Table 3 CARD10 rs6000782 polymorphism in patients with type 1 AIH and healthy controls

	Healthy control (%) n = 214	AIH (%) n = 326	*p* value[a]	OR (95 % CI)
Genotype frequencies			0.186	
A/A	193 (90.2)	284 (87.1)		
A/C	20 (9.3)	42 (12.9)		
C/C	1 (0.5)	0		
Allele frequencies			0.376	
A	406 (94.9)	610 (93.6)		1
C	22 (5.1)	42 (6.4)		1.271 (0.747–2.161)

OR odds ratio, *CI* confidence interval

[a] Genotype frequencies were determined by χ^2 test using 2 × 3 contingency tables between patients with AIH and healthy controls. Allele frequencies were determined by χ^2 test using 2 × 2 contingency tables between patients with AIH and healthy controls

The first application for type 1 AIH by de Boer et al. in AIH patients identified three genes exhibiting significant association in 649 patients and 13,436 healthy controls in Dutch and German populations. The main finding of this study was the strength of the AIH association with HLA, although it also identified associations with the *SH2B3* rs3184504 *A allele and *CARD10* rs6000782 *C allele.

The present study found no association of *CARD10* rs6000782 variants with type 1 AIH in a Japanese population. The major strength of this study is the finding that the association discovers by deBoer in a Caucasian Northern European Dutch and German population is not generalizable to the East Asian Japanese population. While this contrasts with the finding of de Boer et al. in a Caucasian population, it is in agreement with the documented lack of association between *CARD10* variants and AIH in populations of diverse racial backgrounds. Gene–gene interactions or epistasis have been proposed to occur between genes that cluster within specific immune pathways, thus enhancing their effect on disease susceptibility [12]. These have been reported in autoimmune diseases, including a possible interaction between HLA and non-HLA genes [13]. This suggests that further gene–gene interaction studies will be necessary to determine the associations of different susceptibility loci in AIH.

The main finding of the study by de Bore et al. [3] remains the relative strength of the HLA associations. The associations of AIH with variants of *SH2B3* rs3184504 ($p = 7.7 \times 10^{-8}$) and *CARD10* rs6000782 ($p = 3.0 \times 10^{-6}$) did not reach the accepted level of significance required to declare genome-wide significance ($p < 5 \times 10^{-8}$). However, the prior association of *SH2B3* variants with autoimmune diseases suggests that this association is likely to be confirmed with larger cohorts. We did not analyze the association of *SH2B3* rs3184504 and AIH in our current study because it is almost monomorphic in a Japanese population (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=3184504). However, such validation is necessary because attempts to

Table 4 Comparison of demographics between AIH patients with or without rs6000782 C allele

	rs6000782 C allele (+) n = 42	rs6000782 C allele (−) n = 284	*p*
Female, n (%)	38 (90.5)	251 (88.4)	0.465
Age, years, mean ± SD	59.6 ± 13.9	59.5 ± 13.3	0.786
Biochemistry			
AST, IU/L [median (IQR)]	189.0 (85.5–835.0)	264.0 (94.0–707.5)	0.620
ALT, IU/L [median (IQR)]	258.5 (93.0–906.0)	305.0 (104.5–813.0)	0.926
ALP, IU/L [median (IQR)]	402.5 (261.0–566.0)	437.0 (323.5–586.5)	0.242
Total bilirubin, mg/dl [median (IQR)]	1.9 (0.8–4.6)	1.2 (0.8–4.6)	0.680
Albumin, g/dl [median (IQR)]	3.9 (3.4–4.2)	3.9 (3.5–4.2)	0.844
IgG, mg/dl [median (IQR)]	2078.0 (1695.0–2691.5)	2250.0 (1835.5–2945.0)	0.199
Platelets, 10^4/μl [median (IQR)]	19.6 (15.1–22.4)	18.4 (13.9–23.1)	0.712
Serology			
ANA ≧ 1:40, n (%)	36 (85.7)	252 (89.0)	0.339
ASMA ≧ 1:40, n (%)	13 (31.0)	108 (39.3)	0.301
Histology			
Cirrhosis, n (%)	8 (19.0)	43 (15.1)	0.515

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphate, *IgG* immunoglobulin G, *ANA* anti-nuclear antibody, *ASMA* anti-smooth muscle antibody, *IQR* interquartile range

generalize genetic associations across ethnicities have had mixed results. For *CARD10* rs6000782, more validation studies are warranted. Our study only enrolled patients with a definite diagnosis of AIH, in order to prevent any potential case ascertainment bias. This approach limited the sample size and therefore introduced the limitation of reduced statistical power, which might have prevented us from identifying potential associations between *CARD10* rs6000782 and AIH.

Conclusions

Our findings showed that *CARD10* rs6000782 is not likely to be associated with type 1 AIH, at least in a Japanese population. However, because allele frequency distributions differ according to ethnicity, replication in other populations and functional studies should be initiated in order to clarify the contribution of this genetic background in the development of AIH . Genetic variations associated with AIH susceptibility remain for further investigation.

Abbreviations

AIH: autoimmune hepatitis; CARD10: caspase recruitment domain-containing protein 10; HLA: human leukocyte antigen; NHO: National Hospital Organization; SH2B3: SH2B adaptor protein 3; SNPs: single nucleotide polymorphisms.

Authors' contributions

KM carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. YJ, HKoz, MY participated in the sequence alignment. AK, SA, KYa, SN, SH, SB, KYo, MS, HKou, HKa, TK, TH, MN, AN, HY, HN, HO, YN, KA, YO, KS, MT, TS, HT, TM, FM, EM, HS, HY collected the clinical data. HF, MN, participated in the design of the study and performed the statistical analysis. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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RESEARCH ARTICLE

Circulating microRNA Profiles in Patients with Type-1 Autoimmune Hepatitis

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Abstract

Recent studies have demonstrated that micro (mi)RNA molecules can be detected in the circulation and can serve as potential biomarkers of various diseases. This study used microarray analysis to identify aberrantly expressed circulating miRNAs in patients with type 1 autoimmune hepatitis (AIH) compared with healthy controls. Patients with well-documented and untreated AIH were selected from the National Hospital Organization (NHO)-AIH-liver-network database. They underwent blood sampling and liver biopsy with inflammation grading and fibrosis staging before receiving treatment. To further confirm the microarray data, circulating expression levels of miR-21 and miR-122 were quantified by real-time quantitative polymerase chain reaction in 46 AIH patients, 40 patients with chronic hepatitis C (CHC), and 13 healthy controls. Consistent with the microarray data, serum levels of miR-21 were significantly elevated in AIH patients compared with CHC patients and healthy controls. miR-21 and miR-122 serum levels correlated with alanine aminotransferase levels. Circulating levels of miR-21 and miR-122 were significantly reduced in AIH patients with liver cirrhosis, and were inversely correlated with increased stages of fibrosis. By contrast, levels of circulating miR-21 showed a significant correlation with the histological grades of inflammation in AIH. We postulate that aberrantly expressed serum miRNAs are potential biomarkers of AIH and could be implicated in AIH pathogenesis. Alternations of miR-21 and miR-122 serum levels could reflect their putative roles in the mediation of inflammatory processes in AIH.

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Introduction

Micro RNAs (miRNAs) are small endogenous RNA molecules of 19–24 nucleotides that control the translation and transcription of targeting RNAs by base-pairing to complementary sites [1]. Serum miRNA expression is stable, reproducible, and consistent among individuals of the same species, and specific expression patterns have been identified as biomarkers for numerous diseases and cancers [2]. For example, miR-122, the most abundant miRNA in hepatocytes, has a well-defined role in hepatitis C virus (HCV) replication, and serves as a viable therapeutic target [3]. A role for miR-122 is also emerging in other liver diseases [4].

Ample evidence exists for the important regulatory potential of other miRNAs in conditions associated with liver inflammation, the metabolic syndrome, or autoimmune processes [5]. miRNAs regulate the function of both the innate and the adaptive immune system, and altered miRNA expression has been reported in human autoimmune diseases [6]. For instance, a unique miRNA expression profile was demonstrated in the sera from an individual with the autoimmune liver disease primary biliary cirrhosis [7].

Autoimmune hepatitis (AIH) is a rare disease characterized by hypergammaglobulinemia, the production of autoantibodies and a good response to immunosuppression [8]. Different subtypes of AIH may be distinguished from differences in the autoantibody patterns. For example, AIH type 1 is characterized by anti-nuclear antibodies (ANA) and/or anti-smooth muscular antibodies (SMA) [9]. The etiology of AIH is unknown, but it is thought to have both a genetic and an environmental basis [10]. miRNAs are emerging as highly tissue-specific biomarkers with the potential applicability to revolutionize disease diagnosis and treatment [11]. In this study, therefore, we used microarray for the initial screening followed by quantitative reverse transcription PCR (qRT-PCR) validation to analyze serum samples from patients with type I AIH. Our results demonstrated that the unique expression pattern of serum miRNAs can serve as a non-invasive biomarker for AIH.

Materials and Methods

Study population

Patients with well-documented and untreated AIH were enrolled from the National Hospital Organization (NHO)-AIH-liver-network database, a multicenter registry for Japanese patients with AIH [12]. Almost patients were enrolled in Nagasaki Medical Center and Nagoya Medical Center. All patients satisfied the 1999 revised criteria of International Autoimmune Hepatitis Group diagnosis of type-1 AIH [13]. Patients were excluded from the study if there was histological evidence of cholangitis or non-alcoholic steatohepatitis. Patients positive for hepatitis B virus surface antigen or HCV RNA were also excluded. Patients with other causes of liver disease, such as excess alcohol or drug use, were excluded based on reviews of their appropriate history and investigations. As controls, patients with chronic hepatitis C (CHC) (n = 40; female/male = 20/20; genotype 1b:29, 2a:7, 2b:4; mean age, 60.5±9.3 years; aspartate aminotransferase (AST), 76.0±63.0 IU/L; alanine transaminase (ALT), 94.5±96.8 IU/L), and healthy controls (n = 13; female/male = 7/6; mean age, 42.5±14.4 years) were included. The study was approved by the Ethics Committee of the NHO Central Internal Review Board and participating NHO liver-network hospitals. Written informed consent was obtained from each individual.

Clinical and histological assessments

Serum concentrations of type IV collagen were determined with a commercial RIA kit (Panassay IV. C, Daiichi Chemical Co. Ltd. Tokyo, Japan).

Liver biopsy and laboratory tests were obtained at baseline prior to treatment. In the histological diagnosis of AIH, each specimen was assessed for inflammatory grading including the degree of portal inflammation, presence of interface hepatitis, and the degree of parenchymal inflammation, as well as the stage of fibrosis (0, absent; 1, expansion of fibrosis to parenchyma; 2, portal–central or portal–portal bridging fibrosis; 3, presence of numerous fibrous septa; and 4, multi-nodular cirrhosis) according to the criteria of Desmet et al. [14]. Cirrhosis was diagnosed histologically when a loss of normal lobular architecture, reconstruction of hepatic nodules, and the presence of regenerative nodules were observed.

Microarray analysis of serum miRNAs

We prepared a serum pool from five patients with untreated with acute-onset type AIH and stocked paired sera before/after glucocorticoid treatments. A control miRNA pool from five healthy subjects. RNA was isolated from serum samples using QIAzol reagent according to the manufacturer's instructions (Qiagen, Hilden, Germany). Microarray analysis was performed to evaluate miRNA expression patterns in serum pools from patients and controls using 3D-Gene miRNA Oligo chips according to the manufacturer's instructions (Toray Industries, Inc., Tokyo, Japan). Small RNAs from serum were labelled using the miRCURY LNA microRNA Array Power Labelling Kit (Exiqon. Palm Beach FL, USA) and analyzed using 3D-Gene miRNA Oligo chips Ver. 17.0 (Toray Industries Inc.) containing more than 1700 antisense probes printed in duplicate spots, according to the manufacturer's instructions. Signals were analyzed using the 3D-Gene Scanner 3000 (Toray Industries Inc. Tokyo, Japan). microRNAs array data from this study are in agreement with the Minimum Information About a Microarray Experiment (MIAME) and are publicly available through the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/projects/geo/) under the accession number GSE71432.

Quantitative reverse transcription PCR validation study

More serum samples of AIH patients (n = 46), CHC patients (n = 40), and healthy controls (n = 13) were used in a qRT-PCR validation study. We followed the protocol previously reported by Mitchell et al. [15] to determine endogenous levels of *Caenorhabditis elegans* miR-39 miRNA (miRNeasy Serum/plasma Spike-In Control). RNA was isolated from serum samples using Qiazol reagent according to the manufacturer's instructions (Qiagen, Limburg, the Netherlands). *C. elegans* miR-39 (Qiagen) was added to serum samples (1.6×10^8 copies/μl) prior to the RNA isolation procedure for the later normalization of extracellular miR-122 and miR-21 levels. The RNA quality was assessed by microcapillary electrophoresis (2100 BioAnalyser, Agilent Technologies, Waldbronn, Germany).

cDNA was reverse transcribed from 2.5 μl RNA using the TaqMan miRNA reverse transcription kit. qRT-PCR for the detection of hsa-miR-21 and miR-122 was carried out in 20 μl PCR reactions using the TaqMan MicroRNA assay with the StepOnePlus detection system (Applied Biosystems) at 50°C for 2 min then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Statistical methods

Expression levels of selected miRNAs detected by qRT-PCR were normalized to cel-miR-39 and presented as the fold-change (2–ΔΔCt) above the control (Control-1): ΔΔCt = (CtmiRNA-Ctcel-miR-39) patient mean – (CtmiRNA-Ctcel-miR-39) control mean. Results for non-normally distributed continuous variables were summarized as medians (interquartile ranges) and were compared by the Mann-Whitney U test. Paired data were analyzed by non-parametric tests

using the Wilcoxon signed-rank test for the comparison of paired data. Correlation coefficients (r) were calculated using the Spearman correlation.

Results

Demographic data of AIH patients

Table 1 shows demographic data of AIH patents. Among the 46 patients with type-1 AIH, 31 (67.4%) were positive for ANA (>1:40) and 32 (69.6%) for SMA (>1:40). Nine (19.6%) had liver cirrhosis at the time of diagnosis.

Unique miRNA expression pattern in AIH

We used the microarray system to determine those circulating miRNAs that were differentially expressed in AIH patients enrolled in Nagasaki Medical Center, Nagoya Medical Center and Ueda Medical Center. Of 2,555 miRNAs assayed, 811 were expressed at greater than background levels. S1 Fig shows the miRNAs that were expressed at higher levels in the sera from AIH patients compared with healthy subjects. A comparison between AIH patients and controls identified 11 miRNAs that were up-regulated by more than 1.7-fold (Table 2). Furthermore, comparing before and after successful glucocorticoid therapy identified 10 miRNAs that were down-regulated by up to 0.4-fold (S1 Fig and Table 3). Among these isolated miRNAs, miR-21 and miR-122 showed similar expression profiles, and were specifically up-regulated in untreated AIH patients and down-regulated in the remission phase after corticosteroid therapy.

Table 1. Baseline Characteristics of 46 Japanese AIH Type 1 Patients. AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphate; IgG, immunoglobulin G; ANA, anti-nuclear antibody; ASMA, anti-smooth muscle antibody; IQR, interquartile range; IAIHG, International Autoimmune Hepatitis Group.

Characteristics	n = 46
Female, n/total (%)	37/46(80.4)
Age, y, mean ± SD	59.6 ± 12.8
Biochemistry	
AST, IU/L, median (IQR)	290.5(97–724)
ALT, IU/L, median (IQR)	405.5(115–842)
ALP, IU/L, median (IQR)	437.5(347–514)
Total Bilirubin, mg/dl, median (IQR)	1.3(0.9–3.3)
Albumin, g/dl, median (IQR)	3.9(3.5–4.2)
IgG, mg/dl, median (IQR)	1860.0(1584–2370)
Prothrombin time, %, median (IQR)	81.3(72.3–89.0)
Platelets, 10^4/μl, median (IQR)	18.0(14.4–21.2)
Serology	
ANA ≧1:40, n/total (%)	31/46(67.4)
ASMA ≧1:40, n/total (%)	32/46(69.6)
Histology	
Cirrhosis, n/total (%)	9/46(19.6)
IAIHG criteria	
Score, median (IQR)	17(15–18)

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Table 2. Differentially expression miRNAs between control and AIH.

up			down		
no.	miR_name	fold change	no.	miR_name	fold change
1	hsa-miR-122-5p	7.34	1	hsa-miR-223-3p	0.14
2	hsa-miR-1915-5p	2.99	2	hsa-miR-575	0.16
3	hsa-miR-193b-3p	2.83	3	hsa-miR-451a	0.18
4	hsa-miR-1908-3p	2.72	4	hsa-miR-4638-5p	0.25
5	hsa-miR-6073	2.63	5	hsa-miR-4443	0.26
6	hsa-miR-99a-5p	2.62	6	hsa-miR-486-5p	0.28
7	hsa-miR-602	1.95	7	hsa-miR-6765-3p	0.29
8	hsa-miR-1199-5p	1.87	8	hsa-miR-6820-5p	0.29
9	hsa-miR-1290	1.78	9	hsa-miR-4648	0.30
10	hsa-miR-21-5p	1.77	10	hsa-miR-6511a-5p	0.32
11	hsa-miR-4732-5p	1.72	11	hsa-miR-6889-5p	0.37
			12	hsa-miR-1207-5p	0.38
			13	hsa-miR-7150	0.39
			14	hsa-miR-6877-5p	0.40
			15	hsa-miR-4476	0.42
			16	hsa-miR-6763-5p	0.43

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Circulating levels of miR-21 and miR-122 in AIH and CHC patients

qRT-PCR was used to verify the data obtained from microarray analysis. Expression levels of miR-21 and miR-122 were normalized to spike-in cel-miR39 and were shown to be significantly elevated in AIH patients compared with both CHC patients and healthy subjects (S2 Fig).

Table 3. Differentially expression miRNAs between befor and after corticosteroid therapy.

up			down		
no.	miR_name	fold change	no.	miR_name	fold change
1	hsa-miR-23a-5p	3.60	1	hsa-miR-122-5p	0.01
2	hsa-miR-4450	3.15	2	hsa-miR-1290	0.14
3	hsa-miR-4294	2.92	3	hsa-miR-21-5p	0.15
4	hsa-miR-4478	2.87	4	hsa-miR-1246	0.15
5	hsa-miR-4733-3p	2.67	5	hsa-miR-4732-5p	0.18
6	hsa-miR-204-3p	2.64	6	hsa-miR-6073	0.20
7	hsa-miR-6076	2.60	7	hsa-miR-1908-3p	0.26
8	hsa-miR-4525	2.19	8	hsa-miR-602	0.30
9	hsa-miR-4665-5p	2.07	9	hsa-miR-92a-3p	0.33
10	hsa-miR-4769-5p	2.05	10	hsa-miR-1915-5p	0.34
11	hsa-miR-7641	1.92			
12	hsa-miR-4476	1.75			
13	hsa-miR-7150	1.75			
14	hsa-miR-6891-5p	1.65			
15	hsa-miR-7109-5p	1.59			
16	hsa-miR-6889-5p	1.52			

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Reversibility of the increased expressed miRNA corticosteroid therapy

Circulating levels of miR-21 and miR-122 were determined before and after corticosteroid therapy in paired serum samples from AIH patients. miR-21 and miR-122 were both shown to be up-regulated in untreated AIH patients, and down-regulated following glucocorticoid treatment of these same patients (S3 Fig).

Relationship between circulating miR-21 and miR-122 and liver function tests

To investigate the relationship between circulating miR-21 levels and standard liver function parameters, we examined correlations between circulating miR-21 or miR-122 and serum levels of AST or ALT (S4 Fig). A positive correlation was observed between serum miR-21 and miR-122 levels and AST (miR-21: $r = 0.820$, $P<0.0001$; miR-122: $r = 0.628$, $P<0.0001$) and ALT (miR-21: $r = 0.761$, $P<0.0001$; miR-122: $r = 0.662$, $P<0.0001$). However, the correlation rate was higher with respect to serum miR-21 compared with serum miR-122. We could not find a significant correlation between serum miR-21 or miR-122 levels and those of type IV collagen (S5 Fig).

Relationship between circulating miR-21 and miR-122, liver fibrosis and necroinflammation

As shown in S6 Fig, AIH patients with liver cirrhosis (LC) had significantly lower levels of circulating miR-21 and miR-122 compared with those without LC. Moreover, serum miR-21 and miR-122 levels were also shown to be reduced in AIH patients with an advanced fibrosis stage (S7A Fig). To evaluate whether serum miR-21 and miR-122 were correlated with hepatic necroinflammation, we evaluated these circulating miRNAs in AIH patients sub grouped according to the grading of necroinflammation. Liver necroinflammation grading correlated positively and significantly with circulating levels of miR-21, but not with miR-122 (S7B Fig).

Discussion

miRNA changes in the liver have been reported in diseases such as viral hepatitis and hepatocellular carcinoma (HCC) [16, 17]. However, little is known about their detection in the blood from AIH patients. The current study provides the first evidence that AIH is associated with altered circulating miRNA expression. We demonstrated that circulating has-miR-21 and miR-122 were significantly elevated in patients with AIH leading to a unique miRNA expression profile, but not in those with AIH combined with LC. We also detected strong correlations between serum miR-21 and miR-122 levels and ALT, a parameter of ongoing liver damage, and a negative correlation with liver fibrosis scores. The persistent liver damage responsible for perpetuating this process is characterised by the infiltration of immune cells into the liver and the death of hepatocytes. miRNAs are essential for the regulation of liver development, regeneration, and metabolic functions [18]. Hence, alternations in intrahepatic miRNA networks are associated with liver diseases, including hepatitis, steatosis, cirrhosis, and HCC. miR-122 is the most common miRNA with aberrant expression identified in liver disease, serving as a biomarker of liver injury in chronic hepatitis B or C, non-alcoholic fatty-liver disease, and drug-induced liver disease [19–22]. It is also an essential host factor for HCV infection and an antiviral target [23]. Circulating miR-122 levels were previously shown to be massively increased in response to hepatic injury and intrahepatic loss of miR-122 [24]. This loss was not significantly correlated with grades of inflammation in CHC infection, although it was significantly correlated with fibrosis in affected patients [25]. Our findings support

previous studies demonstrating liver-specific loss of miR-122 at late stages of fibrosis in CHC infection. However, our data showed that circulating miR-21 levels were significantly positively associated with grades of inflammation and negatively with fibrosis.

Haider et al. performed the global evaluation of miRNAs as biomarkers for non-neoplastic diseases [26]. They isolated six potential miRNA biomarkers of hepatic injury, of which only miR-122 was demonstrated to be liver-specific [26]. miR-122 significantly increased type I interferon (IFN) expression in hepatocytes, presumably through modulation of suppressor of cytokine signaling 1expression [27]. These findings suggest that miR-122 may also contribute to autoimmunity in liver through the IFN-signaling pathway. It has also been demonstrated that miR-21 is induced after hepatectomy and contributes to liver regeneration by inducing the translation of cyclin D [28]. These data suggest that the higher miR-21 serum levels observed in AIH have similar functions to those seen after acute liver injury and following liver regeneration.

Mechanistically, it is possible for miR-21 to leak from damaged cells similar to ALT. These circulating levels are negatively regulated by hepatic fibrosis, as shown by the correlation between miR-21 and hepatic fibrosis. Our data are in line with these findings, indicating that serum miR-21 levels are related to liver damage activity rather than liver fibrosis in patients with CHC [29]. Thus, miR-21 and miR-122 may both originate from the inflamed liver in AIH, although miR-21, but not miR122, correlates with total bilirubin and the grading of liver inflammation. Therefore, serum levels of miR-21 and miR-122 reflect overlapping, but not identical, disease parameters in AIH patients. This might be related to the differential expression patterns of miR-21 and miR-122, with miR-122 being highly selective for the liver [30], whereas miR-21 shows strong expression in other cells such as lymphocytes [31]. Hepatic immune cell infiltrations or activation may contribute to the elevation of serum miR-21 levels in patients with AIH. miRNAs are crucial to many aspects of immunity, including T-cell immunity [32], so we speculate that circulating miR-21 mirrors the presence of immune-mediated hepatocyte injury in patients with AIH.

miR-21 plays a crucial role in T cells by sustaining the proliferation and repression of apoptosis. It has also been reported to target tumor suppressor genes such as *Btf2*, *PDCD4*, *Pten*, and sprout, which regulate cell death and proliferation [33]. miR21 directly down-regulates the expression of *PDCD4*, which encodes a protein that localizes to the nucleus and plays a role in pathogenic T cell apoptosis and cell proliferation [34, 35]. This leads to acquisition of an activated phenotype in normal T cells following miRNA-21 overexpression. miR-21 regulates aberrant T cell responses through control of *PDCD4* expression in human systemic lupus erythematosus [36], suggesting that miR-21 induction enables T cells to elude apoptosis and enhance the secretion of pro-inflammatory cytokines such as interferon-γ and interleukin-17 by repressing *PDCD4* expression [37].

More recently, it was demonstrated that the miR-21 pathway intrinsically controls Th17 differentiation. $CD4^+$ T cells lacking miR-21 were shown to exhibit a specific Th17 cell defect affecting the transforming growth factor-β pathway during experimental autoimmune encephalomyelitis *in vivo* [37]. Given the ameliorating effect of ant-miR-21 observed in autoimmune model mice, the increased circulating miR-21 demonstrated in AIH patients in the present study could play a critical role in immune-mediated liver injury, which could be a contributing factor in the development of AIH. Recently, a report demonstrated that AP-1 is essential for miR-21 expression because mutations in AP-1 binding sites eliminated miR-21 promoter activity [38]. Moreover, glucocorticoid is thought to regulate miR-21 transcription through inhibiting the action of proinflammatory transcription factors such as AP-I [39].

The present study has a number of limitations that should be considered when interpreting the results. First, we could not assess the relationship between the circulating levels of candidate

miRNAs and fluctuations of lupus disease activity with time. Second, because of the unknown impact of factors that regulate circulating miRNA levels, we were unable to normalize for the miRNA content using a reliable housekeeping miRNA. However, we supplemented the samples with recombinant cel-lin-4, which can be consistently detected by qRT-PCR, and detected no significant differences in raw Ct values of cel-lin-4 among the three groups (AIH, CHC, and control); thus, cel-miR39 was used to normalize the difference in the efficiency of RNA isolation. Additionally our findings may not reflect other populations because we only focused on Japanese individuals. Hence, the sampling of other ethnic groups may result in a different circulating miRNA signature for AIH.

In conclusion, this is the first report to investigate the circulating miRNA profiles of AIH patients using microarray and quantitative real-time PCR analysis. We revealed that differential levels of serum miR-21 and miR-122 were associated with AIH, which may play important roles in disease pathogenesis. The mechanisms underlying the regulation of these aberrant circulating miRNAs remain to be investigated.

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Supporting Information

S1 Fig. Comparison of miRNA expressions in the sera from normal subjects and AIH patients by miRNA microarray. (A) Comparison of normalized signal intensities of various miRNAs in sera. X axis represents untreated AIH patients (n = 5) and Y axis represent healthy controls (n = 5). (B) Comparison of normalized signal intensities of various miRNAs in sera. X axis represents the sera from untreated (pretreatment) and Y axis represents the sera from after successful treatment (posttreatment) in the same AIH patients (n = 5). (GEO accession No: GSE71432)
(TIF)

S2 Fig. Quantitative real-time PCR analysis for miR-21 and miR-122 in patients with AIH, chronic hepatitis C (CHC) and healthy subjects. The vertical lines indicate the range, horizontal boundaries of the boxes represent the first and third quartile. Results were compared by

non-parametric Mann-Whitney test.
(TIFF)

S3 Fig. Changes of serum miR-21 and miR-122 before and 4 weeks after the initiation of corticosteroid therapy. Thirty four AIH patients with paired serum samples (Before and 4 weeks after corticosteroid therapy) were subjected to qRT-PCR analysis for miR-122. The vertical lines indicate the range, horizontal boundaries of the boxes represent the first and third quartile. Paired samples from the same subjects were compared by Wilcoxon signed-rank test.
(TIFF)

S4 Fig. Correlations between serum levels of miR-21or miR-122 and AST (A) or ALT (B). Correlations between serum levels of miR-21or miR-122 and serum AST levels were determined in patients with AIH. The correlation coefficient was determined by Pearson's product statistic and the regression line is represented by the solid line.
(TIF)

S5 Fig. Correlations between serum levels of miR-21or miR-122 and type IV collagen. Correlations between serum levels of miR-21or miR-122 and serum type IV collagen levels were determined in patients with AIH. The correlation coefficient was determined by Pearson's product statistic and the regression line is represented by the solid line.
(TIF)

S6 Fig. Serum levels of miR-21 or miR-122 in AIH patients with or without liver cirrhosis (LC). The vertical lines indicate the range, horizontal boundaries of the boxes represent the first and third quartile. Results were compared by non-parametric Mann-Whitney test.
(TIFF)

S7 Fig. (A) Serum levels of miR-21 or miR-122(B) and relationship with stages of fibrosis. Correlations were assessed by Spearamn's rank correlation. Serum levels of miR-21 and miR-122 correlated inversely and significantly with the stage of fibrosis. (miR-21, $r = -0.309$, $p = 0.013$, miR-122, $r = -0.324$, $p = 0.009$). (B) Serum levels of miR-21 or miR-122 and relationship with grades of necroinflammation. Correlations were assessed by Spearamn's rank correlation. Serum levels of miR-21 correlated positively and significantly with the grade of inflammation. ($r = 0.3385$, $p = 0.001$).
(TIF)

Author Contributions

Conceived and designed the experiments: KM AK M. Nakamura MY HF H. Yatsuhashi. Performed the experiments: KM H. Kozuru YJ. Analyzed the data: KM YJ HF. Contributed reagents/materials/analysis tools: SA K. Yamasaki SN SH SB H. Kamitsukasa YN HO MS HT EM TH H. Yamashita H. Kouno M. Nakamuta KA TM HS KS HN K. Yoshizawa TS AN TK YO FM MT. Wrote the paper: KM AK HF.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Impact of alpha-fetoprotein on hepatocellular carcinoma development during entecavir treatment of chronic hepatitis B virus infection

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Abstract
Background Entecavir (ETV) is one of the first-line nucleoside analogs for treating patients with chronic hepatitis B virus (HBV) infection. However, the hepatocellular carcinoma (HCC) risk for ETV-treated patients remains unclear.
Methods A total of 496 Japanese patients with chronic HBV infection undergoing ETV treatment were enrolled in this study. The baseline characteristics were as follows: age 52.6 ± 12.0 years, males 58 %, positive for hepatitis B e antigen 45 %, cirrhosis 19 %, and median HBV DNA level 6.9 log copies (LC) per milliliter. The mean treatment duration was 49.9 ± 17.5 months.
Results The proportions of HBV DNA negativity (below 2.6 LC/mL) were 68 % at 24 weeks and 86 % at 1 year, and the rates of alanine aminotransferase (ALT) level normalization were 62 and 72 %, respectively. The mean serum alpha-fetoprotein (AFP) levels decreased significantly at 24 weeks after ETV treatment initiation (from 29.0 ± 137.1 to 5.7 ± 27.9 ng/mL, $p < 0.001$). The cumulative incidence of HCC at 3, 5, and 7 years was 6.0, 9.6, and 17.2 %, respectively, among all enrolled patients. In a multivariate analysis, advanced age [55 years or older, hazard ratio (HR) 2.84; $p = 0.018$], cirrhosis (HR 5.59, $p < 0.001$), and a higher AFP level (10 ng/mL or greater) at 24 weeks (HR 2.38, $p = 0.034$) were independent risk

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factors for HCC incidence. HCC incidence was not affected by HBV DNA negativity or by ALT level normalization at 24 weeks.
Conclusions The AFP level at 24 weeks after ETV treatment initiation can be the on-treatment predictive factor for HCC incidence among patients with chronic HBV infection.

Keywords Hepatitis B virus · Entecavir · Risk factors for hepatocellular carcinoma incidence · Alpha-fetoprotein

Abbreviations

AFP	Alpha-fetoprotein
ALT	Alanine aminotransferase
cccDNA	Covalently closed circular DNA
ETV	Entecavir
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
IFN	Interferon
NA	Nucleos(t)ide analog
ROC	Receiver operating characteristic

Introduction

More than 350 million people worldwide have hepatitis B virus (HBV) infection, and persistent hepatic damage following HBV infection is associated with liver disease progression [1–3]. Chronic HBV infection accounts for approximately 52.3 % of hepatocellular carcinoma (HCC) cases worldwide [4], and antiviral treatment such as interferon (IFN) or nucleos(t)ide analogs (NAs) that aims to improve the prognosis of patients with chronic HBV infection has been developed [5]. Entecavir (ETV), one of the first-choice NAs, is a more potent antiviral agent with a higher genetic barrier to resistance than lamivudine; ETV administration over the long term has been reported to enable most patients to maintain a state of viral suppression [6–9]. With regard to the suppressive effect of NAs on HCC, in a randomized controlled trial of patients who were treated with lamivudine or placebo, the lamivudine-treatment group showed a significantly lower HCC rate than the placebo group during the observation period of 32.4 months (3.9 % vs 7.4 %, $p = 0.047$) [10]. In other cohort studies of patients who were treated with lamivudine, HCC incidence has been reported to be significantly lower in those who maintained low HBV DNA levels [less than 4 or 5 log copies (LC) per milliliter], especially in those with cirrhosis [11–13]. In contrast, the suppressive effect of ETV on HCC incidence remains unclear because a randomized controlled study of patients treated with ETV or placebo has not been performed.

To date, many studies have assessed the relationship between clinical factors and HCC incidence, such as male gender, advanced age, presence of cirrhosis, and high HBV DNA levels, during the natural course of chronic HBV infection [14, 15]. Among patients who were treated with IFN, it has been reported that hepatitis B e antigen seroconversion achieved with IFN treatment was associated with lower HCC incidence rates compared with nonseroconversion [16]. However, neither the pretreatment factors nor the on-treatment factors that are associated with HCC incidence among patients receiving ETV have been fully examined. ETV treatment for patients with chronic HBV infection reduces serum HBV DNA levels and may also have anti-inflammatory and antineoplastic effects. That is, among patients receiving ETV, various factors, such as HBV DNA, alanine aminotransferase (ALT), total bilirubin, albumin, and alpha-fetoprotein (AFP) levels, have the possibility to change and be associated with HCC suppression.

In this study, we evaluated the risk factors for HCC, especially the on-treatment factors in patients with chronic HBV infection who were undergoing ETV treatment.

Patients and methods

Study population

This study was a retrospective, multicenter study conducted by Osaka University Hospital and other institutions

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that participate in the Osaka Liver Forum. A total of 840 NA-naïve patients chronically infected with HBV started treatment with 0.5 mg of ETV per day between July 2004 and July 2012. Of these patients, we excluded 51 patients with HBV DNA levels under 3 LC/mL at the baseline, 13 patients who were co-infected with hepatitis C virus (HCV) or with human immunodeficiency virus, one patient who had undergone liver transplantation, and 140 patients with a history of HCC at the baseline. In addition, we excluded 51 patients who had been treated with ETV for less than 1 year and 88 patients who developed HCC within 1 year after the initiation of ETV treatment. As a result, 496 patients were enrolled in this cohort study. This study was conducted according to the ethical guidelines of the Declaration of Helsinki, amended in 2002, and was approved by the Institutional Review Board of Osaka University Hospital (approval number 12380-2).

HCC surveillance and data collection

The patients were followed up once every 3–6 months, and clinical symptoms, HBV DNA and other virological markers, complete blood count, liver biochemistry, and AFP levels were assessed. AFP levels measured between 20 and 28 weeks from the initiation of ETV treatment were regarded as valid AFP levels at 24 weeks. Ultrasonography of the abdomen, computed tomography, and/or magnetic resonance imaging was performed every 3–6 months for HCC surveillance. HCC was diagnosed by the presence of typical hypervascular characteristics evident on the computed tomography and/or magnetic resonance imaging scans. If no typical signs of HCC were observed, either hepatic angiography or fine-needle aspiration biopsy was performed with the patient's consent, or the patient was carefully followed until a diagnosis was possible on the basis of a definite observation. Liver cirrhosis was defined by a shrunken, small liver with a nodular surface as noted on liver imaging and by clinical features of portal hypertension.

Definition of treatment response

The surveillance start date was defined as the time of ETV treatment initiation. HBV DNA was measured by the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Tokyo, Japan) with a linear range of detection from 2.6 to 7.6 LC/mL or by the COBAS Taqman HBV Test v2.0 (Roche Diagnostics) with a linear range of detection from 2.1 to 9.0 LC/mL. The achievement of a virological response by ETV treatment was defined by serum HBV DNA levels that were continuously under 2.6 LC/mL. ALT level normalization was defined by serum ALT levels that were 30 IU/L or less.

Statistical analyses

Statistical analyses were performed using SPSS version 19.0 (IBM, Armonk, NY, USA) and SAS for Windows version 9.3 (SAS Institute, Cary, NC, USA). The continuous variables were expressed as the mean ± standard deviation or standard error of the mean or as the median (range), as appropriate, whereas the categorical variables were expressed as frequencies. The Wilcoxon signed-rank sum test was used to analyze differences between continuous variables before and after treatment. The cutoff value of AFP levels at 24 weeks from the initiation of ETV treatment for prediction of HCC incidence was assessed by the time-dependent receiver operating characteristic (ROC) curve, and the 95 % confidence interval for the area under the ROC curve was constructed using the bootstrap method. The Kaplan–Meier method was used to assess the cumulative HCC incidence, and the groups were compared using the log-rank test. The Cox proportional-hazards model was used to identify the independent factors associated with HCC incidence. The factors that were selected as significant by simple Cox regression analysis were evaluated by multiple Cox regression analysis. The risks were expressed as hazard ratios and 95 % confidence intervals. We considered $p < 0.05$ as significant.

Results

The characteristics of the 496 patients at the baseline and at 24 weeks after ETV treatment initiation are summarized in Table 1. The average age of the patients was 52.6 ± 12.0 years at the baseline, and there were 288 males (58 %) and 92 patients with cirrhosis (19 %). The patients were followed up for an average of 49.9 ± 17.5 months.

The cumulative incidence of virological response (HBV DNA level less than 2.6 LC/mL) at 24 weeks, 1 year, and 3 years after the initiation of ETV treatment was 68, 86, and 95 %, respectively. The median levels of HBV DNA were significantly decreased among noncirrhotic (6.9 LC/mL to less than 2.6 LC/mL, $p < 0.001$) and cirrhotic (6.9 LC/mL to less than 2.6 LC/mL, $p < 0.001$) patients from the baseline to 24 weeks after ETV treatment initiation (Table 1). ALT level normalization (30 IU/L or lower) was achieved in 62 % of patients at 24 weeks and in 72 % of patients at 1 year. The median ALT levels were significantly decreased among noncirrhotic (72.0–25.0 IU/L, $p < 0.001$) and cirrhotic (51.0–29.0 IU/L, $p < 0.001$) patients from the baseline to 24 weeks after ETV treatment initiation. The following parameters were also significantly increased from the baseline to 24 weeks after ETV treatment initiation: platelet counts and serum albumin levels

Table 1 Characteristics of patients at the baseline and 24 weeks after initiation of entecavir (ETV) treatment

	All patients, $n = 496$		Noncirrhotic patients, $n = 404$		Cirrhotic patients, $n = 92$	
	Baseline	24 weeks	Baseline	24 weeks	Baseline	24 weeks
Age (years)	52.6 ± 12.0 (15–82)		51.3 ± 12.1 (15–82)		58.2 ± 9.8 (32–81)	
Gender: male/female	288/208 (58 %)		233/171 (58 %)		55/37 (60 %)	
HBeAg[a]: positive/negative	220/270 (45 %)		181/219 (45 %)		39/51 (43 %)	
Histology[b], activity: A0/1/2/3	3/82/74/14		3/75/63/12		0/7/11/2	
Histology[b], fibrosis: F0/1/2/3/4	8/63/51/32/20		8/63/52/32/0		0/0/0/0/20	
History of IFN therapy: presence	50 (11 %)		44 (11 %)		6 (7 %)	
Platelet count ($\times 10^4/\mu$L)	16.0 ± 5.8	16.5 ± 6.4*	17.3 ± 5.2	17.7 ± 5.3*	10.3 ± 5.8	11.5 ± 7.9
Total bilirubin (mg/dL)	1.01 ± 1.48	0.83 ± 0.45*	0.91 ± 0.95	0.78 ± 0.42*	1.45 ± 2.78	1.09 ± 0.48
Albumin (g/dL)	3.94 ± 0.52	4.11 ± 0.44*	4.03 ± 0.44	4.18 ± 0.39*	3.56 ± 0.64	3.79 ± 0.50*
PT (%)	83.8 ± 16.3		86.7 ± 15.7		72.4±16.3	
ALT (IU/L)	143.7 ± 199.3 (9–1,885)	29.6 ± 16.5* (6–166)	156.1 ± 210.8 (9–1,885)	29.2 ± 16.9* (6–166)	89.2 ± 124.7 (12–763)	31.5 ± 14.0* (10–84)
ALT ≤ 30 (IU/L)	11 %	62 %	10 %	64 %	13 %	53 %
30 < ALT ≤ 60 (IU/L)	31 %	33 %	28 %	31 %	48 %	43 %
60 < ALT (IU/L)	58 %	5 %	62 %	5 %	39 %	4 %
HBV DNA (LC/mL) (median)	6.9	<2.6*	6.9	<2.6*	6.9	<2.6*
HBV DNA < 2.6 (LC/mL)	–	68 %	–	68 %	–	70 %
2.6 ≤ HBV DNA < 4.0 (LC/mL)	4 %	24 %	4 %	21 %	3 %	30 %
4.0 ≤ HBV DNA (LC/mL)	96 %	8 %	96 %	11 %	97 %	0 %
AFP (ng/mL)[c]	29.0 ± 137.1 (1–2,225)	5.7 ± 7.9* (1–126)	29.5 ± 152.7 (1–2,225)	4.9 ± 4.6* (1–126)	27.4 ± 48.0 (1–318)	9.3 ± 14.6* (1–52)
Observation periods (months)	49.9 ± 17.5 (14–109)		49.2 ± 17.6 (14–109)		52.8 ± 16.6 (18–82)	

Data are expressed as the mean ± standard deviation except for hepatitis B virus (*HBV*) DNA (median)

AFP alpha-fetoprotein, *ALT* alanine aminotransferase, *HBeAg* hepatitis B e antigen, *IFN* interferon, *LC* log copies, *PT* prothrombin time

* $p < 0.05$ (Wilcoxon signed-rank sum test)

[a] HBeAg measurement at the baseline was missing in six patients

[b] Liver biopsy was performed in 174 patients

[c] AFP data were missing in 78 noncirrhotic patients and five cirrhotic patients with cirrhosis

among noncirrhotic patients ($p = 0.008$ and $p < 0.001$, respectively) and serum albumin levels in cirrhotic patients ($p < 0.001$).

Mean serum AFP levels decreased significantly from 29.0 ± 137.1 ng/mL at the baseline to 5.7 ± 7.9 ng/mL at 24 weeks after the initiation of ETV treatment ($p < 0.001$). Mean AFP levels were assessed according to the severity of liver disease and decreased significantly from the baseline to 24 weeks in both the noncirrhotic group and the cirrhotic group (noncirrhotic group 29.5 ± 152.7 to 4.9 ± 4.6 ng/mL, $p < 0.001$; cirrhotic group 27.4 ± 48.0 to 9.3 ± 14.6 ng/mL, $p < 0.001$; Table 1). The proportion of patients with AFP levels below 10 ng/mL increased from 73 % at the baseline to 95 % at 24 weeks among noncirrhotic patients and from 48 % at the baseline to 76 % at 24 weeks among cirrhotic patients (Fig. 1).

A total of 42 patients developed HCC during the observation period (16 noncirrhotic patients, 26 cirrhotic patients). The cumulative incidence of HCC at 3, 5, and 7 years was 6.0, 9.6, and 17.2 %, respectively. The mean time point of HCC development was 34.0 ± 18.4 months from the initiation of ETV treatment. AFP levels among patients who developed HCC decreased from 24 weeks (13.1 ± 3.9 ng/mL) (mean ± standard error of the mean) to 48 weeks (10.2 ± 3.0 ng/mL) after the initiation of ETV treatment and increased again from 24 weeks before HCC incidence (7.6 ± 1.6 ng/mL) to the time of HCC incidence (35.4 ± 12.8 ng/mL) (Fig. S1). The cutoff value of AFP levels at 24 weeks from the initiation of ETV treatment for prediction of HCC incidence was set as 10 ng/mL on the basis of the calculated cutoff value (12.1 ng/mL) assessed using the time-dependent ROC curve (Table S1).

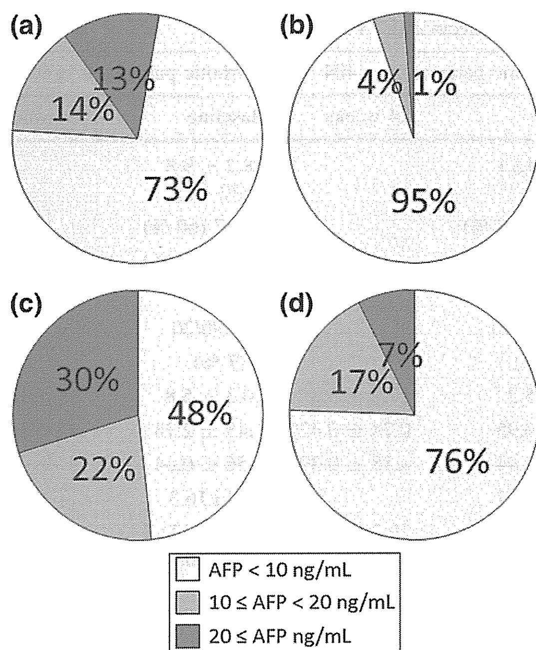

Fig. 1 Distribution of alpha-fetoprotein (*AFP*) levels at the baseline and at 24 weeks after the initiation of entecavir (ETV) treatment according to the severity of liver disease: **a** patients without cirrhosis at the baseline ($n = 326$); **b** patients without cirrhosis at 24 weeks after ETV treatment initiation ($n = 326$); **c** patients with cirrhosis at the baseline ($n = 87$); **d** patients with cirrhosis at 24 weeks after ETV treatment initiation ($n = 87$)

Factors associated with HCC incidence at the baseline

In a univariate analysis, factors at the baseline such as advanced age, cirrhosis, lower platelet counts, and higher total bilirubin, lower albumin, and higher AFP levels were significant, and a multivariate analysis demonstrated that advanced age (55 years or older) and cirrhosis were significant independent risk factors for HCC incidence (Table 2). After a stratified analysis of HCC incidence according to those risk factors at the baseline, the cumulative incidence of HCC at 5 years was 2.5 % in younger patients (younger than 55 years) and 18.6 % in older patients (55 years or older, $p < 0.001$; Fig. 2a). The cumulative incidence of HCC at 5 years was 5.3 % in noncirrhotic patients and was 30.0 % in cirrhotic patients ($p < 0.001$; Fig. 2b).

Factors associated with HCC incidence at 24 weeks after the initiation of ETV treatment

The association between HCC incidence and posttreatment factors at 24 weeks after the initiation of ETV treatment was estimated. In a univariate analysis, advanced age, cirrhosis, lower platelet counts,and lower albumin, higher total bilirubin, and higher AFP levels at 24 weeks were significant, and a multivariate analysis showed that a higher AFP level (10 ng/mL or greater) at 24 weeks was the only additional factor independently associated with HCC incidence other than advanced age and cirrhosis, which were found to be significant risk factors at the baseline (Table 3). The cumulative incidence of HCC at 5 years was 8.2 % among patients with an AFP level below 10 ng/mL at 24 weeks and was 34.2 % among patients with an AFP level of 10 ng/mL or higher at 24 weeks (Fig. 3a). Although the American Association for the Study of Liver Disease practical guidelines for chronic hepatitis B indicate that the aims of treatment for patients infected with HBV are to achieve a reduction in the serum HBV DNA levels and a normalization of serum ALT levels [17], in this study, neither virological response nor biochemical response (ALT level of 30 IU/L or lower) at 24 weeks by ETV treatment affected HCC incidence (Table 3). The cumulative incidence of HCC was almost equivalent between patients with and without virological response at 24 weeks in the analysis among all enrolled patients ($p = 0.685$; Fig. 3b). Additionally, there was no significant difference in the cumulative incidence of HCC between patients with or without normalization of ALT levels at 24 weeks ($p = 0.076$; Fig. 3c). The cumulative incidence of HCC significantly increased with higher AFP levels (10 ng/mL or greater) at 24 weeks even among patients who achieved virological response ($p = 0.023$) or normalization of ALT levels at 24 weeks ($p = 0.002$). The AFP levels at 24 weeks were closely related to HCC incidence irrespective of the virological response or biochemical response at 24 weeks in patients with HBV infection who were undergoing treatment with ETV.

The impact of AFP at 24 weeks on HCC incidence according to baseline factors

Because AFP levels at 24 weeks were found to be a significant factor related to HCC incidence among multiple factors that varied during treatment, the impact of AFP at 24 weeks on HCC incidence was assessed in the subgroups stratified by HCC-related factors at the baseline: age and the severity of liver disease. In the subgroup analysis stratified by age, AFP levels at 24 weeks were significantly related to HCC incidence, and the cumulative incidence of HCC at 5 years was significantly higher in patients with AFP levels of 10 ng/mL or higher at 24 weeks than those with AFP levels below 10 ng/mL, irrespective of age (younger than 55 years, 16.1 % vs 2.2 %, $p = 0.009$; 55 years or older, 45.4 % vs 14.9 %, $p < 0.001$; Fig. 4a, b). In the subgroup analysis that was stratified according to the severity of liver disease, the AFP level at 24 weeks was a significant factor in the cirrhotic group ($p = 0.029$) but not in the noncirrhotic group ($p = 0.377$); the cumulative incidence of HCC at 5 years in the cirrhotic group was

Table 2 Risk factors at the baseline for hepatocellular carcinoma (HCC) incidence in chronic hepatitis B patients receiving ETV treatment (Cox proportional-hazards model)

Factors	Category	Univariate analysis		p	Multivariate analysis		
		HR	95 % CI		HR	95 % CI	p
Age (years)	0:<55	1	2.601–13.243	<0.001	1	1.592–8.560	0.002
	1:≥55	5.869			3.691		
Gender	0:male	1	0.365–1.319	0.265			
	1:female	0.694					
Severity of liver disease	0:no cirrhosis	1	4.050–14.085	<0.001	1	2.415–9.404	<0.001
	1:cirrhosis	7.553			4.765		
HBeAg	0:negative	1	0.412–1.436	0.410			
	1:positive	0.770					
Histology: activity	0:A0-1	1	0.352–3.800	0.810			
	1:A2-3	1.157					
Histology: fibrosis	0:F0-2	1	0.865–5.910	0.096			
	1:F3-4	2.262					
History of IFN therapy	0:none	1	0.032–1.718	0.154			
	1:presence	0.236					
Platelet count ($\times 10^4/\mu L$)	0:<15	1	0.103–0.449	<0.001			
	1:≥15	0.215					
Total bilirubin (mg/dL)	0:<1.0	1	1.235–4.141	0.008			
	1:≥1.0	2.261					
Albumin (g/dL)	0:<4.0	1	0.201–0.725	0.003			
	1:≥4.0	0.381					
PT (%)	0:<80	1	0.301–1.056	0.074			
	1:≥80	0.564					
ALT (IU/L)	0:<80	1	0.345–1.246	0.197			
	1:≥80	0.656					
HBV DNA(LC/mL)	0:<6.5	1	0.748–2.701	0.283			
	1:≥6.5	1.422					
AFP (ng/mL)	0:<10	1	1.040–3.721	0.038			
	1:≥10	1.967					

CI confidence interval, *HR* hazard ratio

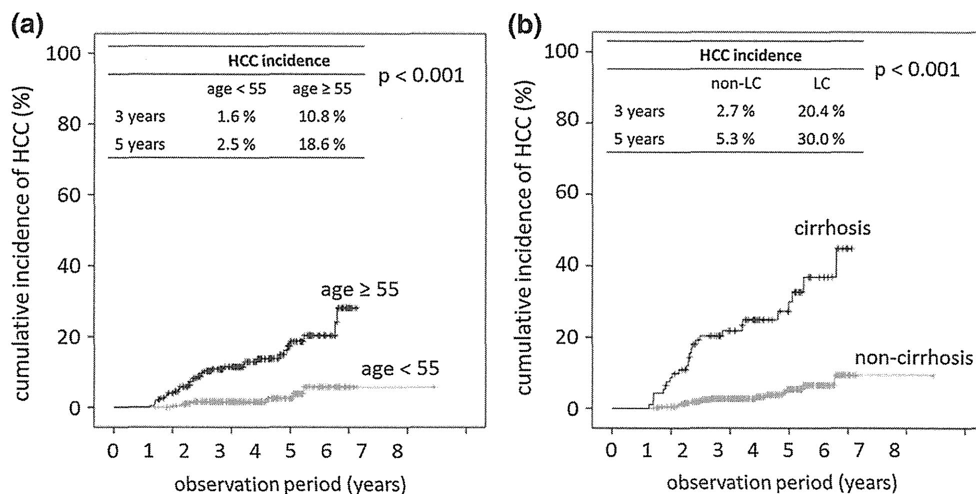

Fig. 2 Cumulative hepatocellular carcinoma (*HCC*) incidence among patients with hepatitis B virus (HBV) infection according to factors at the baseline (log-rank test). **a** Cumulative HCC incidence according to the age at the baseline (*black line* 55 years or older, *gray line* younger than 55 years). **b** Cumulative HCC incidence according to the severity of liver disease (*black line* cirrhosis, *gray line* no cirrhosis)

Table 3 Risk factors at 24 weeks after initiation of ETV treatment for HCC incidence in chronic hepatitis B patients receiving ETV treatment (Cox proportional-hazards model)

Factors	Category	Univariate analysis			Multivariate analysis		
		HR	95 % CI	*p*	HR	95 % CI	*p*
Age (years)	0:<55	1	2.601–13.243	<0.001	1	1.198–6.748	0.018
	1:≥55	5.869			2.843		
Gender	0:male	1	0.365–1.319	0.265			
	1:female	0.694					
Severity of liver disease	0:no cirrhosis	1	4.050–14.085	<0.001	1	2.518–12.411	<0.001
	1:cirrhosis	7.553			5.590		
Platelet count ($\times 10^4/\mu$L) at 24 weeks	0:<15	1	0.114–0.473	<0.001			
	1:≥15	0.233					
Total bilirubin (mg/dL) at 24 weeks	0:<1.0	1	1.360–4.569	0.003			
	1:≥1.0	2.493					
Albumin (g/dL) at 24 weeks	0:<4.0	1	0.201–0.725	0.003			
	1:≥4.0	0.381					
ALT (IU/L) at 24 weeks	0:≤30	1	0.938–3.157	0.080			
	1:>30	1.720					
VR[a] at 24 weeks	0:none	1	0.461–1.664	0.685			
	1:presence	0.875					
AFP (ng/mL) at 24 weeks	0:<10	1	2.589–11.496	<0.001	1	1.066–5.316	0.034
	1:≥10	5.456			2.381		

VR virological response

[a] VR is defined as HBV DNA of less than 2.6 LC/mL

higher in patients with AFP levels of 10 ng/mL or greater at 24 weeks than in those with AFP levels below 10 ng/mL (50.0 % vs 24.7 %; Fig. 4c, d).

Risk analysis for HCC incidence among patients who achieved virological response by ETV treatment

Among patients with HBV infection who achieved virological response by ETV treatment, the risk analysis for HCC incidence was performed in a Cox proportional-hazards model according to the number of the following three risk factors: AFP levels at 24 weeks, age, and the presence of cirrhosis (Fig. S2). When the AFP level remained high (10 ng/mL or higher) at 24 weeks, the cumulative incidence of HCC at 5 years was 6.7 % with no other risk factors (Fig. S2a), 14.8 % with the factor of age of 55 years or older, 27.9 % with the factor of cirrhosis, and 57.7 % with the factors of age of 55 years or older and cirrhosis (Fig. S2b).

Discussion

ETV treatment has been reported to reduce serum HBV DNA levels and ALT levels in patients with chronic HBV infection and to improve hepatitis [18]. On the basis of a study that showed that a higher HBV DNA level at the baseline is associated with a higher HCC incidence in the natural history cohort (the REVEAL study) [15], a reduction of HBV DNA levels by ETV treatment has been considered to have the possibility to suppress HCC incidence among patients with chronic HBV infection. However, it was still unknown whether a lower or an undetectable level of serum HBV DNA, which was achieved by ETV treatment, has a suppressive effect on HCC incidence as shown in the natural course. In the present study, factors associated with HCC incidence during ETV treatment among patients with chronic HBV infection were investigated.

In a previous study that used a historical control group, a significant suppressive effect of ETV on HCC incidence was shown in cirrhotic but not noncirrhotic patients [19]. Furthermore, Wong et al. [20] reported that HCC incidence was significantly lower among patients with cirrhosis who had undetectable levels of HBV DNA compared with those with detectable levels of HBV DNA. In the present study, reduced serum HBV DNA levels were associated with a decrease in the cumulative incidence of HCC only in patients with cirrhosis, and not in those without cirrhosis (Fig. S3). Originally, HBV covalently closed circular DNA (cccDNA) levels in the hepatocyte nuclei were nearly parallel to the serum HBV DNA levels in the natural