psoriasiform dermatitis	2	0.000470563	TGFB1, MKI67
12	Rectum cancer	3	0.0005718	GPX3, CD44, MKI67
13	Vascular disease	11	0.00075445	NPR3, SERPINA5, MARC1, TGFB1, EDNRA, PTGER2, CD44, MMP12, CD28, MKI67, CXCR4
14	Infection by bacteria	8	0.00090366	CD28, SLAMF1, TGFB1, SERPINA5, CD44, MMP12, CD79A, IGLL1/IGLL5
15	Replication of HIV-1	4	0.00121203	CD28, TGFB1, CXCR4, PLA2G10
16	Pulmonary hypertension	3	0.001305595	NPR3, CXCR4, EDNRA
17	Prostatic tumor	9	0.001615688	GPX3, TGFB1, CXCR4, FOLH1, CD44, EDNRA, STAP1, MKI67, TNFRSF11B
18	Uterine serous papillary cancer	6	0.001802487	ABCA8, P2RY14, PTTG1, C7, EDNRA, PEG3
19	Fibrosis of lung	5	0.001814056	TGFB1, PLA2G10, ADA, EDNRA, MMP12
20	Cancer	33	0.001947463	ERAP2, HIST2H2BE, PTTG1, FOLH1, TPD52L1, MLLT4, MELK, MKI67, CD79A, IGLL1/IGLL5, GPX3, CD28, SARNP, TGFB1, ABCC1, C7, E2F5, ADA, MMP12, TNFRSF11B, PEG3, HIST1H4A, CXCR4, CDK7, STAP1, ABCA8, P2RY14, RASGRP1, OIT3, SERPINA5, CD44, EDNRA, PTGER2
21	Endometrial carcinoma	8	0.002077475	GPX3, ABCA8, P2RY14, TGFB1, PTTG1, C7, EDNRA, PEG3
22	Neuroblastoma	3	0.002212756	CXCR4, ABCC1, CD44
23	Edema of tissue	2	0.002294982	TGFB1, SPRED2
24	Carcinoma	28	0.002351502	ERAP2, HIST2H2BE, PTTG1, FOLH1, TPD52L1, MELK, MKI67, IGLL1/IGLL5, GPX3, TGFB1, ABCC1, C7, E2F5, MMP12, TNFRSF11B, PEG3, HIST1H4A, CXCR4, CDK7, STAP1, ABCA8, P2RY14, RASGRP1, OIT3, SERPINA5, CD44, EDNRA, PTGER2
25	Polyuria	3	0.002583567	NPR3, PTTG1, ABCC1

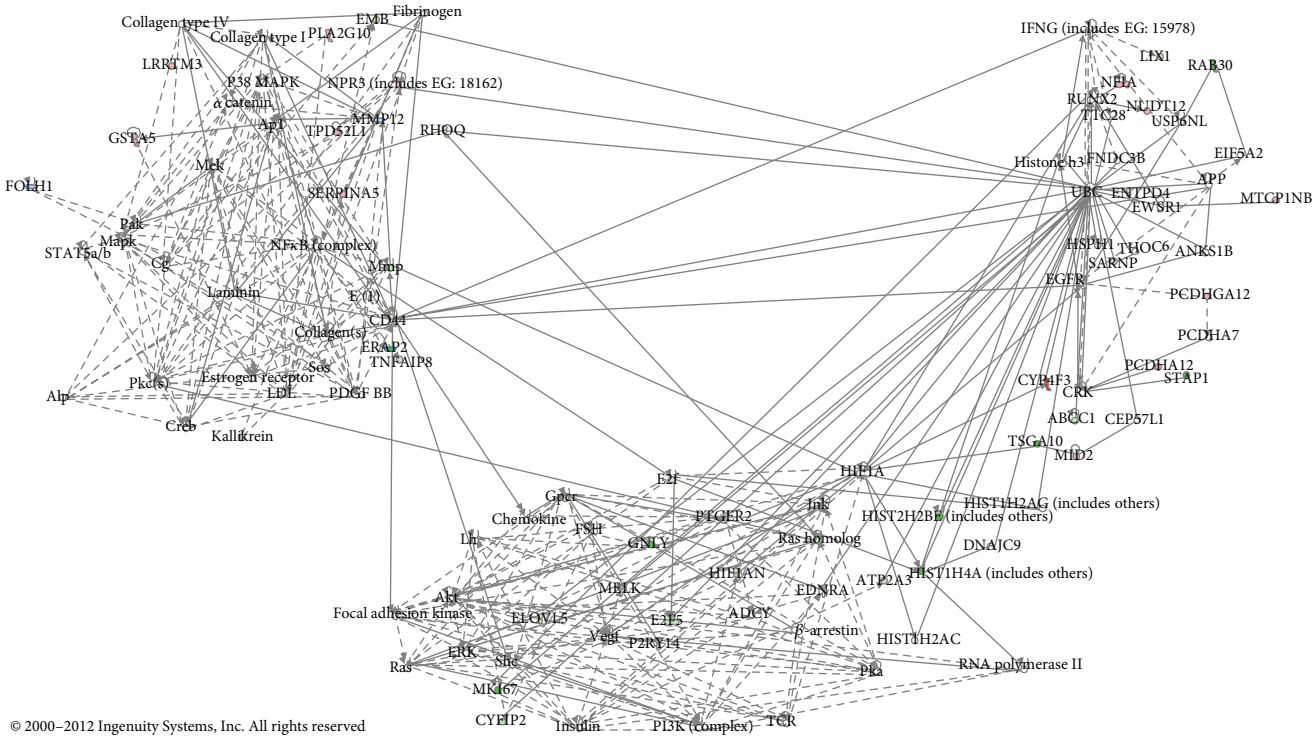
Underlined genes indicate increases in expression. Not underlined genes indicate decreases in expression.
DEGs: differentially expressed genes.

cells, although our results are preliminary and require further analysis. In this context, abnormalities in AA-related synthesis/metabolism and ET signals can be viewed as compensatory reactions of blood cells corresponding to changes in the external environment of blood cells, for example, increases/decreases in the levels of certain factors in the serum.

The present study enrolled four IPH patients and three of four samples were used for gene analyses. We cannot deny the possibility that our results did not cover the patterns of whole IPH patients because the sample number was small. In addition, we conducted comprehensive gene expression analysis of blood samples. Splenomegaly is a characteristic symptom in patients with IPH and is often accompanied by



(a)



(b)

Figure 3: IPA network illustration of the relationships among gene groups showing significantly increased/decreased expression in the presence of IPH. Genes colored pink or red exhibited increased expression, while genes colored green exhibited reduced expression. The straight lines indicate direct relationships among genes, while broken lines indicate indirect relationships. (a) Illustration of the first selected pathway combined with the second pathway. (b) Illustration of the first pathway combined with the third and fifth pathways. Similar to the results from Ingenuity iReport analysis, reduced expression of ENTPD4, ABCC1, ADA, and TGF-β and increased expression of CYP4F3 were observed inside the IPA network.

histological abnormalities, such as splenic sinus hyperplasia and increased collagen fibers. Thus, analysis of splenic tissue specimens may be useful for clarifying the pathophysiology of IPH. However, unlike the liver, in which percutaneous biopsy is relatively easy, the spleen is difficult to be biopsied or resected in healthy individuals and in patients with IPH during routine clinical practice. To date, no reports have described comprehensive gene expression analysis in patients with IPH. Therefore, additional genetic studies are needed to confirm the biological roles of the molecular pathology of IPH determined in the present study. The identification of genetic abnormalities in IPH may lead to the development of genetic therapy.

5. Conclusion

Blood samples from patients with IPH and healthy individuals as controls were subjected to comprehensive gene analysis with DNA microarrays. In cluster analysis, we did not observe any dominant probe groups showing clear differences between the IPH group and the healthy control group. In functional annotation analysis, the cluster of compromised function among patients with IPH included many gene terms primarily related to the immune system. Network analysis revealed suppression of immune function, abnormal nucleic acid metabolism, and abnormal AA-related synthesis/metabolism. These results suggested that abnormal systemic nucleic acid metabolism was a key factor involved in the onset of IPH and that this abnormality induced abnormal differentiation and function in immunocompetent cells. The abnormalities in AA-related synthesis/metabolism and ET signals may represent compensatory reactions in blood cells, corresponding to changes in the external environment of blood cells, such as increases/decreases in the levels of certain factors in the serum.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Liver Cirrhosis: Evaluation, Nutritional Status, and Prognosis

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The liver is the major organ for the metabolism of three major nutrients: protein, fat, and carbohydrate. Chronic hepatitis C virus infection is the major cause of chronic liver disease. Liver cirrhosis (LC) results from different mechanisms of liver injury that lead to necroinflammation and fibrosis. LC has been seen to be not a single disease entity but one that can be graded into distinct clinical stages related to clinical outcome. Several noninvasive methods have been developed for assessing liver fibrosis and these methods have been used for predicting prognosis in patients with LC. On the other hand, subjects with LC often have protein-energy malnutrition (PEM) and poor physical activity. These conditions often result in sarcopenia, which is the loss of skeletal muscle volume and increased muscle weakness. Recent studies have demonstrated that PEM and sarcopenia are predictive factors for poorer survival in patients with LC. Based on these backgrounds, several methods for evaluating nutritional status in patients with chronic liver disease have been developed and they have been preferably used in the clinical field practice. In this review, we will summarize the current knowledge in the field of LC from the viewpoints of diagnostic method, nutritional status, and clinical outcomes.

1. Introduction

The liver is the major organ for the metabolism of three major nutrients: protein, fat, and carbohydrate [1, 2]. Chronic hepatitis C virus (HCV) infection affects about 170 million people worldwide and is the most common cause of chronic liver disease. Of these HCV-infected individuals, 20–30% eventually develop liver cirrhosis (LC) or hepatocellular carcinoma (HCC). In our country, about 30,000 persons per year die from HCC, with 70–80% of these deaths ascribed to HCV [3, 4].

LC results from different mechanisms of liver injury that lead to necroinflammation and fibrosis. Histologically, LC is characterized by diffuse nodular regeneration surrounded by dense fibrotic septa with subsequent collapse of liver structures and thus causes pronounced distortion of vascular architecture in the liver [5].

Increasingly, LC has been seen to be not a single disease entity but one that can be graded into distinct clinical stages related to prognosis [5]. In addition, the economic and social burden of LC is immense considering decreased quality of life, the disability of labor, poorer physical activity, and need for frequent hospitalizations in patients with LC.

In terms of diagnostic methods for LC, several noninvasive methods have been developed and these methods have been used for predicting prognosis in patients with LC; these include serum markers such as aspartate aminotransferase to platelet ratio index (APRI), FIB-4 index, aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio, or modalities such as acoustic radiation force impulse (ARFI), transient elastography (TE), and magnetic resonance elastography [6–13].

On the other hand, subjects with LC often have protein-energy malnutrition (PEM) and poor physical activity. These conditions often result in sarcopenia, which is the loss of skeletal muscle volume and increased muscle weakness. Recent studies have demonstrated that PEM and sarcopenia are predictive factors for poorer survival in patients with LC [14]. Based on these backgrounds, several methods for evaluating nutritional status in patients with chronic liver disease such as indirect calorimetry, dual-energy X-ray absorptiometry (DEXA), bioimpedance analysis (BIA),

and anthropometry have been developed and they have been preferably used in the clinical settings [15].

In this review, we will summarize the current knowledge in the field of liver cirrhosis from the view of diagnostic method, nutritional status, and clinical outcomes.

2. Conventional Classification and Prognostic Assessment of Liver Cirrhosis

Currently, the most commonly used classification of liver function for patients with LC is Child-Pugh classification. This was originally designed to predict mortality during surgery in patients with LC [16]. This has been demonstrated to be useful in determining patient prognosis and thus several staging system for HCC including Japan Integrated Staging (JIS), Barcelona Clinic Liver Cancer (BCLC), and Cancer of Liver Italian program (CLIP) use this system as prognostic determinant [17–20]. The Model for End-Stage Liver Disease (MELD) score was originally developed as a prognostic model of early mortality in LC patients undergoing a transjugular intrahepatic portosystemic shunt (TIPS) [21]. This score includes variables of serum concentrations of bilirubin and creatinine and international normalized ratio for prothrombin time (INR) and, in most liver transplantation centers, MELD score has replaced the Child-Pugh score for priority of organ allocation due to superiority of prognostic ability of MELD score [21, 22]. On the other hand, serum sodium has been demonstrated to be an independent risk factor for mortality in LC patients with or without HCC [23, 24]. Kim et al. reported that the addition of the serum sodium to generate the MELD-Na score was more accurate than MELD for predicting short-term mortality on the waiting list of liver transplantation in LC patients [23]. Moreover, in their study, they estimated that the use of MELD-Na might have prevented 7% of deaths that occurred within 90 days of listing for liver transplantation [23]. In our previous study ($n = 1170$), we demonstrated that lower serum sodium concentration is a useful predictor in HCC patients complicating with LC [24]. Hepatic venous pressure gradient as determined by subtraction of the free-hepatic venous pressure from the wedged hepatic venous pressure has also been demonstrated to be an independent predictor in patients with LC [25]. However, unfortunately, these models did not include nutritional status of the LC patients.

On the other hand, D'Amico et al. have classified compensated LC into clinical stages 1 and 2 and decompensated LC in clinical stages 3 and 4 based on a systematic review of 118 reports [26]. They defined patients in clinical stage 1 as neither varices nor ascites and reported that the 1-year mortality was only 1%, and if patients develop varices (clinical stage 2), the 1-year mortality increases up to 3.4%. Furthermore, with decompensated LC and onset of ascites (clinical stage 3), the 1-year mortality increases up to 20%, and, following a variceal bleeding (clinical stage 4), the 1-year mortality was higher than 50% [26]. Currently, a proposal has been made to include two more additional clinical stages to this classification system: clinical stage 5, LC patients with bacterial infections (such as spontaneous bacterial peritonitis or bacteremia) as 1-year mortality increases from 49% to 66%, and clinical stage 6, patients with renal failure as mortality at 1-year could be around 70% [27, 28]. These classification systems are promising for predicting prognosis in patients with LC.

3. Noninvasive Methods for Predicting LC or LC Related Complications

Noninvasive markers of LC can be radiologic or serum based. Although liver biopsy remains the reference standard for evaluating the extent of liver fibrosis in patients with chronic liver diseases, several noninvasive methods such as TE and ARFI have been developed as alternatives to liver biopsies. Recent reports have focused on assessing the performance of noninvasive methods through long-term follow-up studies with clinical outcomes associated with LC [29–31].

Vergniol et al. reported that noninvasive tests for liver fibrosis (measurement of liver stiffness (FibroScan), FibroTest, APRI, and FIB-4 index) can predict 5-year survival of patients with chronic hepatitis C ($n = 1457$) [29]. Singh et al. demonstrated in their meta-analysis ($n = 7058$) that the degree of liver stiffness using elastography is associated with risk of decompensated cirrhosis, HCC, and death in patients with chronic liver diseases and it might be used in risk stratification [30]. A recent Japanese study demonstrated that measurements of spleen stiffness using ARFI can be used to identify patients with cirrhosis with esophageal varices (EVs) or high-risk EVs [31].

On the other hand, we previously reported that the GSA index as defined by the uptake ratio of the liver to the liver plus heart at 15 min to the uptake ratio of the heart at 15 min to that at 3 min ratio calculated from 99mTc-labeled diethylene triamine pentaacetate-galactosyl human serum albumin (99mTc-GSA) scintigraphy yielded the highest area under the receiver operating curve (AUROC) for predicting histologically proven cirrhosis with a level of 0.786 at an optimal cut-off value of 1.37 (sensitivity: 65.9%; specificity: 79.0%) in HCV-related HCC patient treated with surgical resection (SR) ($n = 213$) and it can be a useful predictor for HCC recurrence after surgery [32]. Furthermore, in non-B and non-C HCC patients treated with SR ($n = 118$), we have shown that the FIB-4 index yielded the highest AUROC for histologically proven cirrhosis with a level of 0.887 at an optimal cut-off value of 2.97 (sensitivity: 92.3%; specificity: 69.6%), and FIB-4 index >2.97 ($P = 0.044$) was a significant independent factor linked to HCC recurrence [33].

Recently, the Wisteria floribunda agglutinin-positive human Mac-2-binding protein (WFA+-M2BP) was demonstrated to be a liver fibrosis glycobiomarker with a unique fibrosis-related glycoalteration [34]. Yamasaki et al. reported that WFA+-M2BP can be applied as a useful surrogate marker for not only liver fibrosis but also the risk of HCC development [35].

4. Nutritional Status and Nutritional Assessment in Liver Cirrhosis

Cirrhosis, which develops over a long period of time, is frequently complicated with PEM [1, 2]. In our data, the proportion of PEM in LC patients was around 30% (unpublished

data). PEM is one of the most common complications in LC patients and it is associated with high morbidity and mortality for patients with LC [1, 2, 36]. However, despite the significant role that the nutritional status has in the prognosis of LC, it is frequently overlooked as the nutritional assessment could be complex in LC patients with fluid retention and/or overweight [37].

Clinicians should consider various aspects of the LC patients including medical history, physical examination, the severity of underlying illness, and biochemical data for the nutritional evaluation [37]. The subjective global assessment (SGA) is one of commonly used instruments for nutritional assessment in patients with LC. In general, nutritional evaluation is performed using SGA, anthropometry including muscle arm circumference and body mass index (BMI), and biological markers such as albumin and prealbumin (transthyretin). The factors of the SGA consist of physical examination component that assesses the loss of subcutaneous fat, peripheral edema, and muscle wasting [38]. The quantity of muscle and subcutaneous tissue is graded subjectively by the examiner. Then, it is classified as normal, mildly, moderately, or severely decreased [38]. Considering multiple components such as body weight loss and clinical symptoms, the patients are graded as well nourished (SGA grade A), moderately malnourished or suspected of being malnourished (SGA grade B), or severely malnourished (SGA grade C) [38]. However, the SGA may be a partially subjective method. In addition, previous studies reported that SGA has shown low sensitivity in LC patients for nutritional diagnosis, as it underestimates the nutritional state in the majority of LC patients [39]. There is one interesting report of comparison between SGA grading system and a tool for controlling nutritional status (CONUT), which was proposed by Ignacio de Ulíbarri et al. [40, 41]. In that report, CONUT had the good agreement with SGA grading system (kappa index: 0.680) [41].

On the other hand, several laboratory tests have been used as a part of the nutritional assessment in patients with LC, including albumin, prealbumin, the prothrombin time, creatinine height index, and indirect evaluation of the of immune function [42–46]. However, as their studies had diverse results, an optimal index for nutritional status in LC patients in terms of availability, reproducibility, practicality, and prognostic performance is required [42–46].

Nutrition and exercise management can improve PEM and sarcopenia in patients with LC [14]. Nutritional management includes sufficient dietary intake and improved nutrient metabolism. However, with the current high prevalence of obesity, the number of obese LC patients has increased, and restriction of excessive caloric intake without the exacerbation of impaired nutrient metabolism is needed for LC patients with obesity [14]. Exercise management can increase skeletal muscle strength and volume.

5. Sarcopenia in Liver Cirrhosis

Sarcopenia is characterized by the depletion of skeletal muscle mass [47, 48]. In general, skeletal mass is maintained by a balance between synthesis and breakdown of protein [49]. LC patients have insufficient glycogen stores because of deterioration of liver function and energy generation pattern in these patients after an overnight fast is reported to be equivalent to that observed in healthy controls after 2 or 3 days of starvation [49]. These catabolic states increase the consumption of amino acids as an energy source and accelerate the breakdown in skeletal muscle to release amino acids, eventually leading to sarcopenia [49]. Recently, some studies have indicated that hyperammonemia can cause sarcopenia [50].

On the other hand, sarcopenia has become a key clinical entity for understanding the impact of aging on health outcomes. In 1989, Rosenberg first introduced the term "sarcopenia" to refer to age-related loss of skeletal muscle mass and volume [51]. Similar to bone, when persons reach around 50 years of age, they lose about 1-2% of their muscle mass per year [52]. Sarcopenia is a common disorder in aged populations contributing to functional decline, disability, and frailty [51, 52]. Several studies reported the increased risk of chronic metabolic disorders and mortality in persons with low muscle mass [53, 54]. Aging-related sarcopenia is defined as primary sarcopenia, whereas LC is a cause of secondary sarcopenia. Hiraoka et al. reported that in their analysed 988 subjects with chronic liver disease and 372 normal control subjects, presarcopenia as defined by less than two standard deviations below the mean psoas muscle area index (psoas muscle area at the mid-L3 level in CT (cm^2)/height $(m)^2$ value in the controls) was observed in 15.3% of patients with chronic hepatitis, 24.4% of those with Child-Pugh A, 37.7% of those with Child-Pugh B, and 37.1% of those with Child-Pugh C and the frequency of presarcopenia was higher in chronic hepatitis regardless of age as compared with normal controls [55].

5.1. Assessment Methods for Sarcopenia. Several methods for sarcopenia assessment in patients with LC have been proposed.

5.1.1. Handgrip Strength. Hirsch et al. demonstrated in their controlled trial that handgrip strength was a useful marker for the assessment of nutritional status in LC patients [56]. Currently, The European Working Group on Sarcopenia in Older People (EWGSOP) recommends measurement of handgrip strength as a practical measure of muscle strength [57]. However, it should be kept in mind that this method has not been well established as considerable variation in the measurement methods has the potential to lead to measurement errors.

5.1.2. Imaging Studies: CT. According to the recent investigations, the psoas muscle area or thickness can be measured on the axial CT scan at the various levels of lumbar spine such as L3 vertebral level, L4 vertebral level, and at the level of umbilicus for assessing sarcopenia [58–61]. These studies showed the good correlations of clinical outcomes and the psoas muscle mass [58–61]. The psoas muscle can be easily identified and easily measured on a CT scan, as it is surrounded by retroperitoneal fat tissue and vertebra, and is not susceptible to the compression of ascites or splenomegaly

[58–61]. Thus, measurement of psoas muscle area at L3 vertebral level has been preferably used for assessing sarcopenia. However, no consensus value for CT-based sarcopenia has been well established in Asian populations.

5.1.3. Bioimpedance Analysis. BIA is a noninvasive technique that measures electrical resistance and reactance [39, 62–64]. In recent studies, electrical BIA has been proposed for body composition analysis in patients with chronic liver disease [39, 62–64]. This method is based on the principle that body fat and no fat mass have specific components, such as water, proteins, and minerals [39, 62–64]. Electrical bioimpedance consists in the delivery of a low-intensity electric current which flows through the body by the ions movements [39, 62–64]. Fernandes et al. reported that the assessment using BIA presented a statistically significant correlation with Child-Pugh classification [39]. On the other hand, BIA did not demonstrate the ability to distinguish between minimal and advanced degrees of hepatic fibrosis in patients with chronic HCV infection [63].

5.1.4. Dual-Energy X-Ray Absorptiometry. DEXA through a low-dose X-ray can be used to measure fat, total body bone mineral, and fat-free soft tissue mass [65, 66]. In healthy persons, excellent agreement is found between data obtained using DEXA and data obtained from the more established reference methods [66]. However, this technique is not accurate for evaluating body composition in LC patients with fluid retention.

5.1.5. Other Methods. Skin-fold thickness measurement using a caliper is the method that quantify fat mass in the upper arm (midarm muscle area) [67]. However, there have been conflicting reports for the accuracy for predicting malnutrition in LC patients because of its interobserver variability, and this method did not correlate with Child-Pugh classification [66, 67].

6. Sarcopenic Obesity

The current global obesity epidemic has created a new condition: the combination of obesity and sarcopenia, described as sarcopenic obesity [68]. As LC patients occasionally have sarcopenia (around 40%) and obesity (around 30%), it can be deduced that a considerable number of LC patients may have sarcopenic obesity [69]. In addition, obesity is often accompanied by nonalcoholic fatty liver disease (NAFLD), and the prevalence of this liver disease is increasing in industrialized countries. NAFLD can progress to nonalcoholic steatohepatitis and LC [70]. The increase in obesity prevalence rates in elderly patients is also of concern, given the associated disease risks such as coronary heart disease and more limited treatment options available in this age group. Sarcopenic obesity has been also found to be related to poorer survival in patients with solid tumors of the respiratory and gastrointestinal tracts [71]. Sarcopenic obesity may become a major condition in LC patients in the future.

Sarcopenic obesity is assuming a significant role as a risk factor due to the double metabolic burden derived from excess adiposity (obesity) and low muscle mass (sarcopenia). Obesity also induces systemic inflammation and insulin resistance and both prompt hypercatabolism and impairs the anabolic effect of muscles, leading to protein breakdown stimulation and muscle synthesis suppression [53]. Skeletal muscle plays a significant role in insulin sensitivity as a primary tissue associated with whole body insulin-mediated glucose uptake [53]. Several studies reported that low skeletal muscle mass is linked to obesity, metabolic syndrome, and dysglycemia, and the reverse was demonstrated in large populations with higher muscle mass associated with better insulin resistance and a lower risk of developing diabetes [53, 54, 72]. Moreover, a recent study demonstrated that sarcopenic obesity is more closely linked to insulin resistance than obesity or sarcopenia alone [54]. Taken together, this new condition may lead to accelerating sarcopenia progression.

On the other hand, sarcopenic obesity is a newly recognized clinical entity following living donor liver transplantation [73]. Choudhary et al. reported that 82 patients are undergoing liver transplantation and 72 patients (88%) developed sarcopenic obesity and metabolic syndrome despite resuming routine exercise after liver transplantation [73]. In LC patients, who receive liver transplantation, appropriate nutrition and exercise after transplantation may be required.

BMI is a simple anthropometric index calculated from individual height and weight and is widely used. However, BMI is limited anthropometrically in that it does not evaluate individual components of body weight such as muscle volume or regional fat distribution. Body mass can be grossly divided into two compartments. These are fat mass and fat-free mass. In a multicompartment body composition model, fat-free mass may be partitioned into skeleton and integument and skeletal muscle and visceral organs and total body water. Total body water is further partitioned into intracellular and extracellular water [74]. Regional fat distribution plays an essential role especially in patients with metabolic syndrome [75]. Taking these into consideration, BMI may not be suitable for evaluating sarcopenic obesity.

7. Nutritional Support in LC Patients with Sarcopenia

The aims of nutritional therapy in cirrhotic patients are the support of liver regeneration, the prevention or correction of specific nutritional deficiencies, and the prevention and/or treatment of the LC related complications [76]. The recommendations in nutritional intervention target the optimal supply of adequate substrates related to requirements linked to protein, energy, lipids, carbohydrates, vitamins, and minerals. Early identification and treatment of malnutrition in LC patients have the potential to lead to better clinical outcome and prevent LC related complications [77].

7.1. Vitamin. Vitamin deficiencies such as vitamin A, B, D, and E in LC patients are in general associated with disorders of liver function and diminished reserves and with increasing severity of the disease. They are related to inadequate dietary

intake and/or malabsorption. Fat soluble vitamin deficiencies are common manifestations in LC patients [78]. Thus, vitamin supplementation may be essential for advanced LC patients [78].

7.2. Minerals. Zinc is an essential trace element required for normal cell growth, development, and differentiation and zinc deficiency is common in LC patients [79]. Zinc supplementation is demonstrated to reverse clinical signs of zinc deficiency in LC patients [79, 80]. Furthermore, zinc supplementation produced metabolic effects and trended toward improvements in liver functional reserve, hepatic encephalopathy, and general nutritional status [80, 81].

7.3. BCAA. In LC patients, the plasma level of branched-chain amino acid (BCAA) is positively correlated with the serum albumin level. Such a correlation is seen only in patients with chronic liver diseases such as cirrhosis [1, 2, 76]. The albumin-BCAA correlation and the inability of cirrhotic patients to maintain an adequate plasma level of BCAA with diet alone serve as the theoretical rationale for the use of BCAA granules for the treatment of cirrhosis. In cirrhotic patients, BCAA uptake in skeletal muscle is increased for ammonia detoxification and energy production and, in turn, the plasma level of BCAA and albumin production decrease [1, 2, 76]. BCAA granules (LIVACT, Ajinomoto Pharma, Tokyo, Japan) contain L-valine, L-leucine, and L-isoleucine at a ratio of 1.2 : 2 : 1. L-leucine induces albumin synthesis in hepatic cells via transcription factors such as mammalian target of rapamycin (mTOR) [1, 76, 82–88]. BCAA granules were originally developed for the treatment of hypoalbuminemia associated with decompensated cirrhosis. However, later studies found a variety of other pharmacological actions of this drug. BCAA granules therapy not only improves hypoalbuminemia but also inhibits cirrhosis-related complications such as esophageal varices and ascites, reduces insulin resistance and oxidative stress, improves fatty acid metabolism, stimulates the immune system, and inhibits angiogenesis [1, 76, 82–88]. The 2010 guidelines for comprehensive treatment of hepatitis virus-related cirrhosis in Japanese patients recommend the use of BCAA granules to preserve liver function and inhibit hepatic carcinogenesis [89]. Furthermore, Hanai et al. recently reported that BCAA supplementation improved the survival of sarcopenic LC patients in their subgroup analysis ($P < 0.01$) [36]. Conversely, the American Society for Parental and Enteral Nutrition (ASPEN) and the European Society for Clinical Nutrition and Metabolism recommend that BCAA supplementation be carried out only in cirrhotic patients with chronic hepatic encephalopathy that is refractory to pharmacotherapy [90, 91]. There may be differences of indications for BCAA therapy in LC patients between Japan and Western countries.

7.4. Carnitine. Carnitine deficiency has been demonstrated to be linked to LC [92]. Administration of L-carnitine, which is a derivative with high bioactivity in carnitine derivatives, has been suggested as a safe alternative treatment for LC patients [93, 94]. In field clinical practice, Malaguarnera et al. reported that LC patients treated with L-carnitine showed greater reductions in serum ammonia levels and improvements of neuropsychological functioning in comparison with placebo [93]. On the other hand, Nakanishi et al. demonstrated that L-carnitine reduces muscle cramps in LC patients [94]. However, whether L-carnitine improves sarcopenia in LC patients remains unclear. Further examination will be needed to confirm these results.

8. Sarcopenia, Patient Performance Status, and HCC Prognosis

Severe muscle wasting or sarcopenia is one of the most common and frequently hidden complications in HCC patients with LC, which negatively has an effect on survival and quality of life. These complications may potentially lead to deterioration of performance status (PS). The PS scale measures how the daily living ability is affected by the underlying disease. The PS scale recommended by the Eastern Cooperative Oncology Group (ECOG) is widely used by clinicians to assess the functional status in patients with various cancers [95]. It also serves as an indicator of cancer therapy and predictor of patient survival. The PS scale is a major survival determinant in patients with HCC and is specifically included in the BCLC staging system as an essential parameter for treatment guidance for HCC [96]. In our previous study of PS on survival in HCC patients ($n = 1003$), a worse PS was significantly associated with age, gender, Child-Pugh classification, HCC stage, JIS score, initial treatment option for HCC, maximum tumor size, alanine aminotransferase value, hypoalbuminemia, hyperbilirubinemia, renal insufficiency, hyponatremia, and prothrombin and poorer PS was an independent predictor linked to OS with a hazard ratio of 1.773 ($P < 0.001$). Thus, we concluded that PS was closely associated with status of HCC patients with LC and could be an important predictor for these populations [20]. In our recent another study, we proposed PS combined JIS system in HCC patients with LC and demonstrated that it can be a useful prognostic system for HCC patients complicating with LC as compared with other classification systems such as original JIS system, BCLC, and CLIP ($n = 1170$) [97].

8.1. HCC and Impact of Sarcopenia. Fujiwara et al. retrospectively investigated the effect of body composition components on survival in HCC patients ($n = 1257$) and demonstrated that sarcopenia, intramuscular fat (IMF) deposition, and high VSR (called visceral adiposity) were significantly associated with mortality, independent of HCC stage or Child-Pugh classification and their multivariate analysis revealed that sarcopenia (hazard ratio (HR), 1.52; 95% confidence interval (CI), 1.18–1.96; $P = 0.001$), IMF deposition (HR, 1.34; 95% CI, 1.05–1.71; $P = 0.020$), and visceral adiposity (HR, 1.35; 95% CI, 1.09–1.66; $P = 0.005$) but not BMI were significant predictive factors linked to survival [98].

8.2. Prognosis in Sarcopenic HCC Patients according to Treatment Modality for HCC

8.2.1. Surgical Resection. The quality of skeletal muscle has attracted much attention as a novel indicator of sarcopenic HCC patients. Recently, Hamaguchi et al. demonstrated in their large study (447 HCC patients) that preoperative quality of skeletal muscle as evaluated by intramuscular adipose tissue content using preoperative CT imaging was well linked to postoperative mortality and HCC recurrence [99].

In addition, a recent study demonstrated that sarcopenia, as assessed by total psoas major volume, was an independent factor predictive of postoperative complications for primary hepatic cancers (HR; 3.06) [100]. Another recent study revealed that, in 109 HCC patients undergoing hepatectomy, sarcopenic HCC patients ($n = 59$) had significantly shorter median overall survival than nonsarcopenic HCC patients (52.3 months *versus* 70.3 months; $P = 0.015$) and, in their multivariate analysis, sarcopenia was revealed to be an independent predictor of poorer overall survival (HR = 3.19; $P = 0.013$) and disease-free survival (HR = 2.60; $P = 0.001$) [101]. Otsuji et al. reported that preoperative sarcopenia increased the morbidity rate including the rate of developing liver failure in patients treated with major hepatectomy with extrahepatic bile duct resection ($n = 256$) [102].

8.2.2. Trancatheter Arterial Therapies. Dodson et al. demonstrated that sarcopenia was an independent predictor of mortality following transcatheter intra-arterial therapy with sarcopenic patients having a twofold increased risk of mortality in patients with liver malignancies ($n = 216$) [103].

8.2.3. Molecular Targeted Therapy. Recently, sarcopenia, regardless of the presence of weight loss, has been identified as an independent adverse predictor for systemic chemotherapy toxicity. Mir et al. reported that in advanced HCC patients with Child-Pugh A ($n = 40$), sarcopenia predicts the occurrence of dose limiting toxicities within the first month of sorafenib therapy [104].

9. Conclusion

Several noninvasive methods for evaluating the degree of liver fibrosis and nutritional status have been developed and these methods have been used for predicting prognosis in patients with LC. LC patients often have PEM and poor physical activity. These conditions often result in sarcopenia, affecting negatively the survival. Sarcopenic obesity, which is recently recognized as novel clinical entity, may lead to accelerating sarcopenia progression. Thus, adequate nutritional support and exercise management may be essential for such patients. In HCC patients complicating LC, sarcopenia is also a significant problem due to its prognostic impact (Figure 1).

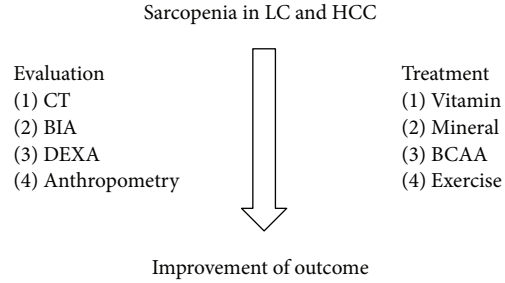

Figure 1

Conflict of Interests

The authors have no conflict of interests to declare.

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Research Article

Semiannual Imaging Surveillance Is Associated with Better Survival in Patients with Non-B, Non-C Hepatocellular Carcinoma

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Since it remains elusive whether and how the imaging surveillance affects the survival in patients with non-B, non-C hepatocellular carcinoma (NBNC-HCC), we conducted this retrospective study which investigated the association between the semiannual surveillance prior to HCC diagnosis and the survival in patients with the initial diagnosis of HCC induced by hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infections ($N = 141$) and non-B, non-C etiology ($N = 30$). It was demonstrated that surveillance was less frequently performed in the NBNC-HCC patients compared to that in HCC patients with HBV and/or HCV infections (B/C-HCC patients), and the survival was unfavorable in NBNC-HCC patients. On the other hand, the survival of NBNC-HCC patients with semiannual surveillance was significantly favorable than those patients without semiannual surveillance, and the survival was similar between B/C-HCCs and NBNC-HCCs with semiannual surveillance. In conclusion, though NBNC-HCC patients compared to B/C-HCC patients had poorer prognosis overall, these NBNC-HCC patients with semiannual surveillance had a better survival almost equivalent to the survival of B/C-HCC patients with semiannual surveillance, demonstrating the clinical utility of the semiannual imaging surveillance program for NBNC-HCCs.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, being the fifth in terms of incidence and third in the total number of deaths [1]. In Japan, the death number from HCC exceeds 30,000 per year [2, 3], which has been caused predominantly by chronic liver inflammation induced by hepatitis viruses B (HBV) and C (HCV) [4–6]. Recently, on the other hand, the number of nonviral non-HBV, non-HCV (NBNC) HCCs has been increasing, and 20% of newly diagnosed HCCs are classified as NBNC-HCCs in Japan [7]. It is speculated that the main reason for the increase is attributable to the increase of patients with nonalcoholic fatty liver disease (NAFLD) or nonalcoholic liver disease (NASH) associated with the increase of patients with metabolic disorders [8, 9].

As NBNC-HCCs increase, it has been gradually disclosed that the survival of the NBNC-patients is unfavorable compared to that of HCC patients caused by HBV and/or HCV infections (B/C-HCCs). Compared to B/C-HCCs, NBNC-HCCs are often found in a far advanced stage and their tumor sizes are large while their liver functions are rather conserved [10]. However, it was reported that the survival of NBNC-HCC patients was comparable to that in B/C-HCCs

if they were found in the early stages of disease [11, 12]. These recent reports suggest that the poor prognosis of NBNC-HCC patients could be attributable to delay in the HCC diagnosis.

Routine imaging surveillance protocols for early detection of HCC are recommended for patients with chronic hepatitis virus infections [13–19]. Actually, many previous studies reported that routine imaging surveillance (ultrasound, dynamic CT, and MRI with gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)) was beneficial for the early HCC diagnosis [20–24] in viral hepatitis patients. Based upon the improved survival of liver cirrhosis patients infected with hepatitis B and C viruses, surveillance with imaging studies with every six months, compared to that with longer intervals or no examination, has been recommended for the early HCC discovery in chronic liver disease patients though the importance of the semiannual surveillance has not been established in NBNC-chronic liver disease patients [14, 19, 25].

In this study, we conducted a retrospective analysis to clarify the effect of semiannual surveillance on the survival of NBNC-HCC patients since effective HCC surveillance protocol has been lacking for NBNC-HCCs so far and it is unknown how the imaging surveillance and its interval would influence the survival of NBNC-HCC patients.

2. Patients and Methods

2.1. Patients. Among the consecutive patients who were admitted to the Yamanashi University Hospital from January 2008 to March 2011, 171 HCC patients for whom information was available regarding the imaging study intervals up to the initial diagnosis of HCC were included in the analysis. HCCs positive for hepatitis B surface antigen (HBsAg) were classified as B-HCC and those positive for HCV antibodies were classified as C-HCC, while HCCs negative for both HBsAg and HCV antibody were defined as NBNC-HCC. In total, 141 patients were classified as B/C-HCC patients (120 C-HCC and 19 B-HCC, two HCCs with B and C) while 30 patients were classified as NBNC-HCC patients. Diagnosis of liver cirrhosis was made histologically or clinically by the occurrence of liver shrinkage, splenomegaly, or ascites or by the presence of esophageal varices.

2.2. Surveillance, HCC Diagnosis, and Treatment. As for the imaging modalities used for HCC surveillance, abdominal ultrasound, abdominal dynamic CT scan, and MRI with Gd-EOB-DTPA were included. The final HCC diagnosis was made when typical features of hypervascular HCC were confirmed by one imaging technique (dynamic CT scan, MRI with Gd-EOB-DTPA, or contrast medium-enhanced ultrasound) and the TNM stage was determined. The treatment method for HCC was basically determined according to the Practice Guideline for HCC established in 2009 [19]. Among the therapeutic modalities, surgical resection and radiofrequency ablations (RFA) were classified as curative therapies while transarterial chemoembolization (TACE), systemic chemotherapy, and other palliation therapies including best supportive care were classified as noncurative therapies.

Table 1: Clinical characteristics of the patients.

Variable	NBNC $n = 30$	B/C $n = 141$	p value
Age (yr)†	71.0 ± 8.1	68.2 ± 8.9	0.12
Male	73% (22/30)	68% (96/141)	0.57
Accompanied by LC	90% (27/30)	84% (118/141)	0.38
Child-Pugh (A/B, C)	17/10	74/44	0.81
Platelet ($\times 10^4$/mL)†	13.0 ± 6.4	11.4 ± 5.2	0.16
ALT (IU/L)‡	25 (8–1858)	35 (11–320)	0.45
AFP (ng/mL)‡	6.6 (2.2–41719)	14.1 (1.6–150300)	0.76
DCP (mAU/mL)‡	48 (9–40753)	22 (7–99760)	0.35
Tumor size (mm)†	31.4 ± 19.4	21.8 ± 16.0	<0.01
Tumor number (n)†	1.8 ± 1.5	1.7 ± 1.3	0.42
Vascular invasion (yes/no)	20% (6/30)	4% (6/141)	<0.01
TNM stage (I, II/III, and IV)	18/12	108/33	0.06
Curative therapy*	63% (19/30)	68% (96/141)	0.18
No surveillance	47% (14/30)	12% (17/141)	<0.01
Semiannual surveillance	40% (12/30)	57% (81/141)	0.08

†Mean ± SD, ‡median (range), and *curative therapy which includes operative resection and radiofrequency ablation.

Discharged patients were observed as outpatients at the Yamanashi University Hospital with regular imaging studies. Clinical variables, including tumor stages, therapeutic modalities, and surveillance intervals prior to the initial HCC diagnosis, were investigated for their association with the survival rate. According to the Declaration of Helsinki, this study was carried out after approval was obtained by the ethical committee, Faculty of Medicine, University of Yamanashi.

2.3. Statistics. Fisher's exact test was used for categorical data and the Mann-Whitney U test or Student's t-test was used for numerical data. Survival rates were analyzed by Kaplan-Meier curve analysis with the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. ROC analysis was used to determine the appropriate cutoff value.

3. Results

3.1. Comparison of Patient Characteristics Based on the Etiology at the Time of HCC Diagnosis. In Table 1, the clinical characteristics of the patients at the time of initial diagnosis are listed according to the status of hepatitis viral infection (NBNC-HCC versus B/C-HCC). There was no statistical difference between the two groups in terms of the patients' age, sex, percentages of liver cirrhosis, Child-Pugh score, platelet count, ALT level, and tumor markers AFP and DCP. However, there was a tendency for NBNC-HCCs to show

Table 2: Univariate and multivariate analysis for variables associated with survival.

Variable	n	Univariate analysis			Multivariate analysis		
		HR	95% CI	p value	HR	95% CI	p value
Age (years)							
≥71	97	1.66	0.67–4.09	0.27			
≤70	74						
Gender							
M	118	3.43	0.79–14.86	0.08			
F	53						
Etiology							
NBNC	30	6.26	2.54–15.45	<0.01	4.32	1.46–12.8	<0.01
B/C	141						
Child-Pugh							
B, C	30	2.02	0.82–4.98	0.13			
A	141						
Platelet (/mL)							
$\leq 12.0 \times 10^4$	104	0.81	0.32–2.02	0.65			
$\geq 12.1 \times 10^4$	67						
ALT (IU/L)							
≥40	65	1.08	0.43–2.70	0.87			
≤39	106						
AFP (ng/mL)							
≥20.1	74	1.66	0.67–4.10	0.27			
≤20.0	97						
DCP (mAU/mL)							
≥40	60	3.08	1.23–7.69	0.02	1.14	0.39–3.32	0.82
≤39	106						
AFP-L3 (%)							
≥10.0	34	2.85	1.12–7.27	0.03	1.80	0.62–5.22	0.28
≤9.9	137						
HCC stage (TNM)							
III, IV	45	3.94	1.58–9.81	<0.01	1.48	0.50–4.31	0.48
I, II	136						
Semiannual surveillance							
No	73	6.84	1.98–23.64	<0.01	4.55	1.21–17.08	0.02
Yes	98						
Curative therapy*							
No	56	8.71	2.88–26.33	<0.01	5.93	1.88–18.67	<0.01
Yes	115						

*Curative therapy includes operative resection and radiofrequency ablation.

more advanced disease determined by TNM classification ($p = 0.06$). As to the imaging surveillance, a proportion of patients without receiving any imaging surveillance was significantly higher in NBNC-HCC than B/C-HCC ($p < 0.01$), and a proportion of patients receiving semiannual surveillance was tended to be low in NBNC-HCC than B/C-HCC ($p = 0.08$).

3.2. Factors Affecting the Survival of Patients after the Initial Diagnosis of HCC. When univariate analysis was performed to determine the variables affecting the survival of all the HCC patients, non-B, non-C etiology ($p < 0.01$, HR 6.26), DCP ≥ 40 mAU/mL ($p = 0.02$, HR 3.08), AFP-L 3% ≥10% ($p = 0.03$, HR 2.85), and HCC stage III/IV ($p < 0.01$, HR 3.94) were found to be significantly associated with poor survival (Table 2). No evident association was found in variables including Child-Pugh score, platelets, ALT level, and total AFP level. As for the imaging surveillance prior to HCC diagnosis, receiving semiannual surveillance prior to HCC diagnosis was associated with better survival ($p < 0.01$, HR 6.84). On the other hand, patients without curative therapy showed significantly short survival ($p < 0.01$, HR 8.71). In

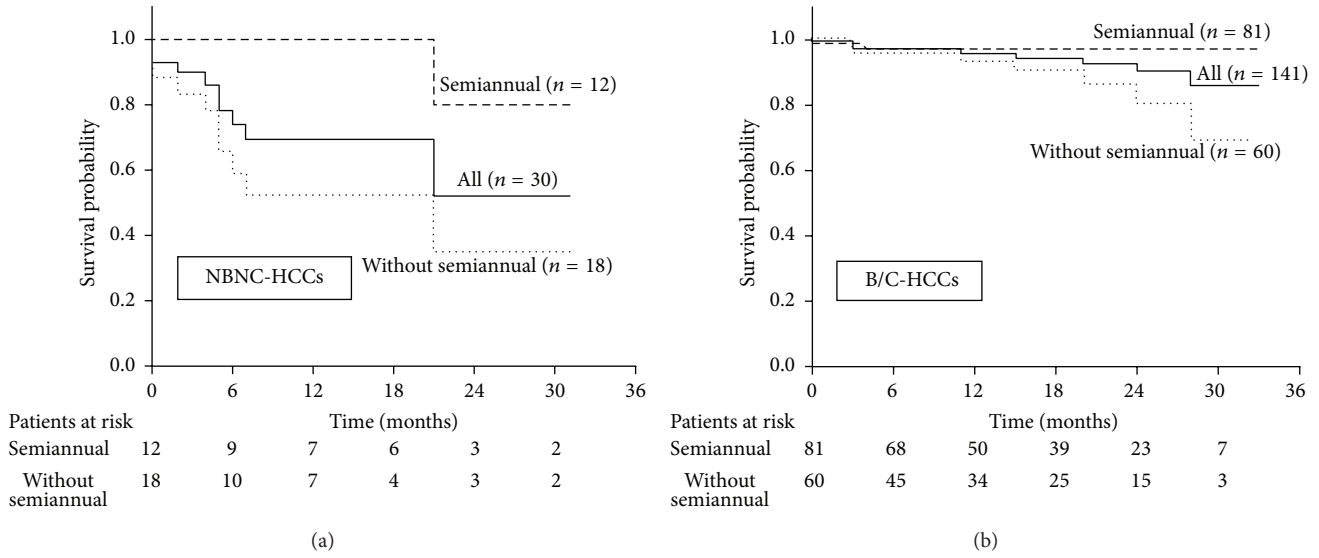

Figure 1: Comparison in the survival of HCC patients with and without semiannual imaging surveillance. Survival Kaplan-Meier curves in all NBNC-HCC patients, in those with semiannual surveillance, and in those without are demonstrated. One-year and 2-year survival in all NBNC-HCC patients are 69% and 52%, respectively, while that in those with semiannual surveillance and that in those without semiannual surveillance are 100% and 80%, and 52% and 35% ($p < 0.01$) (a). Survival Kaplan-Meier curves in all B/C-HCC patients, in those with semiannual surveillance and in those without are demonstrated. One-year and 2-year survival in all B/C-HCC patients are 95% and 90%, respectively, while that in those with semiannual surveillance and that in those without semiannual surveillance are 97% and 97%, and 94% and 80% ($p < 0.01$) (b).

a multivariate analysis, no semiannual surveillance ($p = 0.02$, HR 4.55), NBNC-HCC ($p < 0.01$, HR 4.32), and noncurative therapy ($p < 0.01$, HR 5.93) were extracted as independent variables associated with poor survival (Table 2).

3.3. The Correlation of Semiannual Surveillance and Survival. Kaplan-Meier curve analysis was performed to determine whether semiannual surveillance was associated with the survival rate of NBNC-HCC and B/C-HCC patients. As shown in Figures 1(a) and 1(b), the survival was significantly unfavorable in NBNC-HCC patients compared to that in B/C-HCC patients overall ($p < 0.01$, log-rank test). However, the survival was significantly favorable in the NBNC-HCC patients who had received semiannual surveillance than the patients lacking semiannual surveillance ($p = 0.02$, log-rank test) (Figure 1(a)). Likewise, the survival rate was also significantly high in the B/C-HCC patients who had received semiannual surveillance compared to those patients lacking such surveillance ($p = 0.04$, log-rank test) (Figure 1(b)). When the analysis was limited to patients who had received semiannual surveillance, the survival rate was almost comparable between the NBNC-HCC patients and the B/C-HCC patients ($p = 0.35$, data not shown). On the other hand, when the analysis was limited to patients who had not received semiannual surveillance, the survival rate was significantly lower for the NBNC-HCC patients than the B/C-HCC patients ($p < 0.01$, data not shown).

3.4. Tumor-Related Factors Associated with Semiannual Surveillance and Disease Etiology. Next, tumor-related factors (DCP, AFP, AFP-L3, tumor size, tumor number, vascular invasion, TNM stage, and therapeutic method) were compared between the NBNC-HCC and the B/C-HCC patients according to the status of the semiannual and nonsemiannual surveillance. As shown in Table 3, when tumor-related factors (DCP, AFP, AFP-L3, tumor size, tumor number, vascular invasion, TNM stage, and therapeutic method) were compared between the NBNC-HCC patients and the B/C-HCC patients among those who had received semiannual surveillance, there was no significant difference between these two groups as to factors except for DCP. However, when tumor-related factors were compared between the NBNC-HCC patients and B/C-HCC patients among those lacking semiannual surveillance, DCP value ($p < 0.01$) and vascular invasion ($p < 0.01$) were significantly worse and more advanced in the NBNC-HCC patients and those factors of tumor size and of HCC stage also tended to be worse in NBNC-HCC patients.

On the other hand, when tumor-related factors were compared between NBNC-HCC patients with and without semiannual surveillance, those tumor-related factors of tumor size, vascular invasion, HCC stage, and curative therapy were significantly unfavorable in those lacking semiannual surveillance.

4. Discussion

In this study, we demonstrated that NBNC-HCC patients receiving semiannual imaging surveillance had a significantly better survival than NBNC-HCC patients who lacked such surveillance, and this survival was comparable with that of the B/C-HCC patients with semiannual surveillance.

Table 3: Comparison of tumor-related factors between NBNC-HCCs and B/C-HCCs in each surveillance status.

Variable	Semiannual surveillance		p value (I) versus (II)	Without semiannual surveillance		p value (III) versus (IV)	p value** (I) versus (III)
	(I) NBNC $n = 12$	(II) B/C $n = 81$		(III) NBNC $n = 18$	(IV) B/C $n = 60$		
DCP (mAU/mL)							
≥40	6	18	0.04	13	25	0.02	0.22
≤39	6	63		5	35		
AFP (ng/mL)							
≥20.1	3	33	0.30	8	30	0.68	0.28
≤20.0	9	48		10	30		
AFP-L3 (%)							
≥10.0	1	14	0.43	5	14	0.70	0.19
≤9.9	11	67		13	46		
Tumor size (mm)							
≥21	3	16	0.67	16	39	0.051	<0.01
≤20.0	9	65		2	21		
Tumor number (n)							
1	8	53	0.93	8	32	0.51	0.23
≥2	4	28		10	28		
Vascular invasion							
Yes	0	2	0.58	6	4	<0.01	0.02
No	12	79		12	56		
HCC stage (TNM)							
III, IV	1	12	0.55	11	22	0.07	<0.01
I, II	11	69		7	38		
Curative therapy*							
No	2	27	0.25	9	35	0.53	0.04
Yes	10	54		9	25		

*Curative therapy includes operative resection and radiofrequency ablation.
**Comparison of NBNC-HCCs with versus without semiannual surveillance.

To date, it has not been clear whether regular, semiannual imaging surveillance would improve the survival of NBNC-HCC patients though establishing the role and protocol of the imaging surveillance in NBNC-HCCs is urgently needed and the importance of imaging surveillance has been suspected from previous studies [7, 10, 11]. Namely, there was no previous study in NBNC-HCC patients demonstrating the importance of imaging surveillance program by analyzing those patients in terms of the correlation between the surveillance and the survival improvement. In this study, compared to B/C-HCC patients, we demonstrated that the proportion of patients without any imaging surveillance was much frequent and the survival was significantly worse in NBNC-HCC patients overall. However, when we compared NBNC-HCC patients with and without semiannual imaging surveillance, we found that the survival was significantly favorable for those with semiannual imaging surveillance than those without. Moreover, the survival in NBNC-HCC patients with semiannual imaging surveillance was almost comparable with that in B/C-HCC patients with semiannual surveillance, disclosing the importance and usefulness of the semiannual imaging surveillance for improving the survival in NBNC-HCC patients (Figures 1(a) and 1(b)). In addition, when the B/C-HCC patients with semiannual surveillance were confined to those without previous antiviral therapy, there was a tendency that the survival of NBNC-HCC patients with semiannual imaging surveillance was rather favorable than that of B/C-HCC patients with semiannual surveillance (data not shown).

In multivariate analysis using the Cox proportional hazard model for the survival in all HCC patients, semiannual imaging surveillance was extracted as an independent determinant with the hazard ratio of 4.55, demonstrating the semiannual imaging surveillance to be significantly related to the improvement of the survival independent of liver disease etiology (Table 2). In B/C-HCCs, the importance of surveillance for the survival improvement has been established from various previous studies, and an interval of 6-month screening has been considered as most appropriate [26, 27]. In this study, though the benefit of surveillance for the survival in NBNC-HCC patients was evident, it is still insufficient to conclude that 6-month interval is the most appropriate

interval since patients with surveillance other than 6-month interval were few. However, from log-rank test, difference of the survival was more evident between NBNC-HCC patients with versus without semiannual surveillance than between those patients with versus without any surveillance (data not shown), suggesting the semiannual surveillance would be appropriate while further studies are warranted.

In this multivariate analysis, the etiology of liver disease as NBNC-HCC was also extracted as an independent determinant for the survival. However, we consider that the result does not reflect a biological difference in the malignant potential between NBNC-HCCs and B/C-HCCs, but that the result reflects the poor surveillance status in the NBNC-HCC patients without semiannual surveillance. Namely, the NBNC-HCC patients without semiannual surveillance had a significantly shorter survival than the B/C-HCC patients without semiannual surveillance while no evident survival difference was observed between the NBNC-HCC patients and the B/C-HCC patients if they had received semiannual surveillance (Figure 1). When the surveillance status was investigated further among the patients without semiannual surveillance, 47 out of 60 B/C-HCC patients (72%) had received some surveillance (although the surveillance interval had exceeded 6 months) while only 4 of 18 NBNC-HCC patients (22%) had received the surveillance (data not shown). When tumor-related clinical factors were compared between the NBNC-HCC patients and the B/C-HCC patients, limited to those without semiannual surveillance, NBNC-HCC patients showed more advanced disease in DCP and vascular invasion. Those without semiannual surveillance also tended to have larger tumors and advanced TNM stage (Table 3) while those factors except for DCP were almost comparable between NBNC-HCC patients and B/C-HCC patients if they received semiannual surveillance (Table 3). Collectively, it was considered that cause of the poor prognosis of the NBNC-HCC patients without semiannual surveillance was attributable to insufficient surveillance, resulting in the late HCC diagnosis.

Another important problem in diagnosing NBNC-HCCs is the lack of an appropriate biomarker to diagnose chronic liver disease existing as the background of the NBNC-HCCs [26, 27]. Without the diagnosis of NBNC-chronic liver disease, imaging surveillance cannot be performed. On the other hand, our study demonstrated that 90% (27/30) of NBNC-HCC patients were considered to have liver cirrhosis at the time of HCC discovery judged clinically in most of the patients by the occurrence of liver shrinkage, splenomegaly, or ascites or by the presence of esophageal varices (Table 1). Since an advanced liver fibrosis might underlie HCCs in the liver of NBNC-HCC patients, methods enabling easy and quantitative measurement of liver fibrosis could be candidates to screen and diagnose NBNC-chronic liver disease. In this aspect, several simple methods based on ultrasound or blood biochemical tests such as transient elastography or FIB-4 have been recently developed [28, 29]; these simple and easy modalities might be useful to screen and narrow down the NBNC-HCC candidate patients for the regular imaging surveillance from general population. Actually, only minor proportion of our NBNC-HCC patients was diagnosed as having NBNC-chronic liver disease before HCC development. On the other hand, once the diagnosis of NBNC-chronic liver disease was made, most of these patients received the semiannual imaging surveillance by applying the HCC surveillance guidelines [14, 19, 25] which were made based upon the studies for viral hepatitis.

In conclusion, we demonstrated that semiannual imaging surveillance was significantly associated with the early HCC diagnosis and the favorable survival in NBNC-HCC patients though prospective studies with larger number of patients are still mandatory to confirm our result and to determine the optimal surveillance intervals further.

Conflict of Interests

All authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Virological Mechanisms in the Coinfection between HIV and HCV

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Due to shared transmission routes, coinfection with Hepatitis C Virus (HCV) is common in patients infected by Human Immunodeficiency Virus (HIV). The immune-pathogenesis of liver disease in HIV/HCV coinfected patients is a multifactorial process. Several studies demonstrated that HIV worsens the course of HCV infection, increasing the risk of cirrhosis and hepatocellular carcinoma. Also, HCV might increase immunological defects due to HIV and risk of comorbidities. A specific cross-talk among HIV and HCV proteins in coinfected patients modulates the natural history, the immune responses, and the life cycle of both viruses. These effects are mediated by immune mechanisms and by a cross-talk between the two viruses which could interfere with host defense mechanisms. In this review, we focus on some virological/immunological mechanisms of the pathogenetic interactions between HIV and HCV in the human host.

1. Introduction

Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV) cause considerable global health problems. Coinfection with HCV is frequent in HIV infected individuals, because the viruses share their modes of transmission. So, in the United States, approximately 25% of 1.2 million HIV infected patients are coinfected with HCV [1, 2]. Moreover, in Europe and Asia, rates of HCV coinfections among HIV infected individuals who used injection drugs overcame 90% [2]. Although in the era of Highly Active Antiretroviral Therapy (HAART) worthy achievements have been obtained in the treatment of HIV and HCV infections, HCV-related liver disease remains a significant therapeutic challenge in HIV/HCV coinfected patients.

While HCV is mainly a hepatotropic virus, HIV infects a variety of immune cells, such as CD4+ T lymphocytes and monocytes/macrophages. However, several studies showed that HCV replicates outside the liver [3], while HIV may infect hepatocytes and Hepatic Stellate Cells (HSCs) as well [4].

The disease course of HIV-1 infection is associated with a profound dysregulation of the immune system. During HIV/HCV coinfection, the immune dysregulation is more severe, leading to lower rates of spontaneous control of HCV infection as well as to a faster progression of liver disease [5]. The immunopathogenesis of accelerated hepatic fibrosis is a multifactorial event [6] and several mechanisms have been postulated:

(1) HIV associated immune dysfunction;

(2) defective antiviral CD8+ T cells responses;

(3) reduced CD4/CD8 ratio;

(4) direct activation of HSCs by HIV gp 120 or via proinflammatory mediators [7–9];

(5) HIV and HCV inducing production of Reactive Oxygen Species (ROS) which activate the Mitogen-Activated Protein Kinases (MAPK) pathway and upregulate TGF-β [10, 11];

(6) stimulation of HCV infected hepatocytes by HIV gp 120 that induces HCV replication via TGF-β, which modulates the immune response and favors fibrosis and transformation toward hepatocellular carcinoma [1, 10];

(7) hepatocytes exposed to HCV and HIV envelope proteins that undergo apoptosis and, in particular, HCV E2 and HIV gp 120 that induce apoptosis via a Fas-FasL-dependent pathway [5–7];

(8) HCV core and NS3 proteins that trigger, through Toll-Like Receptor- (TLR-) 2 and Interleukin- (IL-) 1 Receptor-Associated Kinase (IRAK) activity, the release of inflammatory cytokines and chemokines by HSCs [6].

Although the impact of HCV on HIV natural history is debated, the contribution of HCV core in enhancing HIV-1 infection in macrophages has been recently established [12]. Also, it has been suggested by some studies, but not by others, that the immunological response after HAART is impaired in HCV coinfected patients [13].

In this review we focus on the influence of HIV on HCV infection and vice versa. In particular, virological and immune escape mechanisms of HIV/HCV pathogenesis have been reviewed.

2. HIV/HCV Infection as Challenge to the Immune System

HCV and HIV evade the host immune response through intricate processes including signaling interference, effector modulation, and continuing viral genetic variations [1, 14].

Viral RNA is recognized via TLRs or the Retinoic Acid Inducible Gene I (RIG-I) helicase [15]. These interactions activate downstream signaling pathways, inducing type I interferon (IFNα/β) and other antiviral effects, which are the first innate immune response against intracellular pathogens [16].

IFNα/β is produced by plasmacytoid dendritic cells (pDCs), myeloid DCs (mDCs), and hepatocytes [16]. The production of IFN-α induces the expression of several IFN Regulatory Factors (IRFs) and the induction of IFN-Stimulated Genes (ISGs), leading to an antiviral state in cells and promotion of proliferation [17, 18].

HCV acts with several immune escape mechanisms interfering with type I interferon signal transduction molecules [16]. For instance, HCV NS3/4 protease cleaves proteins such as TIR-domain-containing adapter-inducing interferon-β (TRIF) and CARD adapter-inducing interferon-β (CARDIF), which are required for rapid induction of IFN-β through the activation of IRF-3 [19]. Moreover, NS5A, a protein that plays a crucial role in viral RNA replication, virus assembly, and viral pathogenesis [20], and E2, an envelope glycoprotein [21], mediate inhibition of protein kinase RNA-activated (PKR) activity [22].

Similarly, HIV-1 takes part in pathogen-associated molecular pattern via TLRs, because it contains motifs that are recognized by TLR8 [23] and TLR7 [1] and also interfere with PKR activity through Tat protein, a small activator of viral transcription from the Long Terminal Repeat (LTR) promoter [24].

Type I IFN secreted from DCs promotes recruitment and activation of natural killer (NK) cells to the site of infection [20].

NK cells are mediators of antiviral defenses and constitute a significant proportion of liver-infiltrating lymphocytes during HCV infection and an impairment of their function favors viral persistence [20].

In particular, HCV E2 protein can interfere with NK receptors, altering their function through cross-linking of CD81 [25, 26]. In fact, Tseng and Klimpel demonstrated that CD81 cross-linking inhibits cytotoxicity and IFN production of NK cells through immobilized E2 or specific CD81 monoclonal antibody (mAb) [27]. On the side of HIV, the following are well recognized: the decrease in number and dysregulation of CD4+ T cell function, the reduction of antifibrotic activity of NK cells, and production of an atypical *milieu* of ILs (IL-4, IL-5, IL-10, and IL-13) [25, 26]. Altogether these events may contribute to a profound dysregulation of the immune system as follows.

Dysregulation of the immune system in the pathogenesis of liver fibrosis progression (HIV/HCV coinfected patients) is as follows:

(1) lymphocyte apoptosis and CD4+ T cell depletion [1, 28–30];

(2) imbalance among CD4 and CD8 cell responses: this altered ratio is correlated with modified cytokine networks (such as increase in TGF-β and decrease in IFN-γ [6];

(3) "by-stander effect": HIV-1-specific CD8+ T cells are attracted to the liver of HIV/HCV coinfected patients, contributing to release of profibrotic cytokines [29];

(4) aberrant dysregulation of natural killer cells function which leads to altered secretion of cytokines [25–27, 31–33].

Thus, a complex framework is established in the pathogenesis of HIV/HCV coinfections which ends up with a fast progression of liver disease [31].

3. Do HIV Proteins Affect HCV Infection?

Among HIV proteins, a role of gp 120, Rev, Tat, Nef, and Vpr in enhancing HCV replication has been established [10, 34–37].

HIV infection produces effects on hepatocytes and HSCs; indeed, both cells express key HIV coreceptors; the interaction of HIV gp 120 with C–C chemokine receptor type 5 (CCR5) and C–X–C chemokine receptor type 4 (CXCR4) activates specific cell signaling in the liver [6, 28, 29, 38, 39].

The HIV envelope protein gp 120 has been shown to promote hepatocyte apoptosis, hepatocellular secretion of the proinflammatory cytokine IL-8 [38], proinflammatory and profibrogenic effects on HSCs, and directional migration [9].

Moreover, a link between HIV infection and liver fibrogenesis has been demonstrated *in vitro*. In particular, HIV gp 120 induces a significant increase in HSCs chemotaxis and increased expression of the proinflammatory chemokine Monocyte Chemoattractant Protein-1 (MCP-1), IL-6, and tissue inhibitor of metalloproteinase-1 (TIMP-1), thereby leading to increased liver inflammation and fibrogenesis [9].

Concerning its effect on increased HCV replication, as demonstrated by Lin et al. [10], it has been shown that inactivated HIV and gp 120 enhance HCV replication in a CXCR4 or CCR5 engagement-dependent manner. Enhancements of HCV-regulated Transforming Growth Factor- (TGF-) β1 have been also shown in both a replicon and an infectious model of HCV infection [10].

Coinfection with HIV-1 causes increased HCV viral loads, as well as enhanced morbidity in coinfected individuals [40]. HIV contributes to the stimulation of HCV replication and this may change the course of HCV-related liver disease. Mechanisms of HIV effects on HCV replication are not fully clear. However, upregulation of HCV replication may be due to HIV viral proteins, which are secreted from HIV infected cells and diffused into hepatocytes [11, 34].

As demonstrated by Qu et al. [41], HIV Rev protein is a pivotal regulatory protein in the early-to-late switch in the virus life cycle and is involved in the promotion of translocation into the nuclear compartment, translation, and encapsidation of viral RNAs [41]. HIV Rev protein stimulates HCV gene expression through its binding with first internal loop (IIIb) of 5′-Untranslated Region (5′-UTR) HCV RNA [41]. Moreover, Rev regulates Internal Ribosome Entry Site- (IRES-) mediated translation of HCV *via* an enhanced polysomal association of Rev-responsive element- (RRE-) containing RNAs [42].

HIV-1 Tat is a transactivating protein which determines transactivation of viral and cellular genes [43], triggering virus invasion [44, 45]. Tat is released from HIV infected cells and induces its biological effects such as cytokine expression and CCR5 and CXCR4 receptors on neighboring infected or uninfected cells [46]. Qu et al. [35] used two different infectious HCV models to investigate the effects of HIV-1 Tat and interferon gamma-induced protein 10 (IP-10) on HCV replication, demonstrating that both HIV-1 Tat and IP-10 activate HCV replication. Moreover, HIV-1 Tat activates HCV replication by upregulation of IP-10 production, which in turn has been correlated with increased liver damage and higher HCV RNA in HIV/HCV coinfected patients [47].

The viral protein Nef exerts pleiotropic effects during HIV infection and regulates multiple host factors [48]. Nef modifies actin remodeling in various cell systems, alters actin rearrangements, and inhibits immunological synapse formation [49]. Nef also induces the extension of long intercellular conduits allowing its own transfer [50]. Moreover, as demonstrated by Park et al. [34], HIV Nef protein increases HCV replication in the target cells (such as subgenomic replicon cells) most likely through changes in the size and number of lipid droplets. Indeed, it is interesting to observe a drastic increase of HCV replication and an increase of ROS (a critical regulator of hepatic fibrosis progression) when the Nef-expressing cells are treated with ethanol [34, 51–53].

HIV Vpr, a multifunctional protein, mediates many processes such as activation of HIV-1 infection, evasion of the immune system, and induction of infection persistence in the host [54]. Vpr molecular functions include the following: (i) nuclear import of viral Preintegration Complex (PIC); (ii) transcriptional activation of viral and host genes; (iii) regulation of Nuclear Factor kappa B (NF-κB) activity; and (iv) modulation of T-cell apoptosis [54]. Peng et al. [55] provided evidences to support relationships among HIV Vpr, microRNA 122 (miR-122), and HCV replication. However, several lines of evidence showed that Vpr promotes not only HCV RNA replication, but also protein expression, enhancing the HCV 5′ UTR activity through the stimulation of TATA box in the miR-122 promoter [55–58]. Moreover, miR-122 inhibition produces a significant reduction of Vpr-induced HCV 5′ UTR activity [55]. However, miR-122 inhibitor cannot fully abrogate the Vpr-induced HCV replication, suggesting that other mechanisms may create a favorable environment for maximizing virus replication [59, 60].

4. Do HCV Proteins Affect HIV Infection?

Despite the fact that HCV coinfection is able to increase immune activation and CD4 apoptosis [3], the mechanisms by which HCV modulates HIV replication are not completely understood.

HCV is a positive strand RNA virus whose genome encodes for a single polyprotein cleaved by host and cellular proteases to generate four structural (Core, E1, E2, and p7) and six nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [61].

The current knowledge on HCV proteins in the regulation of HIV-1 replication and the molecular mechanism involved will be reviewed below.

NS3 and NS4A proteins associate to form an active enzyme with RNA helicase and serine protease activities involved in the proteolytic processing of NS proteins. NS4A protein is required for proper functioning, while NS3 is a multifunctional protein with serine protease activity at the N-terminal and a Nucleoside-Triphosphatase (NTPase) dependent RNA-helicase activity (NS3 NTPase/helicase) at the C-terminal [62].

As demonstrated for the first time by Simmonds [61], the HCV NS3/4A protein can activate HIV-1 transcription from its LTR region, suggesting that binding activities of the transcription factor activating protein-1 (AP-1) are part of the mechanism involved. The serine protease activity of NS3/4A is essential for such activation effect [66]. Kang et al. developed an *in vitro* model of coinfection [36], showing that NS3 protein of HCV enhances HIV-1 LTR transcription and that Vpu protein regulates transcription of the HIV-1 genome by interacting with NS3/NS4A complex of HCV. Indeed, Vpu removes NS3 from the NS3/NS4A complex of HCV, thus

Table 1: Interactions among proteins of HIV and HCV (relevance for HCV/HIV coinfection models).

HCV proteins	Effect on HIV replication
NS3/NS4A	It interacts with HIV-1 Vpu promoting HIV transcription. Vpu facilitates degradation of NS3/4A and nuclear transfer of NS3 which can activate HIV-1 transcription [36].
Core	It restricts HIV-1 transcription and modulates viral replication in a hepatoma cell line through a repression before accumulation of threshold levels of Tat protein [63].
	It downregulates HIV LTR activity, in presence of high TNF-α level [3].
	It activates the TRAF pathway interacting with HIV-1 Nef, activating the NF-κB pathway via TRAF2, TRAF5, and TRAF6 pathways, and enhancing HIV-1 replication in macrophages [64].
	It induces HIV-1 reactivation in U1 cells through TNF-α and IL-6 [12].
HIV proteins	**Effect on HCV replication**
gp120	It enhances HCV replication in a CXCR4 or CCR5 engagement-dependent manner [9–11, 31, 34–36, 38–40, 65].
Rev	It increases gene expression of HCV by binding to the first internal loop (IIIb) of 5′-Untranslated Region and sites IRES of HCV RNA [42].
Tat	It activates HCV replication by upregulating IP-10 [47].
Nef	It exerts stimulatory effects on HCV replication, modifying the size and numbers of lipid droplets, increasing ROS, and, possibly, accelerating progression of liver disease [51–53].
Vpr	It enhances activity of 5′-Untranslated Region of HCV through stimulation of TATA box in the miR-122 promoter, upregulating miR-122 expression [55, 59, 60].

HCV: Hepatitis C Virus; HIV-1: Human Immunodeficiency Virus 1; NS: Nonstructural protein; CXCR4: C-X-C chemokine receptor type 4; CCR5: C-C chemokine receptor type 5; IRES: Internal Ribosome Entry Site; LTR: Long Terminal Repeat; TNF-α: Tumor Necrosis Factor alfa; IP-10: interferon gamma-induced protein 10; TRAF: TNF Receptor Associated Factor; NF-κB: Nuclear Factor κB; ROS: Reactive Oxygen Species; U1: HIV-1 latently infected U1 monocytic cell line; IL: Interleukin; miR: microRNA.

promoting NS3 nuclear translocation for the activation of HIV-1 transcription [36].

HCV core is mainly a cytoplasmic component, located on the endoplasmic reticulum membranes and around lipid vesicles [67]. It is unique in its pleiotropic effects: in addition to its role in packaging viral RNA, it can indeed modulate cellular transduction pathways, transactivate a number of cellular promoters, regulate viral and cellular gene expression, modulate apoptosis, and inhibit host immunity [68].

Although contributions of HCV core to HIV pathogenesis remain controversial, this core protein has been shown to restrict HIV-1 transcription and modulate viral replication in a hepatoma cell line through a repression before accumulation of threshold levels of Tat protein [63]. Moreover, to better explain the influence of the HCV on HIV replication, Sengupta et al. [3] evaluated HIV LTR in hepatocytes through the analysis of basal and/or Tat-induced activation in presence of HCV core protein, TNF-α, and infectious HCV [3]. These authors demonstrated that HIV LTR activity was downregulated by HCV core protein with high TNF-α levels and that, conversely, it was increased by infectious hepatitis C virions. These data suggest that inhibitory activity of HCV core protein is unchanged and both host cellular where HCV viral proteins influence HIV replication [3].

Khan et al. [64] proposed that HIV-1 Nef and HCV core protein activate the NF-κB canonical pathway in primary macrophages through TNF Receptor Associated Factor (TRAF) 2, TRAF5, and TRAF6 pathways and enhance HIV-1 LTR-driven luciferase expression in a transiently transfected human monocytic cell line through the same pathways. Therefore, this mechanism may promote HIV-1 replication and represent a critical factor for optimal replication of HIV-1 in macrophages of HIV-HCV-coinfected patients. Lastly, Swaminathan et al. [12] analyzed the effect of HCV core on HIV-1 replication in promonocytic cell line THP-1, primary monocyte-derived macrophages (MDMs), and in the HIV-1 latently infected U1 monocytic cell lines. They found that HCV core enhances HIV-1 infection in both THP-1 cells and primary macrophages. Particularly, HCV core protein promotes HIV-1 infectivity in macrophages via TLR2-, JNK-, and MEK1/2-dependent pathways, while a differential activation/regulation of p38 kinase in THP-1 and MDMs was found. Interestingly, although HCV core lacks ability to directly reactivate HIV-1 in latently infected U1 cells, conditioned media (CM) of THP-1 macrophages and primary MDMs of HCV core-stimulated macrophages induced HIV-1 reactivation in U1 cells through TNF-α and IL-6. Therefore, these studies definitely established a role of HCV core in exacerbation of acute and latent HIV-1 infection in macrophages.

Interactions between HIV and HCV proteins and their effect on replication of both viruses are summarized in Table 1.

5. Conclusion

Several *in vitro* and *in vivo* studies indicated that HCV and HIV interact with each other and with innate or adaptive immune system exerting a variety of effects and promoting

a series of hypothesis to be tested in future studies as follows:

(1) HIV infection is associated with a profound dysregulation of the immune system; during HIV/HCV coinfection, this immune dysregulation is more severe, leading to lower rates of spontaneous control of HCV replication and to a faster progression of liver disease [5, 6];

(2) cross-talk among HCV and HIV proteins modulates fibrogenic/inflammatory mediators, immune system response, and replication of both viruses [6];

(3) characterization of viral protein interactions and their effects both on replication of these viruses and on liver function at a cellular level will significantly improve our understanding of HIV/HCV pathogenesis [3, 34, 36, 55, 59, 64, 66].

It is clear from this review that several problems remain to be understood for a better comprehension of the multiple virus-virus and virus-host interactions that can lead to liver fibrosis and enhancement of the pathogenetic effects of both viruses. The development of coculture systems that model the effects of HCV/HIV in hepatocytes will advance our understanding of the pathogenesis of this coinfection. However, it is not obvious that *in vitro* interactions are confirmed *in vivo*. So, a suitable animal model could provide deeper understanding of virus-virus interactions and immunological relationships. Hopefully, recognition of the cause-effect relationships between infection, inflammation, and liver fibrosis progression in HIV/HCV coinfected patients could lead to therapeutic approaches to better control these viruses.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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