

HEPATOLOGY

Prevalence and clinical characterization of patients with acute hepatitis B induced by lamivudine-resistant strains

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Key words

acute hepatitis, genotype, hepatitis B virus, lamivudine.

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Abstract

Background and Aims: Acute hepatitis caused by lamivudine (LMV)-resistant strains has not been reported, and the clinical impact of LMV-resistant strains on acute hepatitis is not known. The aim of this study was to investigate the molecular and clinical characteristics of patients with acute hepatitis B caused by LMV-resistant strains.

Methods: Forty-five patients with acute hepatitis B were studied. Hepatitis B virus (HBV) subgenotypes and LMV-resistance mutations were determined by direct sequencing of the preS and polymerase regions, respectively.

Results: HBV subgenotypes A2 ($n = 18$), B1 ($n = 1$), B2 ($n = 3$), B3 ($n = 2$), C1 ($n = 1$), C2 ($n = 19$) and C6 ($n = 1$) were detected in patients with acute hepatitis. LMV-resistance mutations were detected in two patients. LMV-resistance mutations (L180M, M204I) were detected in a patient with subgenotype C2 who had acute self-limited hepatitis. The other patient with LMV-resistance mutations (L180M, M204V) was infected with subgenotype A2 and had severe hepatitis.

Conclusion: LMV-resistant strains are rare, but they are starting to be found in patients with acute hepatitis B. Surveillance for detecting drug-resistant HBV strains would be important for clinical practice.

Introduction

Approximately 350 million people worldwide are infected with hepatitis B virus (HBV).¹ HBV infection causes a variety of clinical courses, such as self-limited acute hepatitis, fulminant hepatic failure, chronic hepatitis, and progression to cirrhosis and hepatocellular carcinoma.² Therefore, HBV infection is one of the most important global health problems. Most countries have performed universal vaccination to prevent HBV infection, but only high-risk groups, such as health-care workers and household contacts of HBV carriers, have received HBV vaccination in Japan.³ Therefore, acute hepatitis is still a major problem in Japan. The frequencies of HBV strains that are rare in Japan have increased among Japanese patients with acute hepatitis B.⁴⁻⁶ The distributions of the HBV strains in acute hepatitis are variable due to the changing social environment. Along the same lines, a study investigated acute hepatitis B induced by lamivudine (LMV)-resistant HBV strains, but acute hepatitis caused by an LMV-resistant strain has not been found, and the clinical impact of LMV-resistant strains on acute hepatitis is still unknown.⁷ Surveillance of HBV strains associated with acute hepatitis B has been continued, and LMV-

resistant strains have begun to be detected in patients with acute hepatitis B. Thus, the present study reports the clinical characteristics of patients in Japan with acute hepatitis B caused by LMV-resistant HBV strains.

Materials

Forty-five Japanese patients with acute hepatitis B who were treated at Nagoya University Hospital, Ogaki Municipal Hospital, Tosei Hospital, Yokkaichi Hospital, and Fujita Health University Hospital were enrolled in this study between January 2006 and September 2008. The patients were 37 men and eight women, with a mean age of 38.6 ± 12.9 years (range, 18–84 years). There were no patients who had received HBV vaccine. Acute hepatitis B was diagnosed as follows. Each patient had high titers of hepatitis B surface antigen (HBsAg) and immunoglobulin (Ig)M class antibody against HBV core antigen, elevated serum levels of alanine aminotransferase and absence of antibodies against other causative viruses, such as hepatitis A virus, hepatitis C virus, Epstein-Barr virus and cytomegalovirus. It was necessary to discriminate

between initial HBV infection and acute onset or reactivation of chronic HBV infection. Thus, serum HBsAg levels noted in previous medical records, blood donation screening, labor and delivery screening, or employment health screening, were obtained or were followed until negative of HBsAg and/or positive of hepatitis B surface antibody (HBsAb). No patients were using chemotherapeutic and immune modulating agents involved in HBV reactivation. Informed consent was obtained from all patients, and the study was carried out in accordance with the 1975 Helsinki Declaration. Serum was stored at -80°C for virological examinations.

Assay methodology

Hepatitis B virus DNA was isolated from peripheral blood with a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Nested polymerase chain reaction (PCR) analysis and direct sequencing of the preS, polymerase and precore/core regions were performed as reported previously.⁷ In brief, each 50- μL PCR reaction contained 100 nM each primer, 1 ng template DNA, 5 μL GeneAmp 10 \times PCR buffer, 2 μL deoxyribonucleotide triphosphate and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Primers were: preS region sense 5'-TCACCTATTCT TGGGAACAAGA-3' and antisense 5'-GGCACTAGTAAACTG AGCCA-3'; polymerase region, sense 5'-CCTGCTGGTGGCT CCAGTTC-3' and antisense 5'-GGTTGAGTCAGCAAACAC ACTTG-3'; and precore/core region, sense 5'-ATGTCGACAA CCGACCTTGA-3' and antisense 5'-GTATGGTGAGGTGAAC AATG-3'. Amplification conditions consisted of 5 min at 94°C followed by 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was done in the same reaction buffer with the first-round PCR product as template and the following sets of primers: preS region, sense 5'-TCACCTATTCTT GGGAAACAAGA-3' and antisense 5'-AGAAGATGAGGCATAG CAGC-3'; and polymerase region, sense 5'-GGATGTGCTGC GGCGTTT-3' and antisense 5'-ACCCCATCTTTTTGTTTTG TTAGG-3'. PCR products were detected by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator ver. 1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems). The neighbor-joining method⁸ was used for phylogenetic analysis of the preS region to identify HBV subgenotypes. The bootstrap test with 1000 replicates was performed to confirm the reliability of the phylogenetic tree.⁹

Results

The results of the phylogenetic analyses of HBV subgenotypes of the 41 patients are shown in Figure 1. The HBV subgenotypes A2 ($n = 18$), B1 ($n = 1$), B2 ($n = 3$), B3 ($n = 2$), C1 ($n = 1$), C2 ($n = 19$) and C6 ($n = 1$) were detected. The prevalence of subgenotype A2 was increased, as previously reported. LMV resistance-associated mutations were detected within the HBV polymerase region (positions 116–214) by direct sequencing. Alignment of the amino acid sequence of the HBV polymerase region with LMV resistance-associated mutations was analyzed, and LMV-associated mutations could be detected in two patients at acute hepatitis onset.

LMV-resistance mutations (L180M, M204I) were detected in a patient with subgenotype C2. The other patient with subgenotype A2 had LMV-resistance mutations (L180M, M204V). There were no resistant HBV mutants for other nucleoside/nucleotide analogs such as V173L, L180M or M204V/I. The clinical and virological characteristics of patients with LMV-resistant HBV strains are summarized in Table 1.

Discussion

Hepatitis B virus reverse transcriptase is an error-prone enzyme without proofreading capacity, and it is easy for frequent mutations to occur during viral replication. As a result, there are many well-known mutations that are associated with the pathogenesis of HBV infection.¹⁰ LMV-resistant strains that have mutations in the polymerase region are induced by long-term administration of LMV.^{11,12} LMV had been used widely for treatment for chronic hepatitis B and was available from 2000 in Japan. LMV-resistant strains have emerged in patients with chronic hepatitis. However, the prevalence and clinical impact of LMV-resistant strains in patients with acute hepatitis B are unknown. Thus, surveillance of LMV-resistant strains associated with acute hepatitis B had been conducted, but LMV-resistant strains could not be detected in 2006.⁷ The possibility of acute hepatitis B caused by LMV-resistant strains exists, and the surveillance has continued. Of 45 patients with acute hepatitis, two were found to have LMV-associated mutations. We previously hypothesized that LMV-resistant strains may not have enough power to cause acute hepatitis. However, the present study demonstrated that LMV-resistant strains would have infectivity and would be capable of causing acute hepatitis. Less opportunity for infection may explain why previous studies failed to find acute hepatitis caused by LMV-resistant strains.

The infectious source of the LMV-resistant strains could not be confirmed. The subgenotypes of the patients infected with LMV-resistant strains were subgenotype A1 and C2, respectively. The patient infected with subgenotype C2 plus LMV-resistant strain had a history of sex with a prostitute 1 month before admission. Subgenotype C2 was the predominant subgenotype found in Japanese patients with chronic hepatitis B.^{7,13–15} The infectious source would be a chronic hepatitis patient who developed resistant HBV mutants during long-term LMV treatment. The route of infection for the other patient with subgenotype A2 was unknown. HBV subgenotype A2 has been rarely reported in Japanese patients with chronic hepatitis B. However, subgenotype A2 has been increasing and has become responsible for the majority of patients with acute hepatitis B.^{4,7,16} This study also confirmed that HBV subgenotype A2 has become widespread among Japanese patients with acute hepatitis. However, the origin of subgenotype A2 with an LMV-resistant mutation is not clear. The possibility of it coming from a patient with chronic hepatitis B is low, because subgenotype A2 is rarely found in Japanese patients with chronic hepatitis B who receive long-term LMV treatment. The other possible infectious source is a patient co-infected with HIV. Nucleoside/nucleotide analogs (NA) such as LMV were effective for both HBV and HIV. NA were used not only for treatment of HBV but also for treatment of HIV, and LMV-resistant strains have been reported.¹⁷ HBV genotype A and HIV co-infection have been found among male

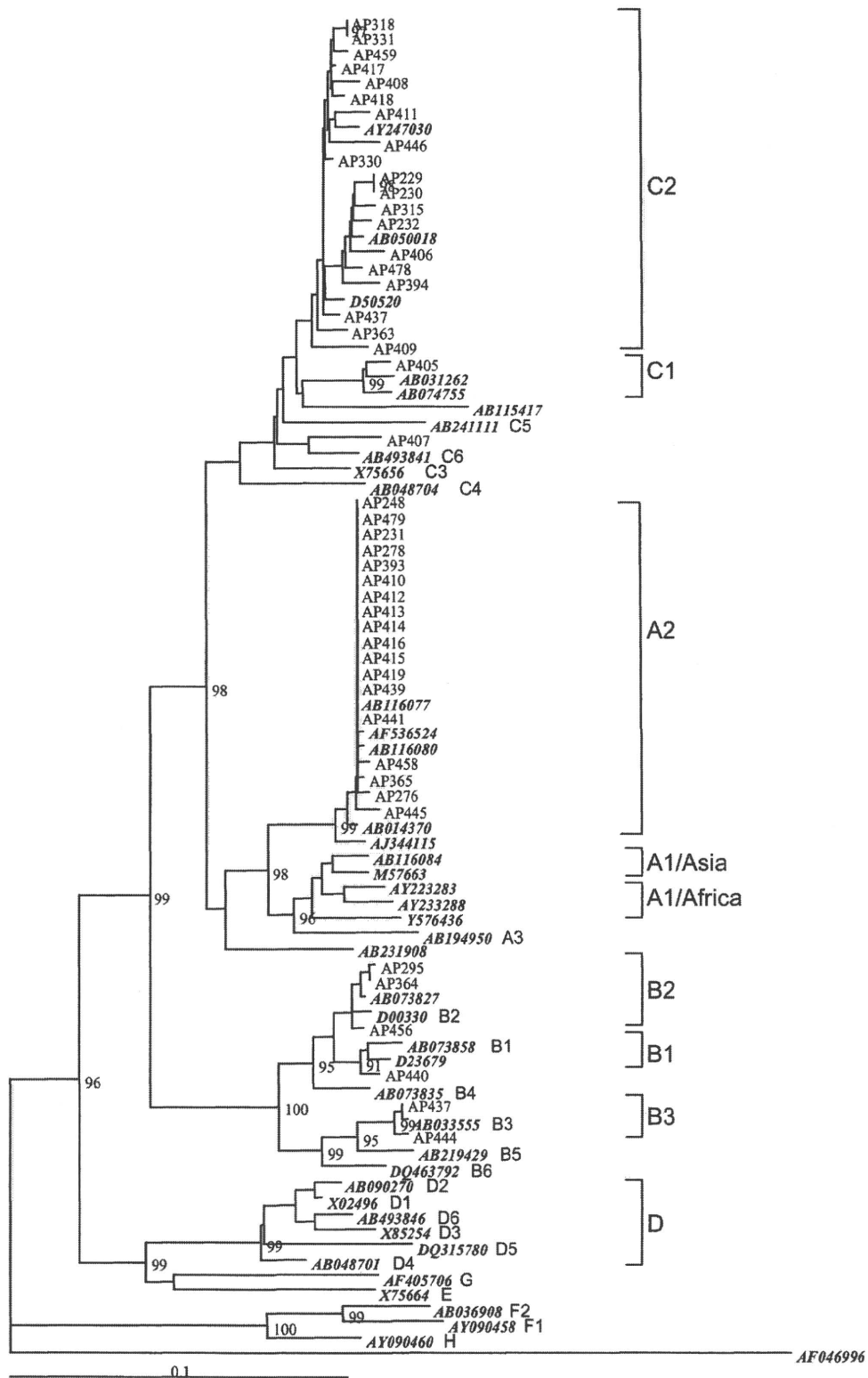


Figure 1 Results of phylogenetic analysis of 45 sequences from the preS region of hepatitis B virus (HBV) of acute hepatitis patients and 42 reference strains from a database and shown by accession number. Strains isolated from patients with acute hepatitis are indicated as AP. Phylogenetic analysis was performed by the neighbor-joining method with Woolly monkey HBV (AF046996) as out-group. Percentages of bootstrap values greater than 90% are shown on the nodes. The scale bar indicates genetic distance.

Table 1 Clinical characteristics

	Case 1	Case 2
Age (years)	32	32
Sex	Male	Male
ALT (IU/L)	4429	2820
AST (IU/L)	2709	1620
T Bil (mg/dL)	3.0	4.1
HBeAg	Positive	Positive
HBV (log copies/mL)	5.2	7.4
BCP1762/1764	T/A	A/G
PC1896	G	G
Route	STD	Unknown
Subgenotype	C2	A2

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; STD, sexually transmitted disease; T Bil, total bilirubin.

patients who have sex with men in Japan.¹⁸ Because the patient infected with subgenotype A2 that was LMV-resistant was not co-infected with HIV, this was also inconclusive. The other possibility was that the infectious source could have been a foreign patient with subgenotype A2 in whom an LMV-resistant strain emerged. This study has the following limitations: a small number of patients, patients without symptom were not recruited, the identification of the infectious source. Thus, further studies such as a nationwide survey including blood banks to investigate asymptomatic patients, the need to make conclusion of the prevalence of patients with acute hepatitis B induced by LMV-resistant strains in Japan.

The patient with LMV-resistant mutations with subgenotype C2 developed self-limited hepatitis, while the other patient with LMV-resistant mutations with subgenotype A1 developed severe acute hepatitis. Basal core promoter (BCP) and precore (PC) variants have been shown to be associated with the severity of the clinical course of acute hepatitis. In particular, mutations at BCP/PC of HBV subgenotype C2 and B1 can increase the risk of progression to fulminant hepatic failure. The clinical impacts of basal core promoter and precore variants in other genotypes are unclear.^{7,16} In the present study, both patients with acute hepatitis caused by LMV-resistant strains had wild-type BCP/PC variants. The wild-type BCP/PC variants were linked with mild self-limited hepatitis in the patient with subgenotype C2. The clinical impact of LMV-resistant strains on acute hepatitis appears to be not serious for subgenotype C2. Meanwhile, the mutations in the BCP/PC regions were not associated with the severity of acute hepatitis in the patient with subgenotype A2. Therefore, LMV-resistant mutations in subgenotype A2 might be associated with the severity of the clinical course. However, the present sample size was too small to allow evaluation of the clinical course in acute hepatitis B with LMV-resistant strains and to determine whether LMV-resistant strains have different effects on each subgenotype. Further studies are needed to clarify the influence of LMV-resistant strains on the clinical course of acute hepatitis B.

Lamivudine has begun to be used to treat patients with acute hepatitis to prevent progression to fulminant hepatic failure or chronic hepatitis. Some reports have shown the safety and effectiveness of LMV for the treatment of acute hepatitis B.^{19,20}

However, one clinical study that has been published did not confirm its efficacy.²¹ Thus, the administration of LMV in acute hepatitis B is controversial. The use of LMV for all acute hepatitis was not of benefit and was not recommended for use in all patients. However, selected patients who have a high risk for progression to fulminant hepatic failure and chronic infection may benefit from LMV to prevent disease progression. There is a small possibility that acute hepatitis B can be caused by LMV-resistant strains, but previous studies did not consider LMV-resistant strains before they started to use LMV. Caution must be exercised when determining whether LMV should be used to treat acute hepatitis B because of the possibility of the development of LMV-resistant strains. In the present study, the patient with LMV-resistant mutations who progressed to severe hepatitis was treated with LMV and steroid. Despite the limited efficacy of LMV in suppressing viral replication of LMV-resistant strains, this patient recovered from severe acute hepatitis. Patients with severe acute hepatitis have a high risk for progression to fatal liver failure. However, patients not treated with LMV may have a full recovery and not progress to fulminant liver failure, either because of the efficacy of other treatment, such as steroid, or because the patients' immune reaction could clear the HBV infection. It is difficult to judge the clinical role of LMV-resistant strains in acute hepatitis based on this case. The present study included insufficient information about the magnitude of screening for LMV-resistant strains in acute hepatitis.

Lamivudine is associated with a high incidence of resistance.²² Thus, the first-line agent for HBV infection has been changed from LMV to adefovir or entecavir because of their powerful antiviral effect and the lower likelihood of drug resistance mutations emerging. The emergence of drug resistance during long-term adefovir or entecavir therapy in chronic hepatitis B was not frequent compared to that with LMV.^{23,24} With adefovir or entecavir, the incidence of LMV-resistant strains would be remarkably decreased, but the risk for other HBV drug-resistant strains still remains. Clinical use of anti-HBV agents such as adefovir, entecavir, telbivudine, clevudine and tenofovir has started, and multiple anti-HBV drug-resistant strains could occur in patients undergoing long-term treatment in the near future. Therefore, maintaining surveillance to detect drug-resistant strains of HBV may have a small impact, but it is important for clinical practice.

In conclusion, LMV-resistant mutations were previously rare but now appear to be prevalent among patients in Japan with acute hepatitis B. LMV-resistant strains must be considered in patients with acute hepatitis B.

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The Progression of Liver Fibrosis Is Related with Overexpression of the miR-199 and 200 Families

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Abstract

Background: Chronic hepatitis C (CH) can develop into liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Liver fibrosis and HCC development are strongly correlated, but there is no effective treatment against fibrosis because the critical mechanism of progression of liver fibrosis is not fully understood. microRNAs (miRNAs) are now essential to the molecular mechanisms of several biological processes. In order to clarify how the aberrant expression of miRNAs participates in development of the liver fibrosis, we analyzed the liver fibrosis in mouse liver fibrosis model and human clinical samples.

Methodology: In a CCL₄-induced mouse liver fibrosis model, we compared the miRNA expression profile from CCL₄ and olive oil administrated liver specimens on 4, 6, and 8 weeks. We also measured expression profiles of human miRNAs in the liver biopsy specimens from 105 CH type C patients without a history of anti-viral therapy.

Principle Findings: Eleven mouse miRNAs were significantly elevated in progressed liver fibrosis relative to control. By using a large amount of human material in CH analysis, we determined the miRNA expression pattern according to the grade of liver fibrosis. We detected several human miRNAs whose expression levels were correlated with the degree of progression of liver fibrosis. In both the mouse and human studies, the expression levels of miR-199a, 199a*, 200a, and 200b were positively and significantly correlated to the progressed liver fibrosis. The expression level of fibrosis related genes in hepatic stellate cells (HSC), were significantly increased by overexpression of these miRNAs.

Conclusion: Four miRNAs are tightly related to the grade of liver fibrosis in both human and mouse was shown. This information may uncover the critical mechanism of progression of liver fibrosis. miRNA expression profiling has potential for diagnostic and therapeutic applications.

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Introduction

Chronic viral hepatitis is a major risk factor for hepatocellular carcinoma (HCC) [1]. Worldwide 120–170 million persons are currently chronically Hepatitis C Virus (HCV) infected [2]. Due to repetitive and continuous inflammation, these patients are at increased risk of developing cirrhosis, subsequent liver decompensation and/or hepatocellular carcinoma. However, the current standard of care; pegylated interferon and ribavirin combination therapy is unsatisfied in the patients with high titre of HCV RNA and genotype 1b. Activated human liver stellate cells (HSC) with chronic viral infection, can play a pivotal role in the progression of liver fibrosis [3]. Activated HSC produce a number of profibrotic cytokines and growth factors that perpetuate the fibrotic process through paracrine and autocrine effects.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that control gene expression by degrading target mRNA or suppressing their translation [4]. There are currently 940 identifiable human miRNAs (The miRBase Sequence Database - Release ver. 15.0). miRNAs can recognize hundreds of target genes with incomplete complementarity; over one third of human genes appear to be conserved miRNA targets [5][6]. miRNA is associated several pathophysiologic events as well as fundamental cellular processes such as cell proliferation and differentiation. Aberrant expression of miRNA can be associated with the liver diseases [7][8][9][10]. Recently reported miRNAs can regulate the activation of HSCs and thereby regulate liver fibrosis. miR-29b, a negative regulator for the type I collagen and SP1, is a key regulator of liver fibrosis [11]. miR-27a and 27b allowed culture-activated rat HSCs to switch to a more quiescent HSC phenotype,

with restored cytoplasmic lipid droplets and decreased cell proliferation [12].

In this study, we aimed to reveal the association between miRNA expression patterns and the progression of liver fibrosis by using a chronic liver inflammation model in mouse. We also sought to identify the miRNA expression profile in chronic hepatitis (CH) C patients according to the degree of liver fibrosis, and to clarify how miRNAs contribute to the progression of liver fibrosis. We observed a characteristic miRNA expression profile common to both human liver biopsy specimens and mouse CCL₄ specimens, comprising the key miRNAs which are associated with the liver fibrosis. This information is expected to uncover the mechanism of liver fibrosis and to provide a clearer biomarker for diagnosis of liver fibrosis as well as to aid in the development of more effective and safer therapeutic strategies for liver fibrosis.

Results

The expression level of several mouse miRNAs was increased by introducing mouse liver fibrosis

In order to identify changes in the miRNA expression profile between advanced liver fibrosis and non-fibrotic liver, we intraperitoneally administered CCL₄ in olive oil or olive oil alone twice a week for 4 weeks and then once a week for the next 4 weeks. Mice were sacrificed at 4, 6, or 8 weeks and then the degree of mouse liver fibrosis was determined by microscopy (Figure S1). miRNA expression analysis was performed from the liver tissue collected at the same time. Histological examination revealed that the degree of liver fibrosis progressed in mice that received CCL₄ relative to mice receiving olive oil alone (Figure 1A). Microarray analysis revealed that in CCL₄ mice, the expression level of 11 miRNAs was consistently higher than that in control mice (Figure 1B).

miRNA expression profile in each human liver fibrosis grade

We then established human miRNAs expression profile by using 105 fresh-frozen human chronic hepatitis (CH) C liver tissues without a history of anti-viral therapy, classified according to the grade of the liver fibrosis (F0, F1, F2, and F3 referred to METAVIR fibrosis stages)(Figure 2, Table S2). Fibrosis grade F0 was considered to be the negative control because these samples were derived from patients with no finding of liver fibrosis. In zebrafish, most highly tissue-specific miRNAs are expressed during embryonic development; approximately 30% of all miRNAs are expressed at a given time point in a given tissue [13]. In mammals, the 20–30% miRNA call rate has recently been validated [14]. Such analysis revealed that the diversity of miRNA expression level among specimens was small. Therefore, we focused on miRNAs with a fold change in mean expression level greater than 1.5 ($p < 0.05$) in the two arbitrary groups of liver fibrosis.

Expression of several miRNAs was dramatically different among grades of fibrosis. In the mice study 11 miRNAs were related to the progression of liver fibrosis (mmu-let-7e, miR-125-5p, 199a-5p, 199b, 199b*, 200a, 200b, 31, 34a, 497, and 802). In the human study 10 miRNAs were extracted, and the change in their expression level varied significantly between F0 and F3 (F0<F3: hsa-miR-146b, 199a, 199a*, 200a, 200b, 34a, and 34b, F0>F3: hsa-miR-212, 23b, and 422b). The expression level of 6 miRNAs was significantly different between F0 and F2 (F0<F2: hsa-miR-146b, 200a, 34a, and 34b, F0>F2: hsa-miR-122 and 23b). 5 extracted miRNAs had an expression level that was significantly different between F1 and F2 (F1<F2: hsa-miR-146b, F1>F2: hsa-miR-122, 197, 574, and 768-5p). The expression level of 9 miRNAs changed significantly between F1 and F3 (F1<F3:

hsa-miR-146b, 150, 199a, 199a*, 200a, and 200b, F1>F3: hsa-miR-378, 422b, and 768-5p). The miRNAs related to liver fibrosis were extracted using two criteria: similar expression pattern in both the human and the mice specimens and shared sequence between human and mouse. We compared the sequences of mouse miRNAs as described on the Agilent Mouse MiRNA array Version 1.0 (miRbase Version 10.1) and human miRNAs as described on the Agilent Human MiRNA array Version 1.5 (miRbase Version 9.1). The sequences of mmu-miR-199a-5p, mmu-miR-199b, mmu-miR-199b, mmu-miR-200a, and mmu-miR-200b in mouse miRNA corresponded to the sequences of hsa-miR-199a, hsa-miR-199a*, hsa-miR-199a, hsa-miR-200a, and hsa-miR-200b in human miRNA, respectively (Table S3).

Validation of the microarray result by real-time qPCR

The 4 human miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b) with the largest difference in fold change between the F1 and F3 groups were chosen to validate the microarray results using stem-loop based real-time qPCR. The result of real-time qPCR supported the result of that microarray analysis. The expression level of these 4 miRNAs was significantly different between F0 and F3 and spearman correlation analysis also showed that the expressions of these miRNAs were strongly and positively correlated with fibrosis grade ($n = 105$, $r = 0.498$ (miR-199a), 0.607 (miR-199a*), 0.639 (miR-200a), 0.618 (miR-200b), p -values < 0.0001) (Figure 3).

Over expression of miR-199a, 199a*, 200a, and 200b was associated with the progression of liver fibrosis

In order to reveal the function of miR-199a, miR-199a*, miR-200a, and miR-200b, we investigated the involvement of these miRNAs in the modulation of fibrosis-related gene in LX-2 cells. The endogenous expression level of these 4 miRNAs in LX2 and normal liver was low according to the microarray study (Figure S2). Transforming growth factor (TGF) β is one of the critical factors for the activation of HSC during chronic inflammation [15] and TGF β strongly induced expression of three fibrosis-related genes include a matrix degrading complex comprised of $\alpha 1$ procollagen, matrix remodeling complex, comprised of metalloproteinases-13 (MMP-13), tissue inhibitors of metalloproteinases-1 (TIMP-1) in LX-2 cells (Figure 4A). Furthermore, overexpression of miR-199a, miR-199a*, miR-200a and miR-200b in LX-2 cells resulted significant induction of above fibrosis-related genes compared with control miRNA (Figure 4B). Finally we validated the involvement of TGF β in the modulation of these miRNAs. In LX-2 cells treated with TGF β , the expression levels of miR-199a and miR-199a* were significantly higher than in untreated cells; the expression levels of miR-200a and miR-200b were significantly lower than in untreated cells. Thus, our in vitro analysis suggested a possible involvement of miR-199a, 199a*, 200a, and 200b in the progression of liver fibrosis.

Discussion

Our comprehensive analysis showed that the aberrant expression of miRNAs was associated with the progression of liver fibrosis. We identified that 4 highly expressed miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b) that were significantly associated with the progression of liver fibrosis both human and mouse. Coordination of aberrant expression of these miRNAs may contribute to the progression of liver fibrosis.

Prior studies have discussed the expression pattern of miRNA found in liver fibrosis samples between previous and present study. In this report and prior mouse studies and the expression pattern of

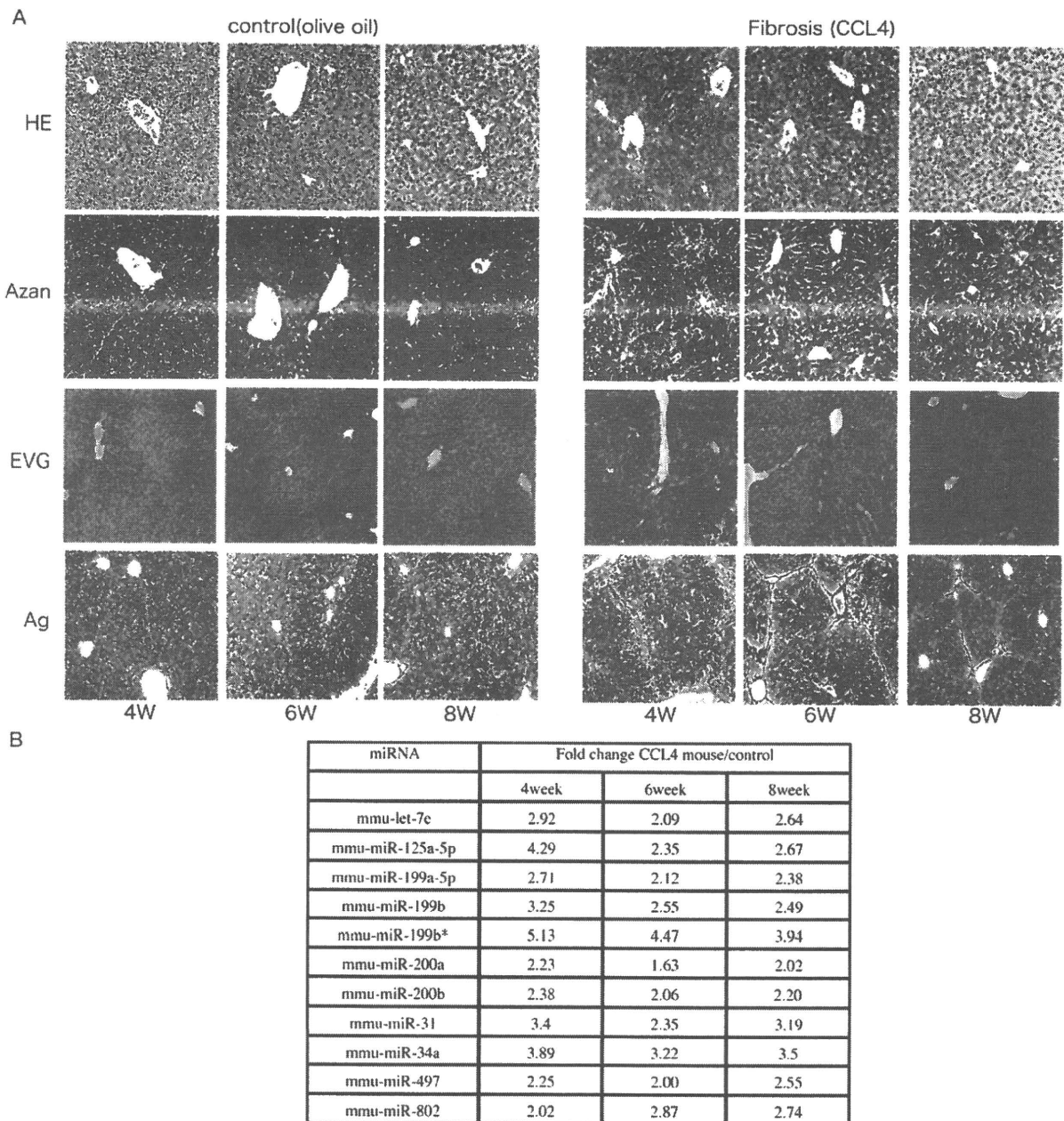


Figure 1. The change of liver fibrosis in mouse model. A. Representative H&E-stained, Azan-stained, Ag-stained, and EVG-stained histological sections of liver from mice receiving olive oil alone or CCL₄ in olive oil. Magnification is $\times 10$. B. The expression level of mmu-miRNA in mouse liver with olive oil or CCL₄ at 4W, 6W, and 8W respectively, by microarray analysis. doi:10.1371/journal.pone.0016081.g001

3 miRNAs (miR-199a-5p, 199b*, 125-5p) was found to be similar while the expression pattern of 11 miRNAs (miR-223, 221, 24, 877, 29b, 29a, 29c, 30c, 365, 148a, and 193) was partially consistent with fibrosis grade [16]. In low graded liver fibrosis, the low expression pattern of 3 miRNAs (miR-140, 27a, and 27b) and the high expression pattern of 6 miRNAs in rat miRNAs (miR-29c*, 143, 872, 193, 122, and 146) in rat miRNA was also similar to our mouse study (GEO Series accession number GSE19865) [11] [12] [17].

The results in this study and previously completed human studies reveal that the expression level of miR-195, 222, 200c, 21,

and let-7d was higher in high graded fibrotic liver tissue than in low graded fibrotic liver tissue. Additionally, the expression level of miR-301, 194, and 122 was lower in the high graded fibrotic liver tissue than in low graded fibrotic liver tissue [18] [19] [20] (GEO Series accession number GSE16922). This difference in miRNA expression pattern may be contributed to (1) the difference of microarray platform, (2) difference of analytic procedure, and (3) the difference of the species (rat, mouse, and human).

The miR-199 and miR-200 families have are circumstantially related to liver fibrosis. TGF β -induced factor (TGIF) and SMAD

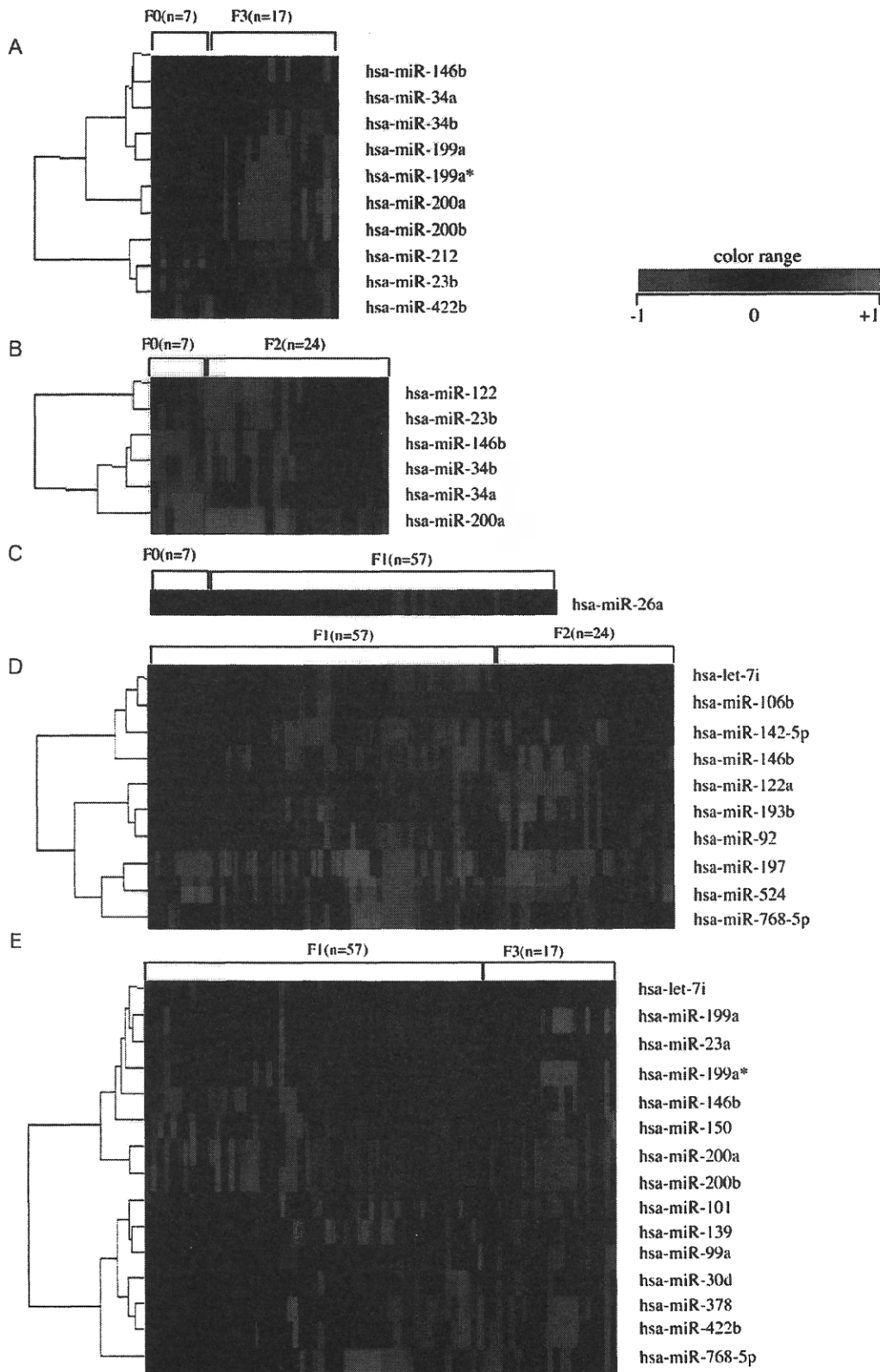


Figure 2. Liver fibrosis in human liver biopsy specimen. A, B, C, D, and E. miRNAs whose expression differs significantly between F0 and F3, F0 and F1, F0 and F2, F1 and F2, and F1 and F3, respectively. Relative expression level of each miRNA in human liver biopsy specimen by microarray. Data from microarray were also statistically analyzed using Welch's test and the Bonferroni correction for multiple hypotheses testing. Fold change, p-value are listed in Table S2. doi:10.1371/journal.pone.0016081.g002

specific E3 ubiquitin protein ligase 2 (SMURF2), both of which play roles in the TGF β signaling pathway, are candidate targets of miR-199a* and miR-200b, respectively, as determined by the Targetscan algorithm. The expression of miR-199a* was silenced in several proliferating cell lines excluding fibroblasts [21]. Down regulation of miR-199a, miR-199a* and 200a in chronic liver injury tissue was associated with the hepatocarcinogenesis [9]. miR-199a* is also one of the negative regulators of the HCV replication [22]. According to three target search algorithms (PicTar, miRanda, and Targetscan), the miRNAs that may be associated with the liver fibrosis can regulate several fibrosis-related genes (Table S4). Aberrant expression of these miRNAs may be closely related to the progress of the chronic liver disease.

Epithelial-mesenchymal transition (EMT) describes a reversible series of events during which an epithelial cell loses cell-cell contacts and acquires mesenchymal characteristics [23]. Although EMT is not a common event in adults, this process has been implicated in such instances as wound healing and fibrosis. Recent reports showed that the miR-200 family regulated EMT by targeting EMT accelerator ZEB1 and SIP1 [24]. From our

observations, overexpression of miR-200a and miR-200b can be connected to the progression of liver fibrosis.

The diagnosis and quantification of fibrosis have traditionally relied on liver biopsy, and this is still true at present. However, there are a number of drawbacks to biopsy, including the invasive nature of the procedure and inter-observer variability. A number of staging systems have been developed to reduce both the inter-observer variability and intra-observer variability, including the METAVIR, the Knodell fibrosis score, and the Scheuer score. However, the reproducibility of hepatic fibrosis and inflammatory activity is not as consistent [25]. In fact, in our study, the degree of fibrosis of the two arbitrary fibrosis groups was classified using the miRNA expression profile with 80% or greater accuracy (data not shown). Thus, miRNA expression can be used for diagnosis of liver fibrosis.

In this study we investigated whether common miRNAs in human and mouse could influence the progression of the liver fibrosis. The signature of miRNAs expression can also serve as a tool for understanding and investigating the mechanism of the onset and progression of liver fibrosis. The miRNA expression profile has the potential to be a novel biomarker of liver fibrosis.

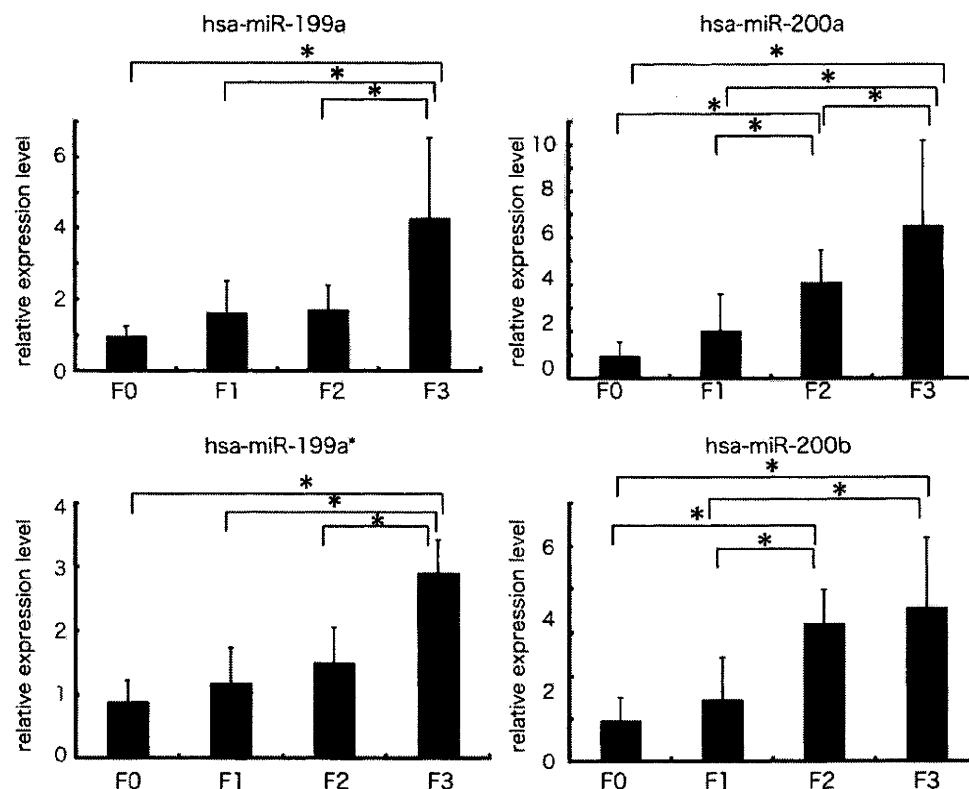


Figure 3. The expression level of miR-199 and 200 families in human liver biopsy specimen by real-time qPCR. Real-time qPCR validation of the 4 miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b). Each column represents the relative amount of miRNAs normalized to the expression level of U18. The data shown are the means \pm SD of three independent experiments. Asterisks indicates to a significant difference of $p < 0.05$ (two-tailed Student-t test), respectively. doi:10.1371/journal.pone.0016081.g003

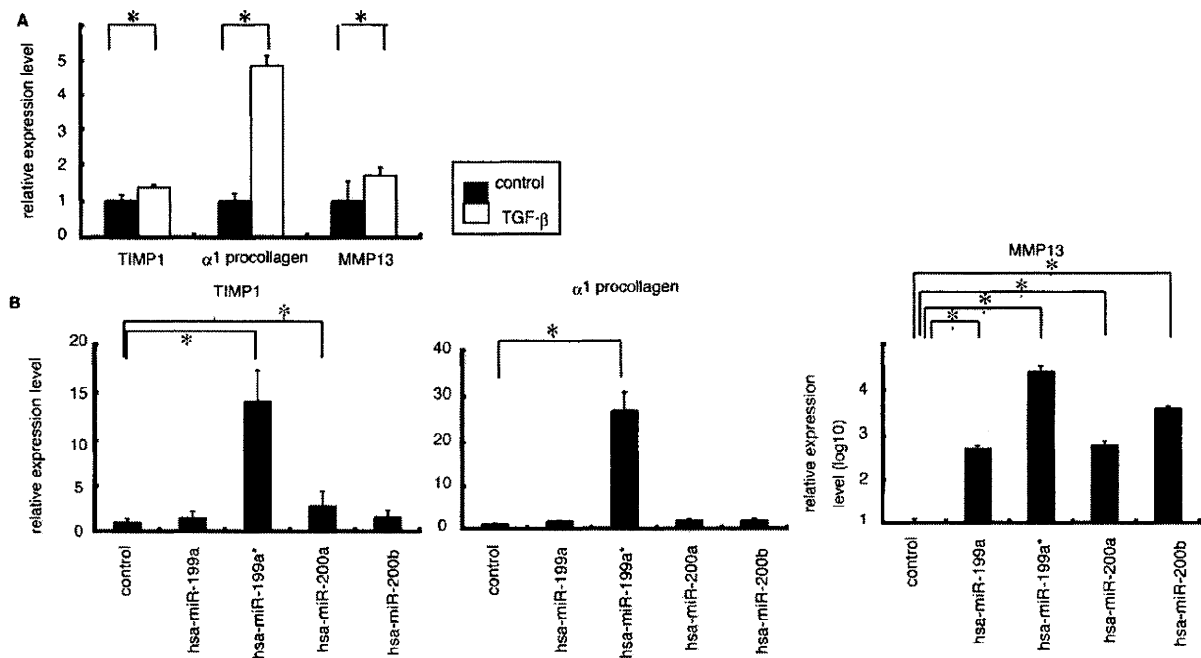


Figure 4. The relationship between expression level of miR-199 and 200 families and expression level of three fibrosis related genes. A. Administration of TGF β in LX2 cells showed that the expression level of three fibrosis related genes were higher than that in non-treated cells. The data shown are the means \pm SD of three independent experiments. Asterisk was indicated to the significant difference of $p < 0.05$ (two-tailed Student-t test). B. The expression levels of 3 fibrosis related genes in LX2 cells with overexpressing miR-199a, 199a*, 200a, or 200b, respectively were significantly higher than that in cells transfected with control miRNA ($p < 0.05$; two-tailed Student t-test). doi:10.1371/journal.pone.0016081.g004

Moreover miRNA expression profiling has further applications in novel anti-fibrosis therapy in CH.

Materials and Methods

Sample preparation

105 liver tissues samples from chronic hepatitis C patients (genotype 1b) were obtained by fine needle biopsy (Table S1). METAVIR fibrosis stages were F0 in 7 patients, F1 in 57, F2 in 24 and F3 in 17. Patients with autoimmune hepatitis or alcoholic liver injury were excluded. None of the patients were positive for hepatitis B virus associated antigen/ antibody or anti human immunodeficiency virus antibody. No patient received interferon therapy or immunomodulatory therapy prior to the enrollment in this study. We also obtained normal liver tissue from the Liver Transplantation Unit of Kyoto University. All of the patients or their guardians provided written informed consent, and Kyoto University Graduate School and Faculty of Medicine's Ethics Committee approved all aspects of this study in accordance with the Helsinki Declaration.

RNA preparation and miRNA microarray

Total RNA from cell lines or tissue samples was prepared using a *mirVana* miRNA extraction Kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. miRNA microarrays were manufactured by Agilent Technologies (Santa Clara, CA, USA) and 100 ng of total RNA was labeled and hybridized using the Human microRNA Microarray Kit protocol for use with Agilent microRNA microarrays Version 1.5 and Mouse microRNA Microarray Kit protocol for use with Agilent microRNA microarrays Version 1.0. Hybridization signals were detected with a DNA microarray scanner G2505B (Agilent Technologies) and

the scanned images were analyzed using Agilent feature extraction software (v9.5.3.1). Data were analyzed using GeneSpring GX 7.3.1 software (Agilent Technologies) and normalized as follows: (i) Values below 0.01 were set to 0.01. (ii) In order to compare between one-color expression profile, each measurement was divided by the 75th percentile of all measurements from the same species. The data presented in this manuscript have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE16922 (human) and accession number GSE19865 (mouse).

Real-time qPCR for human miRNA

For detection of the miRNA level by real-time qPCR, TaqMan[®] microRNA assay (Applied Biosystems) was used to quantify the relative expression level of miR-199a (assay ID. 002304), miR-199a* (assay ID. 000499), miR-200a (assay ID. 000502), miR-200b (assay ID. 002251), and U18 (assay ID. 001204) was used as an internal control. cDNA was synthesized using the Taqman miRNA RT Kit (Applied Biosystems). Total RNA (10 ng/ml) in 5 ml of nuclease free water was added to 3 ml of 5 \times RT primer, 10 \times 1.5 μ l of reverse transcriptase buffer, 0.15 μ l of 100 mM dNTP, 0.19 μ l of RNase inhibitor, 4.16 μ l of nuclease free water, and 50U of reverse transcriptase in a total volume of 15 μ l. The reaction was performed for 30 min at 16 $^{\circ}$ C, 30 min at 42 $^{\circ}$ C, and 5 min at 85 $^{\circ}$ C. All reactions were run in triplicate. Chromo 4 detector (BIO-RAD) was used to detect miRNA expression.

Animal and Chronic Mouse Liver Injury Model

Each 5 adult (8-week-old) male C57BL/6J mice were given a biweekly intra-peritoneal dose of a 10% solution of CCL₄ in olive oil (0.02 ml/g/ mouse) for the first 4 weeks and then once a week

for the next 4 weeks. At week 4, 6 or 8, the mice were sacrificed. Partial livers were fixed, embedded in paraffin, and processed for histology. Serial liver sections were stained with hematoxylin-eosin, Azan staining, Silver (Ag) staining, and Elastica van Gieson (EVG) staining, respectively. Total RNA from mice liver tissue was prepared as described previously. All animal procedures concerning the analysis of liver injury were performed in following the guidelines of the Kyoto University Animal Research Committee and were approved by the Ethical Committee of the Faculty of Medicine, Kyoto University.

Cell lines and Cell preparation

The human stellate cell lines LX-2, was provided by Scott L. Friedman. LX-2 cells, which viable in serum free media and have high transfectability, were established from human HSC lines [26]. LX-2 cells were maintained in D-MEM (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, plated in 60 mm diameter dishes and cultured to 70% confluence. Huh-7 and HeLa cells were also maintained in D-MEM with 10% fetal bovine serum. HuS-E/2 immortalized hepatocytes were cultured as described previously [27]. LX-2 cells were then cultured in D-MEM without serum with 0.2% BSA for 48 hours prior to TGF β 1 (Sigma-Aldrich, Suffolk, UK) treatment (2.5 ng/ml for 20 hours). Control cells were cultured in D-MEM without fetal bovine serum.

miRNA transfection

LX-2 cells were plated in 6-well plates the day before transfection and grown to 70% confluence. Cells were transfected with 50 pmol of Silencer[®] negative control siRNA (Ambion) or double-stranded mature miRNA (Hokkaido System Science, Sapporo, Japan) using lipofectamine RNAiMAX (Invitrogen). Cells were harvested 2 days after transfection.

Real-time qPCR

cDNA was synthesized using the Transcriptor High Fidelity cDNA synthesis Kit (Roche, Basel, Switzerland). Total RNA (2 μ g) in 10.4 μ l of nuclease free water was added to 1 μ l of 50mM random hexamer. The denaturing reaction was performed for 10min at 65°C. The denatured RNA mixture was added to 4 μ l of 5 \times reverse transcriptase buffer, 2 μ l of 10 mM dNTP, 0.5 μ l of 40U/ μ l RNase inhibitor, and 1.1 μ l of reverse transcriptase (FastStart Universal SYBR Green Master (Roche) in a total volume of 20 μ l. The reaction ran for 30 min at 50°C (cDNA synthesis), and five min at 85°C (enzyme denaturation). All reactions were run in triplicate. Chromo 4 detector (BIO-RAD, Hercules, CA, USA) was used to detect mRNA expression. The primer sequences are follows; MMP13 s; 5'-gaggtctcgagaaatgcagt-3', as; 5'-atgccatcgtgaagtctggt-3', TIMP1 s; 5'-cttgctctcactgatgg-3', as; 5'-acgctgtataagggtggtct-3', α 1-procollagen s; 5'-aacatgacaaaacaaaagt-3', as; 5'-catt-

gtttctctgtctcttctgg-3', and β -actin s; 5'-ccactggcatcgtgatggac-3', as; 5'-tcattgccaatggatgacct-3'. Assays were performed in triplicate, and the expression levels of target genes were normalized to expression of the β -actin gene, as quantified using real-time qPCR as internal controls.

Statistical analyses

Statistical analyses were performed using Student's *t*-test; *p* values less than 0.05 were considered statistically significant. Microarray data were also statistically analyzed using Welch's test and Bonferroni correction for multiple hypotheses testing.

Supporting Information

Figure S1 Time line of the induction of chronic liver fibrosis. Upward arrow indicated administration of olive oil or CCL₄. Downward arrow indicates when mice were sacrificed. (TIF)

Figure S2 Comparison of the expression level of miR-199 and 200 families in several cell lines and human liver tissue. Endogenous expression level of miR-199a, 199a*, 200a, and 200b in normal liver and LX2 cell as determined by microarray analysis (Agilent Technologies). Endogenous expression level of same miRNAs in HeLa, Huh-7 and, immortalized hepatocyte: HuS-E/2 by previously analyzed data [9]. (TIF)

Table S1 Clinical characteristics of patients by the grade of fibrosis. (DOCX)

Table S2 Extracted human miRNAs related to liver fibrosis. (DOCX)

Table S3 Corresponding human and mouse miRNAs. (DOCX)

Table S4 Hypothetical miRNA target genes according to in silico analysis. (DOCX)

Author Contributions

Conceived and designed the experiments: YM KS. Performed the experiments: YM HT YH NK. Analyzed the data: MT MK. Contributed reagents/materials/analysis tools: YM HT YH NK. Wrote the paper: YM MT AT FM NK TO.

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Clinical utility of highly sensitive *Lens culinaris* agglutinin-reactive alpha-fetoprotein in hepatocellular carcinoma patients with alpha-fetoprotein <20 ng/mL

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The *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) has been used as a diagnostic and prognostic marker of hepatocellular carcinoma (HCC). The analytical sensitivity of a conventional method for AFP-L3% is not sufficient in patients with a low AFP level. This study was performed to determine the clinical utility of a newly developed highly sensitive AFP-L3% (hs-AFP-L3%) assay in patients with an AFP level <20 ng/mL. In the cohort study, serum samples obtained from 270 patients with newly diagnosed HCC before treatment and 396 patients with chronic liver disease at Ogaki Municipal Hospital, in both of which the AFP level was <20 ng/mL, were measured for conventional AFP-L3% (c-AFP-L3%), hs-AFP-L3% and des-gamma-carboxy prothrombin (DCP). Diagnostic sensitivity and specificity of hs-AFP-L3% at a cut-off level of 5% were 41.5% and 85.1%, respectively, significantly increasing the sensitivity from 7.0% for c-AFP-L3%. Multivariate analysis identified hs-AFP-L3% as an independent factor associated with reduced long-term survival. The survival rate of patients with high hs-AFP-L3% (≥5%) before treatment was significantly poorer than that of patients with low hs-AFP-L3% (<5%) ($P < 0.001$). In patients with AFP <20 ng/mL, measurements of AFP-L3% by the highly sensitive method before treatment were more useful for diagnosis and prognosis of HCC than by the conventional method. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.01875.x, 2011)

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world, and the third most common cause of cancer-related death.⁽¹⁾ A diagnosis of HCC is typically marked with a poor prognosis, largely because early HCC is difficult to diagnose. Three tumor markers, alpha-fetoprotein (AFP),⁽²⁻⁵⁾ *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3)⁽⁶⁻⁸⁾ and des-gamma-carboxy prothrombin (DCP),⁽⁹⁻¹¹⁾ are currently available as serological markers of HCC for surveillance, diagnosis and patient outcome prediction. Alpha-fetoprotein has been widely used as an *in vitro* diagnostic, and different cut-off values have been proposed by previous studies.⁽¹²⁻¹⁴⁾ However, total AFP is not always specific for HCC, especially when HCC is in the early stages. In contrast, a fucosylated fraction of AFP (AFP-L3) is highly specific for HCC. A combination assay of AFP-L3% and DCP results in an improvement in diagnostic sensitivity compared with AFP-L3% or DCP alone.⁽¹⁵⁾ Furthermore, higher levels of AFP-L3% prior to treatment are also associated with poorer prognosis.^(8,16,17)

The percentage of AFP-L3 is determined by AFP-L3 concentration divided by the total AFP concentration in serum. Therefore, the analytical sensitivity for AFP-L3% can be affected inversely by the total AFP concentration. In addition, the clinical

usefulness of AFP-L3% has been hampered by insufficient analytical sensitivity of the conventional assay system in patients with a low AFP level, which is a liquid-phase binding assay on an auto-analyzer (LiBASys).^(18,19)

Recent technical improvements to the highly sensitive analytical methods, by using novel and advanced microfluidics-based separation science, have improved the analytical sensitivity of this assay.⁽²⁰⁾ This new generation of assays [micro-total analysis system (μ TAS)] has enabled the accurate measurement of AFP-L3% at very low AFP concentrations. In patients with an AFP level of ≥ 20 ng/mL, μ TAS AFP-L3% correlated well with LiBASys AFP-L3%.⁽²⁰⁾ Therefore, we conducted a retrospective cohort study to determine the clinical utility of the new highly sensitive AFP-L3% (hs-AFP-L3%) assay for diagnosis of HCC of our patient population under surveillance who were at risk of HCC and with AFP <20 ng/mL. In addition, we evaluated the clinical usefulness of this new AFP-L3% as a prognostic factor in patients with HCC with AFP <20 ng/mL.

Methods

Patients. Between January 1995 and December 2007 there were 1004 patients diagnosed with primary HCC at Ogaki Municipal Hospital. The AFP levels of all patients had been measured, and 461 patients (46%) had AFP <20 ng/mL. Of these 461 patients, 270 met the eligibility criteria (availability of stored serum samples, patient informed consent and Child-Pugh class A or B). Patients with Child-Pugh class C were not enrolled in the present study, because the influence of poor liver function on patients' survival was too dominant to accurately evaluate HCC-related death in the prognosis part of this study.

Hepatocellular carcinoma was diagnosed by histological examination or the appropriate imaging characteristics using criteria similar to the guidelines accepted by the American Association for the Study of Liver Diseases.⁽²¹⁾ Tumor stage on imaging findings was assessed on the basis of the TNM classification of the Liver Cancer Study Group of Japan.⁽²²⁾

Control samples were obtained from 396 patients with chronic liver disease without HCC, which were collected during routine HCC surveillance in the same period. These patients had AFP <20 ng/mL and met the eligibility criteria of stored samples availability and patient informed consent. There were 98 patients classified by histological confirmation of chronic liver disease, and 298 patients were diagnosed by imaging findings and biochemical tests. To ensure that controls did not have

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HCC, these patients were followed for 3 years after serum sampling by ultrasonography, computed tomography or magnetic resonance imaging to ensure that none had developed HCC.

Individual decisions regarding treatment were made principally on the basis of the treatment guidelines for HCC in Japan.⁽²³⁾ Patients were initially assessed for eligibility for surgical treatment. When patients declined or were deemed ineligible for surgical treatment, they underwent non-surgical treatment. Patients were first offered locoregional ablative therapy (LAT) with percutaneous ethanol injection or, more recently, radiofrequency ablation. Patients who were not eligible for either resection or locoregional ablative therapy were offered transcatheter arterial chemoembolization (TACE). No patient underwent liver transplantation as a treatment.

Each HCC patient's follow-up period began between 1995 and 2007 and continued until death or December 2008, whichever came first. The follow-up period ranged from 0.3 to 101.6 months (a median of 28.2 months).

The study protocol was approved by the Institutional Ethics Review Board and was in compliance with the Declaration of Helsinki.

Assays of AFP, AFP-L3% and DCP. AFP, AFP-L3% and DCP were measured in the same serum sample obtained at the time of HCC diagnosis before any treatment (for HCC group) or from a sample obtained at least 3 years before the examination that confirmed the absence of HCC (for the control group). The measurements of hs-AFP-L3% and DCP were achieved by using a microchip capillary electrophoresis and liquid-phase binding assay on a μ TASWako i30 auto analyzer (Wako Pure Chemical Industries, Ltd, Osaka, Japan).⁽²⁰⁾ The measurements of conventional AFP-L3% (c-AFP-L3%) were performed using a column chromatography and liquid-phase binding assay on a LiBASys auto analyzer (Wako Pure Chemical Industries, Ltd).^(18,19) Analytical sensitivity of μ TAS is 0.3 ng/mL AFP, and the percentage of AFP-L3 can be measured when AFP-L3 is over 0.3 ng/mL. Analytical sensitivity of LiBASys is 0.8 ng/mL AFP, but AFP-L3% can not be calculated at AFP <10 ng/mL. Therefore the correlation between μ TAS and LiBASys was poor at AFP <20 ng/mL.

Statistical analyses. To evaluate the diagnostic value of hs-AFP-L3% and DCP, sensitivity and specificity were calculated. For the evaluation of prognosis, the long-term survival of

Table 1. Demographics of the study population

Characteristics	Patients with HCC (n = 270)	Patients without HCC (n = 396)
Gender (%)		
Male/Female	209 (77)/61 (23)	207 (52)/189 (48)
Age	67.9 \pm 8.8	63.5 \pm 12.2
Mean \pm SD		
Infection of hepatitis virus (%)	38 (14)/193 (71)/7 (3)/32 (12)	82 (21)/217 (55)/12 (3)/85 (21)
HBV/HCV/HBV + HCV/none		
Child-Pugh class (%)	215 (80)/55 (20)/0	323 (82)/73 (18)/0
A/B/C		
Platelet count ($\times 10^3/\text{mm}^3$)	12.4 (7.6, 17.1)	11.3 (8.8, 17.6)
Median (25%, 75% quartile)		
ALT (IU/L)	41.5 (27.0, 64.8)	31.0 (20.0, 57.3)
Median (25%, 75% quartile)		
AST (IU/L)	48.0 (34.0, 68.5)	36.0 (26.0, 61.0)
Median (25%, 75% quartile)		
Total bilirubin (mg/dL)	0.8 (0.5, 1.0)	0.7 (0.5, 1.0)
Median (25%, 75% quartile)		
Albumin (g/dL)	3.7 (3.3, 4.1)	3.9 (3.4, 4.2)
Median (25%, 75% quartile)		
AFP (ng/mL)	6.7 (3.6, 10.3)	2.8 (1.8, 4.9)
Median (25%, 75% quartile)		
c-AFP-L3% (%)	0.5 (0.5, 0.5)	0.5 (0.5, 0.5)
Median (25%, 75% quartile)		
hs-AFP-L3% (%)	4.2 (0.5, 7.1)	0.5 (0.5, 0.5)
Median (25%, 75% quartile)		
DCP (mAU/mL)	33 (18, 182)	19 (15, 27)
Median (25%, 75% quartile)		
Tumor stage†		
I	89	NA
II	127	NA
III	47	NA
IV	7	NA
Tumor size		
≤ 2 cm	123	NA
>2 and ≤ 3 cm	63	NA
>3 and ≤ 5 cm	52	NA
>5 cm	32	NA
Tumor number		
Single	189	NA
Multiple	81	NA

†According to TNM staging by the Liver Cancer Study Group of Japan. AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-gamma-carboxy prothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

Table 2. Sensitivity and specificity. (a) Patients with AFP <20 ng/mL (HCC, n = 270; control, n = 396), and (b) patients with AFP <10 ng/mL (HCC, n = 199; control, n = 357)

	Cut-off (%)	Sensitivity (%)	Specificity (%)
(a) AFP <20 ng/mL			
c-AFP-L3%	5	7.0	98.5
	7	5.2	98.7
	10	4.1	99.0
hs-AFP-L3%	5	41.5	85.1
	7	26.7	92.4
	10	14.8	98.2
(b) AFP <10 ng/mL			
c-AFP-L3%	5	0.0	100.0
	7	0.0	100.0
	10	0.0	100.0
hs-AFP-L3%	5	36.2	88.5
	7	23.6	93.8
	10	11.6	98.3

AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma.

patients with HCC was determined by the Kaplan–Meier method, and the log-rank test was used to compare the survival rates. The Cox proportional hazards model was used for multivariate analysis for factors that influenced patient survival. The values were considered significant when the *P* value was <0.05. All analyses were performed using JMP6 statistical software (SAS Institute, Cary, NC, USA).

Results

Clinical features of patients. The demographics, etiology of liver disease, hepatic functional reserve ranked by Child–Pugh classification, tumor stage, tumor size and tumor number of the study patients are summarized in Table 1. Overall, there were 270 patients with HCC, including 89 in stage I, 127 in stage II, 47 in stage III and seven in stage IV. The majority of HCC (80.0%) were diagnosed stages I and II. The levels of median and quartile for AFP concentration, c-AFP-L3%, hs-AFP-L3%, DCP and other laboratory tests are shown in Table 1. The numbers of HCC patients and non-HCC patients with AFP <10 ng/mL were 199 out of 270 (73.7%) and 357 out of 396 (90.2%), respectively. In addition to evaluating AFP-L3% in patients with AFP <20 ng/mL, we further evaluated the patient group with AFP <10 ng/mL.

Sensitivity and specificity. The receiver-operating characteristic (ROC) curve was constructed to evaluate the area under the curve (AUC) for AFP-L3%. In all patients (HCC, *n* = 270; control, *n* = 396), the AUC of μ TAS hs-AFP-L3% and prior-generation methodology LiBASys c-AFP-L3% were 0.707 and 0.537 respectively (*P* < 0.05) (data not shown). In patients with AFP <10 ng/mL (HCC, *n* = 199; control, *n* = 357), the AUC of μ TAS hs-AFP-L3% was 0.668, but that of LiBASys c-AFP-L3% was not measured because AFP-L3% was out of the reportable range (data not shown). These indicated that the diagnostic accuracy of μ TAS hs-AFP-L3% was superior to that of LiBASys c-AFP-L3%.

Overall sensitivity and specificity calculated for patients with AFP <20 ng/mL and for those with AFP <10 ng/mL using three cut-off values (5%, 7% and 10%) are shown in Table 2. In

Table 3. Sensitivity by tumor characteristics. (a) Patients with AFP <20 ng/mL (n = 270), and (b) <10 ng/mL (n = 199)

	Analyte (n)	c-AFP-L3% (%)	hs-AFP-L3% (%)	DCP (%)	hs-AFP-L3% and DCP (%)
	Cut-off	10%	5%	40 mAU/mL	5% + 40 mAU/mL
(a)					
Staget					
I	89	4.5	34.8	20.2	44.9
II	127	2.4	42.5	57.5	71.7
III	47	6.4	53.2	53.2	74.5
IV	7	14.3	28.6	71.4	85.7
Size					
≤2 cm	123	4.1	36.6	24.4	48.8
>2 and ≤3 cm	63	3.2	46.0	52.4	65.1
>3 and ≤5 cm	52	3.8	44.2	63.5	80.8
>5 cm	32	6.3	46.9	78.1	90.6
Number					
Single	189	3.2	39.2	43.9	60.8
Multiple	81	6.2	46.9	46.9	70.4
(b)					
Staget					
I	66	0.0	28.8	18.2	40.9
II	97	0.0	37.1	58.8	70.1
III	30	0.0	53.3	56.7	73.3
IV	6	0.0	16.7	83.3	83.3
Size					
≤2 cm	90	0.0	31.1	23.3	45.6
>2 and ≤3 cm	43	0.0	37.2	46.5	55.8
>3 and ≤5 cm	42	0.0	40.5	69.0	81.0
>5 cm	24	0.0	45.8	87.5	95.8
Number					
Single	146	0.0	33.6	43.8	58.2
Multiple	53	0.0	43.4	50.9	69.8

†According to TNM staging by the Liver Cancer Study Group of Japan. AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

patients with AFP <20 ng/mL, when the cut-off value for AFP-L3% was set at 5%, the sensitivity and specificity of c-AFP-L3% were 7.0% and 98.5%, respectively. Those of hs-AFP-L3% were 41.5% and 85.1%, respectively. Sensitivity of hs-AFP-L3% was significantly higher than that of c-AFP-L3% ($P < 0.05$). Focusing on patients with AFP <10 ng/mL, the sensitivity of hs-AFP-L3% was 36.2%, which was still much higher than that for c-AFP-L3%. A cut-off value of 10% has been reported for diagnosis of HCC using the earlier generation methodology.⁽⁸⁾ For this study, to maintain the specificity at 85% or more, we chose a cut-off value of 5% for hs-AFP-L3% and 10% for c-AFP-L3%.

Sensitivity with respect to tumor characteristics. Patients were classified by tumor stage (I, II, III and IV), tumor size (<2, 2–3, 3–5 and >5 cm) and tumor number (single tumor and multiple tumors). In patients with AFP <20 ng/mL, sensitivities by tumor characteristics are shown for c-AFP-L3% (cut-off 10%), hs-AFP-L3% (cut-off 5%), DCP (cut-off 40 mAU/mL) and hs-AFP-L3%-DCP combined in Table 3. Sensitivities of hs-AFP-L3% in stages I and II were 34.8% and 42.5%, respectively, whereas those of c-AFP-L3% were only 4.5% and 2.4%, respectively. Those of DCP in stage I and II were 20.2% and 57.5%, respectively. Combination of hs-AFP-L3% and DCP resulted in an improvement in sensitivity compared with hs-AFP-L3% or DCP alone. Those of the combination in stage I and II were 44.9% and 71.7%, respectively. Focusing on patients with AFP <10 ng/mL, sensitivity using the combination in stages I and II were 40.9% and 70.1%, respectively.

In patients treated by hepatectomy, 13 patients had well-differentiated HCC by postoperative pathological examination. Hs-AFP-L3% was elevated ($\geq 5\%$) in four patients (30.8%). Hypervascularity of the tumor was not detected with computed tomography during hepatic arteriography, the most sensitive imaging modality to detect hypervascularity, in five patients. Hs-AFP-L3% was elevated in one of these hypovascular HCC (20.0%).

Survival rates of patients with HCC. We evaluated the significance of hs-AFP-L3% on the survival rate of HCC patients (Fig. 1). Statistical significance were not observed between the patients with high c-AFP-L3% ($\geq 10\%$) and the patients with low c-AFP-L3% ($<10\%$) ($P = 0.175$). The survival rate of patients with high hs-AFP-L3% ($\geq 5\%$) was significantly lower than that of patients with low hs-AFP-L3% ($<5\%$) by the log-rank test ($P < 0.001$). Statistical significance was not observed between the patients with high DCP (≥ 40 mAU/mL) and the patients with low DCP (<40 mAU/mL) ($P = 0.197$). Focusing on patients with AFP <10 ng/mL, statistical significance was still observed between the patients with high hs-AFP-L3% ($\geq 5\%$) and the patients with low hs-AFP-L3% ($<5\%$) ($P = 0.035$).

Univariate and multivariate analyses for prognostic factors for HCC. Table 4 shows the results of univariate and multivariate analyses of prognostic factors evaluated by Cox proportional hazards model in patients with AFP <20 ng/mL. The factors in the analysis were c-AFP-L3%, hs-AFP-L3%, DCP, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin, type of treatment, tumor stage, age and gender. In the univariate analysis, the hazard ratios of hs-AFP-L3%, total bilirubin, albumin, type of treatment and tumor stage were statistically significantly high ($P = 0.001$, <0.001 , 0.001 , 0.001 and 0.006 , respectively). Those of c-AFP-L3% and DCP were not statistically significant ($P = 0.218$ and 0.202 , respectively). In the multivariate analysis, hs-AFP-L3% and non-resection were independent prognostic factors with significantly high hazard ratios ($P = 0.026$ and <0.001 , respectively). For patients with AFP <10 ng/mL, hs-AFP-L3% was identified as a prognostic factor by univariate analysis ($P = 0.045$) but not by multivariate analysis ($P = 0.457$) (data not shown).

Survival rates of patients stratified by the type of treatment. In patients with AFP <20 ng/mL and classified into stages I and II, survival rates evaluated by treatment and by hs-AFP-L3% status are shown in Figure 2. All patients with any treatments ($n = 216$) are shown in Figure 2a, patients with

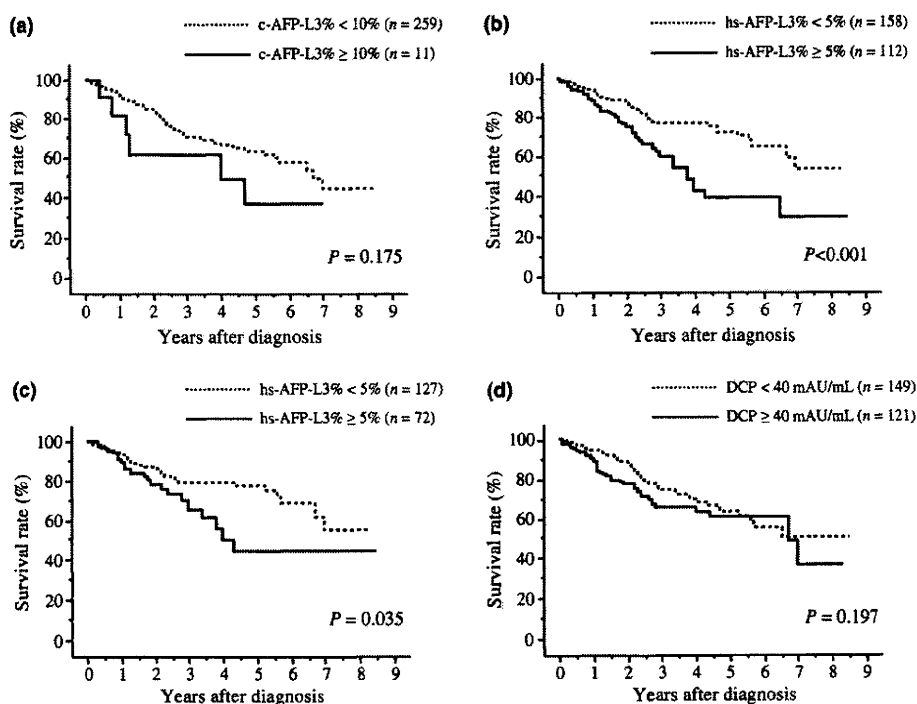


Fig. 1. Survival rates. (a) c-AFP-L3% in patients with AFP <20 ng/mL ($n = 270$), (b) hs-AFP-L3% in patients with AFP <20 ng/mL ($n = 270$), (c) hs-AFP-L3% in patients with AFP <10 ng/mL ($n = 199$), and (d) Des-gamma-carboxy prothrombin (DCP) in patients with AFP <20 ng/mL ($n = 270$). c-AFP-L3%, conventional AFP-L3%; hs-AFP-L3%, highly sensitive AFP-L3%.

Table 4. (a) Univariate and (b) multivariate analyses for prognostic factors of hepatocellular carcinoma in patients with alpha-fetoprotein <20 ng/mL

Variables	Hazard ratio (95% confidence interval)	P value	
(a) Univariate analyses			
c-AFP-L3% $\geq 10\%$	1.765 (0.683–3.739)	0.218	
hs-AFP-L3% $\geq 5\%$	2.195 (1.401–3.450)	0.001	
DCP ≥ 40 mAU/mL	1.335 (0.855–2.080)	0.202	
ALT ≥ 40 IU/L	1.132 (0.725–1.792)	0.587	
AST ≥ 40 IU/L	1.370 (0.845–2.310)	0.207	
Total bilirubin ≥ 1 mg/dL	2.466 (1.543–3.901)	<0.001	
Albumin <3 g/dL	2.868 (1.567–4.923)	0.001	
Treatment (LAT + TACE/resection)	4.893 (2.876–8.832)	<0.001	
Stage†	III + IV/I + II	2.111 (1.247–3.440)	0.006
Age		1.009 (0.983–1.037)	0.504
Gender	Male/Female	1.185 (0.902–1.616)	0.232
(b) Multivariate analysis			
hs-AFP-L3% $\geq 5\%$	1.697 (1.066–2.709)	0.026	
Total bilirubin ≥ 1 mg/dL	1.575 (0.961–2.558)	0.071	
Albumin <3 g/dL	1.650 (0.878–2.930)	0.116	
Treatment (LAT + TACE/resection)	3.627 (2.066–6.708)	<0.001	
Stage†	III + IV/I + II	1.675 (0.982–2.753)	0.058

†According to TNM staging by the Liver Cancer Study Group of Japan. AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-gamma-carboxy prothrombin; LAT, locoregional ablative therapies; TACE, transcatheter arterial chemoembolization.

resection ($n = 103$) in Figure 2b, patients with LAT ($n = 56$) in Figure 2c and patients with TACE ($n = 57$) in Figure 2d. The difference in the survival rate of patients with resection was not found patients with high hs-AFP-L3% and with low hs-AFP-L3% ($P = 0.813$). In the case of LAT, the survival rate of patients with high hs-AFP-L3% was significantly lower than that of patients with low hs-AFP-L3% ($P = 0.037$). The survival rate of patients with high hs-AFP-L3% tended to be lower than that of patients with low hs-AFP-L3%, but the difference was not statistically significant in the case of TACE. The survival rate of patients with resection was significantly higher than that of patients with ablation and TACE regardless of the hs-AFP-L3% level ($P = 0.002$) (data not shown).

Discussion

Alpha-fetoprotein, AFP-L3% and DCP are used as markers for HCC, and their utility in the diagnosis of HCC and the evaluation of tumor progression and prognosis has been reported. Alpha-fetoprotein is the most widely used marker for monitoring HCC development. However, elevated AFP is not typically observed in patients with a small tumor or early stage HCC. Recent advances in diagnostic imaging techniques have allowed for the detection of small tumors and early stage HCC,^(24–28) and the establishment of surveillance programs for HCC in the high-risk group have also contributed to diagnosis of early stage HCC.^(29,30) These trends have resulted in an increase in the number of HCC patients diagnosed by imaging without elevation of AFP. Thus, HCC patients with low AFP represent the appropriate study population in a successful HCC surveillance program. Among the tumor markers, AFP-L3% is highly specific for HCC, and elevated AFP-L3% correlates with tumor progression, poor tumor differentiation and unfavorable prognosis.^(8,11,31–33)

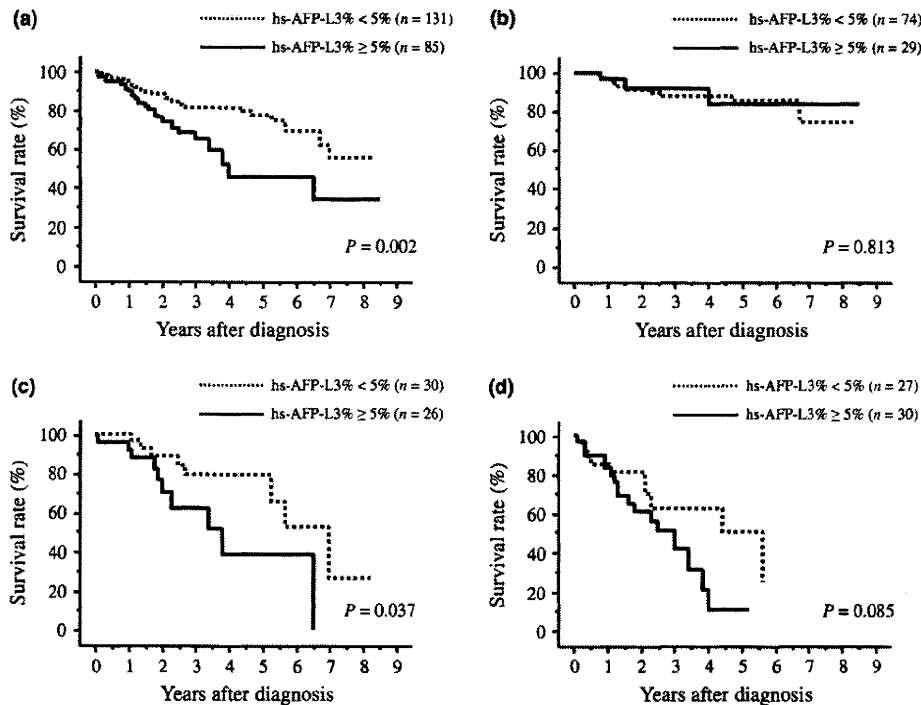


Fig. 2. Survival rates of patients stratified by the type of treatment in patients with alpha-fetoprotein (AFP) <20 ng/mL and classified into stages I and II. (a) All patients with any treatments ($n = 216$), (b) patients treated by surgical resection ($n = 103$), (c) patients treated by locoregional ablative therapies ($n = 56$), and (d) patients treated by transcatheter arterial chemoembolization ($n = 57$).

However, measurement of AFP-L3% by the conventional assay system has not always been reliable in patients with AFP <20 ng/mL due to low analytical sensitivity. Therefore, the clinical utility of conventional AFP-L3% has limited use in the diagnosis and prediction of outcome of this subpopulation. The present study focused on patients with AFP <20 ng/mL, and further, the subgroup with AFP <10 ng/mL, and revealed that the hs-AFP-L3% assay could diagnose earlier stage HCC than the c-AFP-L3 assay (cut-off 5%). The combination assay with DCP resulted in a significant improvement in diagnostic sensitivity. Parallel measurement of hs-AFP-L3% and DCP will identify additional HCC patients in the early stage because the markers are complementary for different subgroups of HCC.

Regarding prognosis, in patients with AFP <20 ng/mL, the survival rate of patients with elevated hs-AFP-L3% ($\geq 5\%$) was significantly lower than that of patients with low hs-AFP-L3% (<5%). Univariate and multivariate analysis identified hs-AFP-L3% as an independent factor associated with long-term survival. Furthermore, high hs-AFP-L3% ($\geq 5\%$) in the present study suggested an unfavorable prognosis, even when focusing on patients with stages I and II. In patients with stages I and II HCC treated by surgical resection, there was no statistically significant difference in survival between patients with high hs-AFP-L3% ($\geq 5\%$) and those with low hs-AFP-L3% (<5%). The survival rate of patients treated by hepatic resection was much higher than that of patients with LAT or TACE. Thus, hepatic resection demonstrated favorable effects on survival compared with the other treatments, which might confound the clinical utilities of hs-AFP-L3%. In patients with AFP <10 ng/mL,

hs-AFP-L3% was not identified as a prognostic factor by multivariate analysis, probably for the same reason. Although in our previous study using conventional AFP-L3% there was no difference in survival between patients with high AFP-L3% and those with low AFP-L3% in the patients treated surgically,⁽³⁴⁾ postoperative AFP-L3% has been reported as a predictive marker for recurrence and long-term survival.⁽³⁵⁾ To evaluate the prognosis of patients with resection, measurements of hs-AFP-L3% using samples after treatment should be performed.

The lower survival rate of patients with elevated hs-AFP-L3% and high rate of elevation in early stage HCC indicated that hs-AFP-L3 will be useful in identifying early stage HCC but with poorer prognosis, for which early diagnosis and treatment would be important. It may be advisable that hs-AFP-L3% should be included as a routine screening tool for HCC in the surveillance of patients at high risk of the development of HCC, together with imaging modalities.

In conclusion, the present study shows that hs-AFP-L3% was a useful marker for the diagnosis of early stage HCC in patients with AFP <20 ng/mL, and parallel measurement with DCP improved sensitivity. In addition, measurement of hs-AFP-L3% before treatment could help predict patient prognosis.

Disclosure Statement

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