Biology Open (2015) 000, 1–9 doi:10.1242/bio.20148532

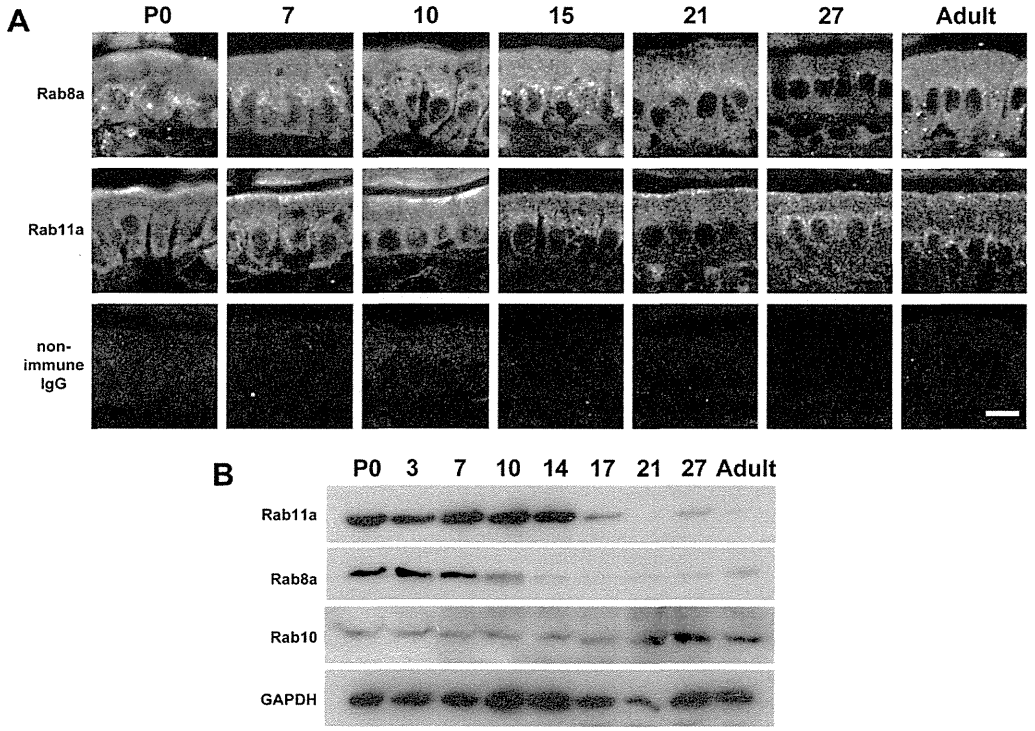

Fig. 7. Localisation of Rab8a and Rab11a and quantification of Rab11a, Rab8a, and Rab10 in the small intestine during postnatal development. (A) Localisation of Rab8a (top), Rab11a (middle) and non-immune rabbit IgG (bottom), as determined by immunofluorescence, during postnatal (P) days in wild-type epithelial cells of the small intestine. (B) Levels of Rab11a, Rab8a, and Rab10 in the wild-type small intestine during postnatal development. Scale bar: 10 μm.

Millipore, San Diego, CA, USA); Golgin-97 (1:100; Yoshimura et al., 2004); Lamp2 (1:100; clone Abl-93; Developmental Studies Hybridoma Bank, Iowa, USA); Lamin B (1:500 for WB; Santa Cruz Biotechnology, Dallas, TX, USA); low density lipoprotein receptor (LDL-R; 1:1000 for WB, 1:100 for IF, R&D Systems, Minneapolis, MN, USA); microtubule-associated protein 1A (MAP1A; 1:100; Shiomura and Hirokawa, 1987); Na^+-K^+ ATPase (1:1000 for WB; clone C464.6; Upstate Biotechnology, 1:100 for IF; Homareda et al., 1993); Phalloidin–Tetramethylrhodamine B isothiocyanate (1:1000; Sigma-Aldrich, St. Louis, MO, USA); Rab8a (1:1000 for WB, 1:100 for IF; Sato et al., 2007); Rab10 (1:500 for WB; Cell Signaling Technology, Danvers, MA, USA); Rab11b (1:500 for WB; Aviva Systems Biology, San Diego, CA, USA); synaptophysin (1:1000; EMD Millipore, Billerica, MA, USA), and Tbr1 (1:500; EMD Millipore, Billerica, MA, USA). Alexa 488- or Alexa 594-labelled species-specific secondary antibodies (1:400; Life Technologies, Carlsbad, CA, USA) were used.

The rabbit polyclonal antibody against Rab11a was raised using a bacterially expressed GST-fused Rab11a protein fragment (C-terminal 30 amino acids) encoded by pGEX4T1. The antisera were affinity purified prior to use in the experiments (1:500 for WB and 1:100 for IF).

The stained sections were analysed by confocal microscopy (FV1000D, Olympus, Tokyo, Japan or LSM510 META, Carl Zeiss Japan, Tokyo, Japan), as previously described (Sato et al., 2007).

Nissl staining

Paraformaldehyde-fixed brains were sectioned in 16 μm thick with cryostat (Leica, Germany). The sections were washed with water and then stained with Toluidine blue O (Waldeck GmbH&Co. KG, Division Chroma, Muenster, Germany) solution (0.01% Toluidine blue O, 0.06 M sodium citrate and 0.08 M Na_2HPO_4) for 15 min at room temperature. After washing in water, the sections were analysed by using a BX61 light microscope (Olympus Corporation, Tokyo, Japan).

Peptide *N*-glycosidase F (PNGase F) treatment

Small intestinal tissue derived from control or IKO mice was lysed by the buffer containing 10 mM Tris-HCl, pH 7.8, 0.5 M NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Nonidet P-40) with protease inhibitor cocktail (Roche, Basel, Switzerland). After centrifugation (15,000 g) for 10 min at 4℃, the supernatant was boiled in 0.1 M 2-mercaptoethanol and 0.5% SDS for 10 min. After boiling, the 50 μg of protein was incubated for 16 h at 37℃ with 100 mM Tris-HCl (pH 8.6), 1% Nonidet P-40 and 40 mU/ml PNGase F (TAKARA, Shiga, Japan). The sample was subjected to SDS-PAGE and analysed by Western blotting.

Measurement of a starvation marker

Approximately 100 μl of the blood was sent to SRL Inc. (Tokyo, Japan) and analysed the measure of total ketone bodies (acetoacetic acid and 3-hydroxy-butyrate).

Electron microscopy

Mice were perfused or immersed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The tissues were then dissected, fixed for another 2 h at RT, and treated with 1% OsO_4 in 0.1 M cacodylate buffer followed by 0.5% uranyl acetate in water. The samples were dehydrated and embedded in Epon, and the thin sections were post-stained with uranyl acetate and lead citrate, as previously described (Harada et al., 1990; Harada et al., 1994). The sections were then examined by electron microscopy (H7650; Hitachi, Tokyo, Japan) at 80 kV.

Image processing and quantification

Images were processed using Adobe Photoshop® (Adobe Systems, Inc., CA, USA) version 7.0.

Analysis of human samples by immunohistochemistry

We used small intestine samples from an early-onset microvillus inclusion disease patient. The male patient was diagnosed with microvillus inclusion disease by electron-microscopic examination of the jejunal epithelial cells at 4 months of age. Detailed descriptions of this patient are available in previous papers (Kagitani et al., 1998). This study was approved by the Ethics Committee of Osaka University School of Medicine based on the written, informed consent of each subject. The small intestine samples were obtained following small intestine transplantations in the patients, and they were fixed in formaldehyde and embedded in paraffin.

The paraffin-embedded sections of diseased and control human small intestine tissue were prepared on license from the ethics committee of Osaka university hospital. The paraffin slides were rehydrated in a descending ethanol series following deparaffinisation with Clear Plus. After rinsing with 1× phosphate-buffered saline (PBS; pH 7.4), the slides were treated with blocking solution (PBS containing 3% bovine serum albumin (Sigma) and 5% normal goat serum (Gibco)) for 1 hour at room temperature (RT). The slides were then incubated overnight at 4℃ with a polyclonal rabbit anti-Rab11a antibody (1:100) and a polyclonal rabbit anti-alkaline phosphatase antibody (1:100; Rockland Immunochemicals Inc., PA, USA) in blocking solution. The slides were washed with PBS and then treated with a biotin-conjugated goat anti-rabbit IgG secondary antibody (1:200; Vector Laboratories Inc., CA, USA) for 30 min at RT in PBS. Subsequently, the slides were incubated in 1.5% H_2O_2 for 30 min at RT to eliminate endogenous peroxidases. Following amplification with the avidin-biotin complex (ABC kit; Vector Laboratories), visualisation of the reaction products was carried out with 50 mM Tris-buffered saline (TBS; pH 7.4) containing 1.25% DAB and 0.75% hydrogen peroxide. The slides were immersed in 50 mM TBS to stop the reaction, followed by hematoxylin treatment as a counter-stain. Finally, following dehydration, the slides were coverslipped and sealed with Entellan (Merck, Darmstadt, Germany). Stained samples of non-treated small intestines were also prepared as controls. Control samples were examined using normal rabbit IgG (1:100; Dako, Glostrup, Denmark) instead of the primary antibody during the incubation process. We performed hematoxylin-eosin (HE) staining using standard histological procedures. All slides were analysed using a BX61 light microscope (Olympus Corporation, Tokyo, Japan).

Acknowledgements

We thank A. Goto, H. Togawa, M. Natsume, A Izumi, and A Watanabe for technical assistance, H. Homareda and N. Nakamura for providing us with antibodies, and S. Mushiake and E. Morii for providing human tissue samples.

Competing interests

The authors have no competing interests to declare.

Author contributions

T.S. generated and analysed knockout mice, S.Y. conducted Western blot analyses. T.I. conducted brain morphological analyses. M.K. and N.A. conducted electron microscopy analyses. M.W. raised rabbit antisera against Rab11a. S.M., E.Mo., and Y.K. provided, sectioned, and stained human samples. E.Mi. and A.H. designed experiments, conducted morphological analyses, and prepared the manuscript.

Funding

This work was supported by the Grants-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science (KAKENHI no. 24390046 to A.H. and from the Mitsubishi Foundation and Takeda Science Foundation to A.H. and S.Y.).

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J Gastroenterol
DOI 10.1007/s00535-014-1010-7

The Japanese Society of Gastroenterology

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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Received: 22 August 2014 / Accepted: 21 October 2014

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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Electronic supplementary material The online version of this article (doi:10.1007/s00535-014-1010-7) contains supplementary material, which is available to authorized users.

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Published online: 11 November 2014

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).

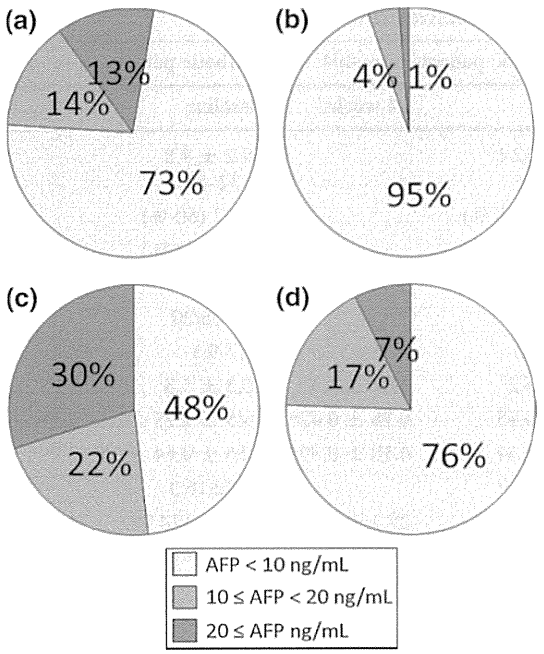

Fig. 1 Distribution of alpha-fetoprotein (*AFP*) levels at the baseline and at 24 weeks after the initiation of entecavir (ETV) treatment according to the severity of liver disease: **a** patients without cirrhosis at the baseline ($n = 326$); **b** patients without cirrhosis at 24 weeks after ETV treatment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

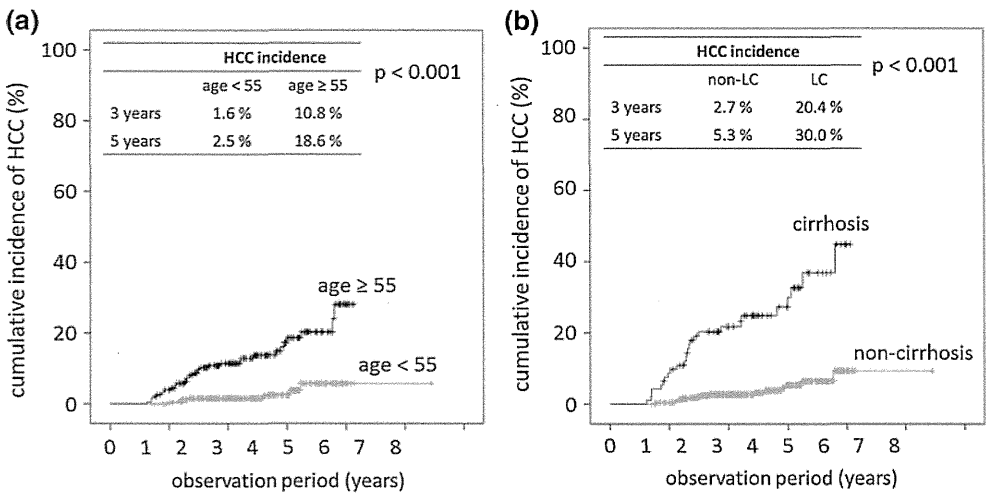

Fig. 2 Cumulative hepatocellular carcinoma (*HCC*) incidence among patients with hepatitis B virus (HBV) infection according to factors at the baseline (log-rank test). **a** Cumulative HCC incidence according to the age at the baseline (*black line* 55 years or older, *gray line* younger than 55 years). **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

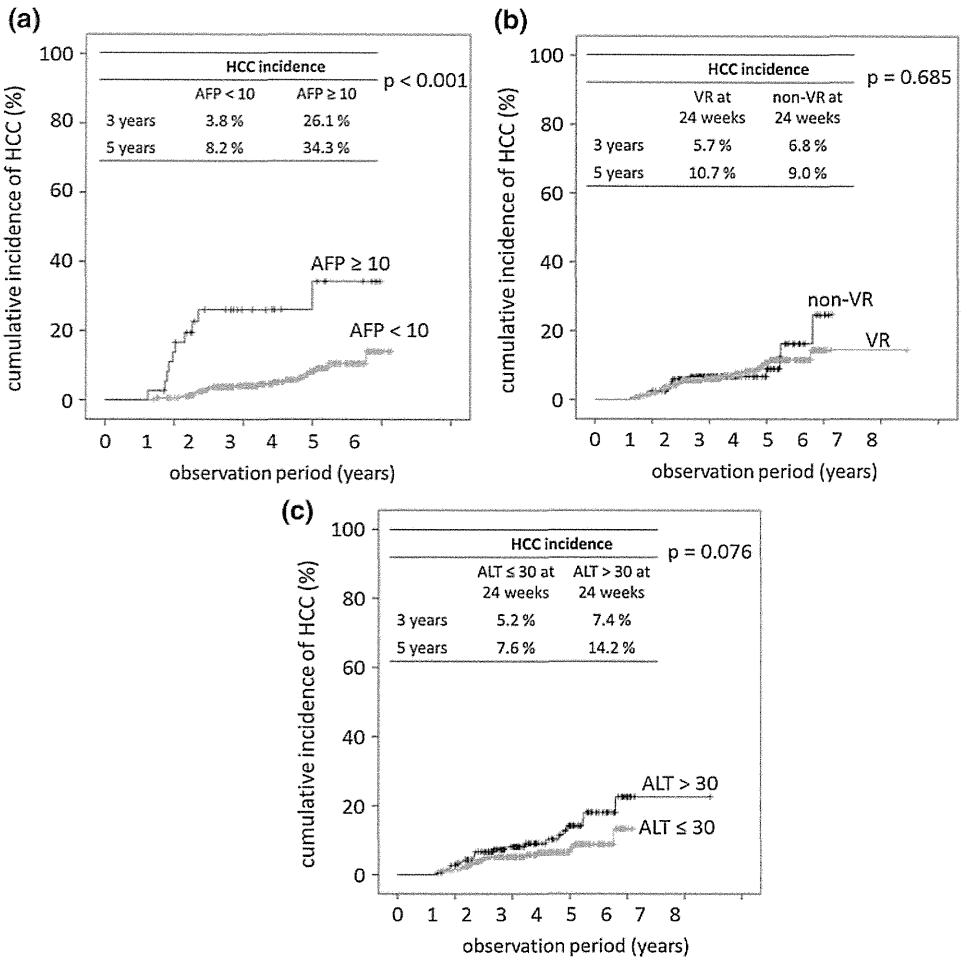

Fig. 3 Cumulative HCC incidence among patients with HBV infection according to factors at 24 weeks after ETV treatment initiation (log-rank test). Virological response (*VR*) is defined as HBV DNA of less than 2.6 log copies per milliliter. **a** Cumulative HCC incidence according to AFP levels at 24 weeks (*back line* AFP level of 10 ng/mL or greater at 24 weeks, *gray line* AFP level below 10 ng/mL at 24 weeks). **b** Cumulative HCC incidence according to virological response at 24 weeks (*black line* no VR at 24 weeks, *gray line* VR at 24 weeks). **c** Cumulative HCC incidence according to biochemical response at 24 weeks [*black line* alanine aminotransferase (*ALT*) level above 30 IU/L at 24 weeks, *gray line* ALT level of 30 IU/L or lower at 24 weeks]

course. However, low levels of serum HBV DNA achieved by ETV treatment do not always indicate low intracellular HBV cccDNA levels [21, 22]. Therefore, it is possible that an insufficient decrease of intracellular HBV DNA levels cannot bring the apparent HCC suppression in noncirrhotic liver with low malignant potential. A longer observation period is required to clarify the suppressive effect on HCC incidence among noncirrhotic patients. The relationship between HBV cccDNA levels in the liver and HCC incidence should also be examined.

In this study, in the analysis of the relationship between on-treatment factors and HCC incidence, only higher AFP levels (10 ng/mL or higher) at 24 weeks after the initiation of ETV treatment were found to be associated with HCC incidence. This is the first study to investigate the significance of AFP levels as a representative marker for the potential of HCC development among patients with chronic HBV infection undergoing ETV treatment. Originally, AFP was known as a tumor-associated antigen in HCC and as a target for immunotherapy. AFP has been used in the surveillance of HCC and in the evaluation of treatment response in HCC patients. The use of AFP as a marker to identify HCC among patients with HBV infection has previously been shown in patients with a natural course of the disease [23]. In recent reports that have focused on AFP levels for HCC diagnosis in patients undergoing ETV treatment, elevated AFP levels at 6 months before or at the time of HCC incidence were shown to be useful in detecting existing HCC [24, 25]; that is, elevated AFP levels implied the existence of cancer cells. However, the present study clarified that a high AFP level at 24 weeks did not suggest the existence of cancer cells, but indicates a potential for HCC incidence before the initiation of carcinogenesis. A possible reason is as follows. The AFP levels among patients who developed HCC decreased from 24 to 48 weeks after the initiation of ETV treatment and increased again from 24 weeks before HCC incidence to the time of HCC incidence. Furthermore, it took a considerably long time before HCC incidence, on average 32.6 months of the observation period (Fig. S1). With regard to the relationship between serum AFP levels and HCC incidence among HCV-infected patients, AFP levels at 24 weeks after the end of IFN treatment have been associated with HCC [26, 27]. AFP levels after the

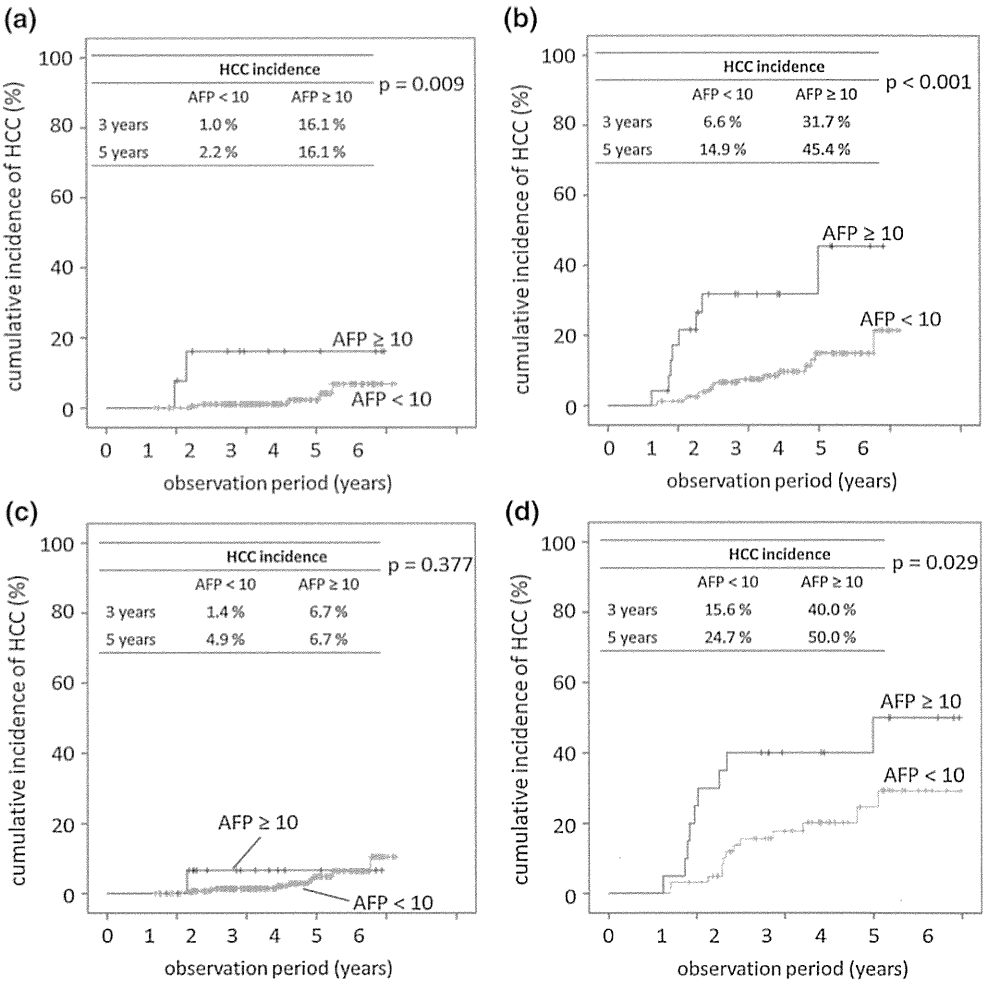

Fig. 4 Cumulative HCC incidence among patients with HBV infection according to AFP levels at 24 weeks after ETV treatment initiation, stratified with baseline factors (log-rank test). **a** Patients younger than 55 years. **b** Patients 55 years or older. **c** Patients without cirrhosis. **d** Patients with cirrhosis. *Black line* AFP level of 10 ng/mL or higher at 24 weeks, *gray line* AFP level below 10 ng/mL at 24 weeks

initiation of treatment of both HBV infection and HCV infection appear to have important implications for HCC incidence.

What the AFP levels at 24 weeks actually represent in patients undergoing ETV treatment is uncertain. The AFP level is a surrogate marker that appears to predict a disease condition from various pathological factors including inflammation, fibrosis, and liver regeneration, which involve carcinogenesis. Moreover, a previous study reported that the activation of natural killer cells by dendritic cells was inhibited when they were co-cultured with AFP; this result suggests an association between HCC development and the maintenance of high AFP levels [28]. Therefore, AFP is thought to be an important biomarker that can reflect various aspects of liver disease.

American Association for the Study of Liver Disease practice guidelines for the management of HBV have defined the goal of NA treatment as to decrease serum HBV DNA levels to undetectable levels to suppress HCC development. In this study, the HBV DNA levels and ALT levels were rapidly lowered in most patients. However, this study shows that the virological and biochemical treatment responses had no association with HCC development, whereas advanced age, liver cirrhosis, and a higher AFP level at 24 weeks after the initiation of ETV treatment were independent risk factors that were significantly associated with HCC development. It is considered that decreasing serum HBV DNA levels to undetectable levels is the necessary, but not sufficient condition to suppress HCC development. In fact, the HCC incidence rate even in patients undergoing ETV treatment who achieved virological response at 24 weeks with the three factors of age of 55 years or older, liver cirrhosis, and AFP level of 10 ng/mL or higher increased to as high as approximately 60 % at 5 years (Fig. S2). Accordingly, the undetectable HBV DNA level in patients with chronic HBV infection undergoing ETV treatment is in itself of little consequence and does not mean a riskless environment.

The limitation of this study is that analysis including other HCC-related factors, such as hepatitis B surface antigen levels, precore and core promotor mutations, and family history of HCC or alcohol consumption, was not

performed. Especially, further investigation is needed to clarify the relationship between the change in hepatitis B surface antigen levels during treatment and HCC incidence in patients with HBV infection.

In conclusion, in the consecutive surveillance for HCC after the initiation of ETV treatment, monitoring the change in AFP levels at 24 weeks is essential, especially among patients of advanced age or with cirrhosis.

Acknowledgments The authors thank Atsuo Inoue (Osaka General Medical Center), Masami Inada (Toyonaka Municipal Hospital), Ikuo Suzuki (Saiseikai Senri Hospital), Akira Takeda (Ashiya Municipal Hospital), Hiroyuki Ogawa (Nishinomiya Municipal Central Hospital), Mitsunari Yamamoto (Kinki Central Hospital of Mutual Aid Association of Public School Teachers, Itami, Hyogo, Japan), and Yukiko Saji (Itami City Hospital) for their support. This work was supported by a Grant-in-Aid for Research on Hepatitis from the Ministry of Health Labour and Welfare of Japan, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

Conflict of interest Professor Tetsuo Takehara received research grants from Merck Sharp and Dohme K.K. Co., Ltd., Chugai Pharmaceutical Co., Ltd. and Bristol Myers Squibb.The other authors have nothing to disclose.

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J Gastroenterol
DOI 10.1007/s00535-014-1023-2

The Japanese Society of Gastroenterology

Association of serum IFN-λ_3 with inflammatory and fibrosis markers in patients with chronic hepatitis C virus infection

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Received: 22 September 2014 / Accepted: 20 November 2014

Abstract

Background Hepatitis C virus (HCV) is one of the major causes of liver cancer. The single nucleotide polymorphisms within the *IFNL3* gene, which encodes interferon (IFN)-λ_3, are strongly associated with the response to pegylated IFN-α (PEG-IFN-α) plus ribavirin (RBV) therapy in chronic hepatitis C (C-CH) patients. However, the roles of IFN-λ_3 in chronic HCV infection are still elusive. In this study, we aimed to identify clinical and immunological factors influencing IFN-λ_3 and evaluated whether serum IFN-λ_3 levels are involved or not involved in the response to PEG-IFN-α plus RBV therapy.

Methods We enrolled 119 C-CH patients with HCV genotype 1 infection who underwent 48 weeks of PEG-IFN-α plus RBV therapy. As controls, 23 healthy subjects and 56 patients with non-HCV viral hepatitis were examined. Serum IFN-λ_3 was quantified by chemiluminescence enzyme immunoassay, and 27 cytokines or chemokines were assayed by the multiplexed BioPlex system.

Results Serum IFN-λ_3 levels were higher in C-CH patients or acute hepatitis E patients than in healthy volunteers. Such levels did not differ between the *IFNL3* genotypes. In C-CH patients, serum IFN-λ_3 was positively correlated with aspartate aminotransferase, alanine aminotransferase, α-fetoprotein, histological activity, fibrosis index, IFN-γ-inducible protein 10, and platelet-derived growth factor. Multivariate analysis showed that *IFNL3* single nucleotide polymorphisms, fibrosis score, and macrophage inflammatory protein 1α were involved in the

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Electronic supplementary material The online version of this article (doi:10.1007/s00535-014-1023-2) contains supplementary material, which is available to authorized users.

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Published online: 14 December 2014

sustained viral clearance in PEG-IFN-α plus RBV therapy; however, serum IFN-λ_3 levels were not involved.
Conclusion Serum IFN-λ_3 levels are increased in C-CH patients regardless of the *IFNL3* genotype. IFN-λ_3 is a biomarker reflecting the activity and fibrosis of liver disease, but is not correlated with the responsiveness to PEG-IFN-α plus RBV therapy.

Keywords Hepatitis C virus · IL-28B · Interferon-λ_3 · Chemokine · Pegylated interferon-α plus ribavirin

Abbreviations

APRI	Aspartate aminotransferase platelet ratio index
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
B-CH	Chronic hepatitis B
C-CH	Chronic hepatitis C
FIB-4	Fibrosis-4
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HV	Healthy volunteer
IFN	Interferon
IP-10	Interferon-γ-inducible protein 10
MIP	Macrophage inflammatory protein
PDGF-BB	Platelet-derived growth factor BB
PEG-IFN-α	Pegylated interferon-α
RANTES	Regulated on activation, normally T cell expressed, and secreted
RBV	Ribavirin
SNP	Single nucleotide polymorphism
SVR	Sustained virological response

Introduction

Hepatitis C virus (HCV) is one of the leading causes of liver cirrhosis and hepatocellular carcinoma (HCC), with nearly 170 million people infected worldwide [1]. A combination therapy with pegylated interferon (IFN)-α (PEG-IFN-α) and ribavirin (RBV) has been used for chronic hepatitis C (C-CH) patients as the standard of care, achieving sustained virological response (SVR) in 42–52 % of genotype 1 patients [2]. Even in the coming era of all oral and IFN-free regimens for the treatment of C-CH patients [3–5], PEG-IFN-α plus RBV therapy could hold promise for elderly patients with advanced fibrosis and high risk of HCC.

Genome-wide association studies, including ours, have demonstrated that single nucleotide polymorphisms (SNPs) upstream of the promoter region within the *IFNL3* gene (also known as *IL28B*), which encodes a type III IFN (IFN-λ_3), are strongly associated with the response to PEG-IFN-α plus RBV therapy in C-CH patients [6–9]. Although such significant impact of the *IFNL3* genotype on the outcome of the combination therapy is well acknowledged, the biological and clinical roles of IFN-λ_3 in chronic HCV infection are still elusive. Furthermore, it is controversial if patients with the *IFNL3* major genotype are capable of producing larger amounts of IFN-λ_3 than those with the minor genotype.

The IFN-λ family consists of several subtypes, such as IFN-λ_1 (IL-29), IFN-λ_2 (IL-28A), and IFN-λ_3 (IL-28B), which are biologically active for the suppression of HCV replication [10, 11]. On initial exposure to HCV, primary human hepatocytes in vitro produced IFN-λ and subsequently induced antiviral IFN-stimulated genes [12]. It is thus rational to consider that the more IFN-λ family members are produced in the exposed hosts, the more likely they are to protect the hosts from HCV virulence in the primary infection. However, in chronically HCV-infected patients, it has not been proven that such a scenario could be applicable for the outcome of the disease.

To gain insight into the role of IFN-λ_3 in chronic HCV infection, we aimed to clarify the factors influencing serum IFN-λ_3 levels, including *IFNL3* genotype, clinical parameters, and various cytokines and chemokines. For application in clinical practice, we evaluated whether serum IFN-λ_3 levels are associated or not associated with the response to PEG-IFN-α plus RBV therapy for C-CH patients.

Materials and methods

Study subjects

One hundred nineteen Japanese patients with C-CH (genotype 1b and high viral load) were enrolled in the study. All patients were negative for hepatitis B virus (HBV) and human immunodeficiency virus (HIV) and did not have any other chronic liver diseases, such as alcoholic, autoimmune, and fatty liver disease. The presence of HCC was ruled out by ultrasonography or computed tomography examinations. The patients had been followed at the National Center for Global Health and Medicine Kohnodai Hospital, the National Hospital Organization Nagasaki Medical Center, Shin-Kokura Hospital, and Musashino Red Cross Hospital. They were treated with PEG-IFN-α_{2b} (subcutaneously once a week; 1.5 μg/kg body weight) or PEG-IFN-α_{2a} (180 μg once a week) plus RBV (600–1,000 mg daily depending on body weight) for 48 weeks according to the guidelines of the Japan Society of Hepatology [13]. Virological response to the combination therapy was defined according to the practical

guidelines of the American Association for the Study of Liver Diseases [14]. All patients attained adherence to PEG-IFN-α plus RBV therapy exceeding 80 % of the estimated total dose. Liver biopsy was performed before the start of the therapy. Histological activity and fibrosis were determined according to the METAVIR scoring system [15]. Serum samples were collected from the patients before PEG-IFN-α plus RBV treatment started and were stored at −80 °C. In some patients, the samples were obtained 24 weeks after the cessation of the therapy (at the end of follow-up).

As controls, serum was obtained from 23 healthy subjects without HCV, HBV, and HIV infection (male-to-female ratio, 5:5, mean age ± standard deviation, 45 ± 12 years). In the comparison of serum IFN-λ levels between C-CH patients and patients with other types of liver diseases, 11 patients with chronic HBV infection (three HBeAg-positive patients and eight HBeAg-negative patients) were examined as well. They were not treated with IFN or nucleot(s)ide analogues for HBV infection. In addition, we compared serum IFN-λ_3 levels among patients with acute viral hepatitis of various causes, such as acute hepatitis A, acute hepatitis B, or acute hepatitis E, the diagnosis of which was determined by serological examinations at Teine Keijinkai Hospital and Kurume University Hospital. The serum samples were obtained from the patients at the time of active liver inflammation [alanine aminotransferase (ALT) levels more than two times the upper limit of the normal range]. As representatives for noninvasive fibrosis markers, the fibrosis-4 (FIB-4) score and the aspartate aminotransferase (AST) platelet ratio index (APRI) were calculated as reported previously [16, 17].

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board at the National Center for Global Health and Medicine (approval ID and date, NCGM-G-001379-00, March 14, 2013) and the ethical committee of each institute. Written informed consent was obtained from all patients.

IFNL3 genotyping

The subjects were evaluated for SNPs near the *IFNL3* gene (rs8099917) using the Invader Plus assay (Invader Chemistry, Madison, WI, USA) as previously reported [18]. The TT, TG, and GG genotypes were determined accordingly.

Measurement of serum IFN-λ_3

Serum levels of IFN-λ_3 were evaluated by the newly developed chemiluminescence enzyme immunoassay system as reported previously [19]. The system enables one to quantify serum IFN-λ_3 specifically without any overlap from IFN-λ_1 and IFN-λ_2. The threshold of the assay is 10 pg/mL and its range is 10–1,000 pg/mL.

Simultaneous measurement of multiple chemokines and cytokines

To quantify multiple chemokines and cytokines simultaneously in the limited volume of the samples, we used the BioPlex 3D system (BioPlex Pro Human GI 27Plex; Bio-Rad, Hercules, CA, USA) for the study. In this system, 27 chemokines and cytokines were measurable, such as basic fibroblast growth factor, eotaxin, granulocyte colony stimulating factor, granulocyte–macrophage colony stimulating factor, IL-1β, IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, IFN-γ-inducible protein 10 (IP-10), monocyte chemotactic protein 1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor BB (PDGF-BB), regulated on activation, normally T cell expressed, and secreted (RANTES), TNF-α, and vascular endothelial growth factor. The detection range and thresholds are given in Table S1. For the measurement of IP-10, ELISA (R&D Systems, MN, USA) was performed as well.

Statistical analyses

Continuous variables were compared between groups using the Wilcoxon signed-rank test and the Mann–Whitney *U* test, and categorical data were compared using the χ^2 test or Fisher's exact test. The correlations between cytokines, chemokines, and clinical markers were evaluated by Spearman's correlation coefficient. A p value below 0.05 was considered to be significant. Logistic regression was used for multivariate analyses. All statistical analyses were performed with PRISM and SPSS.

Results

Serum IFN-λ_3 levels are increased in patients with chronic HCV infection

The clinical backgrounds of C-CH patients are shown in Table 1. First, we compared serum IFN-λ_3 levels among patients with C-CH or chronic hepatitis B (B-CH) and uninfected healthy volunteers (HVs). Such levels in the C-CH group were significantly higher than those in the B-CH group or the HV group (Fig. 1a). The levels in the B-CH group were increased, but the significance of this was much less than in the C-CH group (Fig. 1a). When we compared serum IFN-λ_3 levels in B-CH patients between

Table 1 Clinical backgrounds of the patients with chronic hepatitis C virus (*HCV*) infection

Factors	Values
Number	119 (69 male, 50 female)
Age (years)	56.5 ± 10.1
WBC (/mm^3)	5,120 ± 1,575
Hb (g/dL)	14.4 ± 1.5
Plt ($\times 10^4$/mm^3)	17.7 ± 5.2
TP (g/dL)	7.5 ± 0.5
Alb (g/dL)	4.2 ± 0.4
AST (U/L)	54.7 ± 38.3
ALT (U/L)	71.5 ± 54.2
T-bil (mg/dL)	0.8 ± 0.3
T-chol (mg/dL)	176.6 ± 37.0
AFP (ng/mL)	9.7 ± 13.4
HCV RNA (log IU/mL)	6.3 ± 0.6
Activity (A0/A1/A2/A3)	1/68/33/2
Fibrosis (F1/F2/F3/F4)	48/36/16/4
IFNL3 rs8099917 (TT/non-TT)	100:19

Alb albumin, *AFP* α-fetoprotein, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *Hb* hemoglobin, *Plt* platelets, *T-bil* total bilirubin, *T-chol* total cholesterol, *TP* total protein, *WBC* white blood cells

HBeAg-positive and HBeAg-negative patients, we found no difference between them (2.5 ± 0.9 pg/mL vs 1.8 ± 1.7 pg/mL, respectively). Next, we compared serum IFN-λ_3 levels between patients with the *IFNL3* TT genotype and those with the TG/GG (non-TT) genotype in the C-CH group. Although some patients in the TT group showed relatively higher levels of IFN-λ_3 than those in the non-TT group, this difference between the TT and non-TT groups did not reach significance (Fig. 1b). Third, we compared serum IFN-λ_3 levels before and after the combination therapy in the relevant cases. In patients who successfully eradicated HCV (SVR), serum IFN-λ_3 levels were significantly decreased at 24 weeks after the therapy. In contrast, such levels did not change in those patients who failed to eradicate HCV (transient virological response and no virological response groups, respectively) (Fig. 1c). Fourth, we compared serum IFN-λ_3 levels among patients with various causes of acute viral hepatitis. Unfortunately, serum samples from acute hepatitis C patients were not available in this study. The IFN-λ_3 levels in the acute hepatitis E group were higher than those in the HVs (Fig. 1d). The IFN-λ_3 levels in the acute hepatitis B group tended to be higher than those in the HVs; however, statistical analysis was not performed because of the limited number of samples ($N = 2$). No significant difference was observed between the acute hepatitis A and HV groups. These results indicate that serum IFN-λ_3 levels are increased in patients with C-CH or acute hepatitis E.

Serum IFN-λ_3 levels may be related to liver inflammation or fibrosis in patients with C-CH

To explore the clinical significance of IFN-λ_3 in chronic HCV infection, we simultaneously examined 27 chemokines and cytokines in serum by means of the BioPlex system, which allows one to measure multiple factors at high sensitivity in a small volume of samples (10 μL per sample). In comparison with the results for HVs, we found that the levels of some chemokines in the C-CH group were higher than those in the HV group, such as IP-10, MIP-1α, MIP-1β, RANTES, and PDGF-BB (Figs. 2, S1).

Next, we examined whether serum IFN-λ_3 levels are correlated or not correlated with clinical parameters or immunological markers in the C-CH group. The IFN-λ_3 levels were weakly and positively correlated with AST, ALT, and α-fetoprotein levels and histological activity (Table 2). These results indicate that the increase of serum IFN-λ_3 levels in patients with C-CH is related to liver inflammation. The FIB-4 score and the APRI are representatives of noninvasive markers of liver fibrosis. The levels of serum IFN-λ_3 were positively correlated with the APRI, but not with the FIB-4 score (Table 2). With regard to the chemokines displaying higher values in the C–CH group, the levels of IP-10 and PDGF-BB were positively correlated with the IFN-λ_3 levels (Table 2). Such chemokines are reported to be involved in the early stage of liver fibrosis [20–22]. Thus, serum levels of IFN-λ_3 may be related to the fibrotic markers as well. To clarify the mechanisms causing the increase of serum IFN-λ_3 levels in B-CH patients, we examined the correlations between serum IFN-λ_3 levels and clinical markers and fibrosis indices. Serum IFN-λ_3 levels were correlated with the levels of AST ($r = 0.64$, $p = 0.03$) and total cholesterol ($r = -0.76$, $p = 0.03$), FIB-4 score ($r = 0.65$, $p = 0.03$), and APRI ($r = 0.76$, $p = 0.007$) (Table S2). In addition, serum IFN-λ_3 levels tended to be higher in HBV-positive patients with liver cirrhosis or HCC (3.0 ± 3.1 pg/mL in liver cirrhosis patients and 4.1 ± 4.7 pg/mL in HCC patients, respectively) (Fig. S2). These results show that serum IFN-λ_3 levels are related to liver inflammation and fibrosis not only in C-CH patients but also in B-CH patients.

Pretreatment serum IFN-λ_3 is not related to SVR to PEG-IFN-α plus RBV therapy in patients with C-CH

Because the *IFNL3* genotype is a strong predictor of the efficacy of PEG-IFN-α plus RBV therapy for C-CH, we sought to examine the clinical value of serum IFN-λ_3 in patients who underwent the combination therapy. In a comparison of the clinical and immunological factors between the SVR and non-SVR groups, univariate analysis

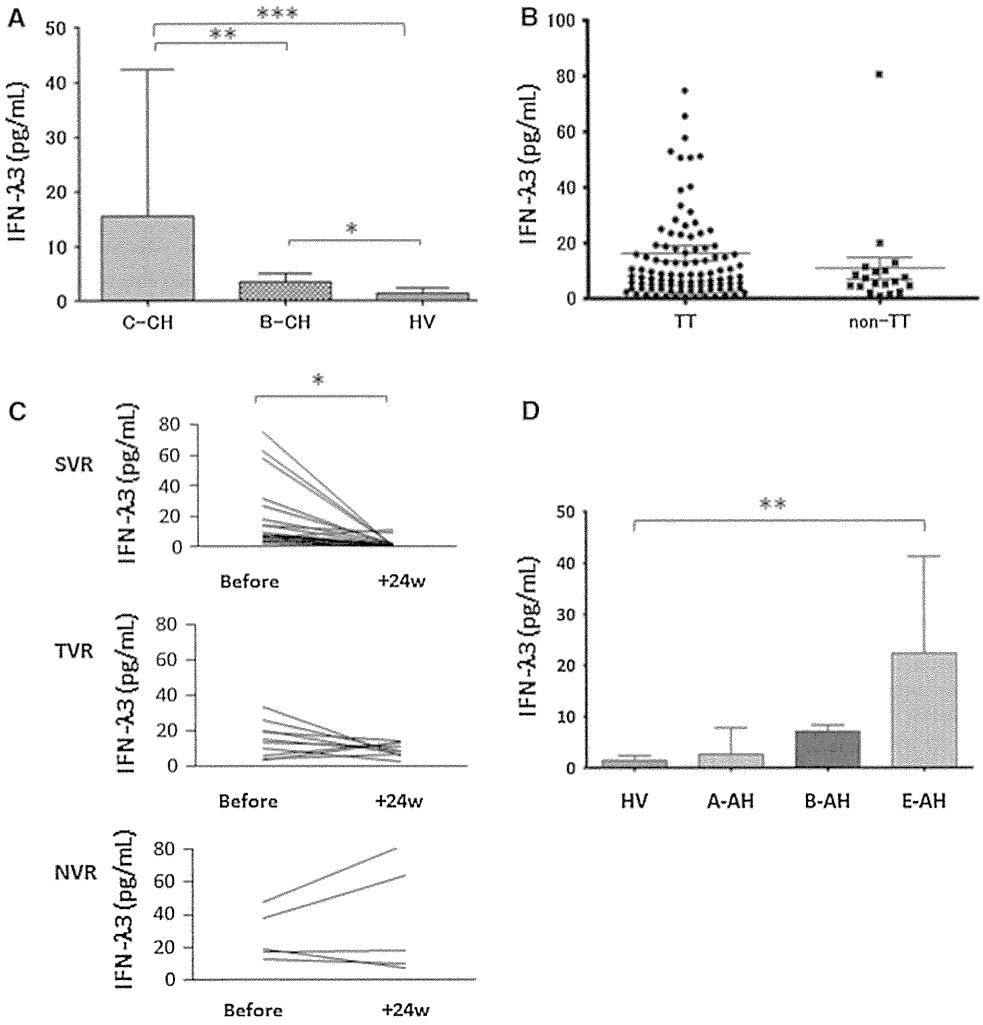

Fig. 1 Serum interferon-λ_3 (*IFN-λ3*) levels are increased in patients with chronic hepatitis C virus infection or acute hepatitis E virus infection. **a** Serum IFN-λ_3 levels in patients with chronic hepatitis C (*C–CH*; $N = 119$), patients with chronic hepatitis B (*B-CH*; $N = 11$), and healthy volunteers (*HV*; $N = 23$) were quantified by the chemiluminescence enzyme immunoassay (CLEIA) method as described in "Materials and methods." *One asterisk* $p < 0.05$, *two asterisks* $p < 0.01$, *three asterisks* $p < 0.0001$ by the Mann–Whitney *U* test. **b** Serum IFN-λ_3 levels in the C-CH group were compared between the patients with the *IFNL3* TT (rs8099917) genotype ($N = 100$) and those with non-TT (TG/GG) genotype ($N = 19$). **c** Serum IFN-λ_3 levels in C-CH patients were compared before and 24 weeks after the pegylated interferon-α plus ribavirin therapy. *SVR* sustained virological response ($N = 21$), *TVR* transient virological response ($N = 10$), *NVR* nonvirological response ($N = 5$), *one asterisk* $p < 0.05$ by Wilcoxon's signed-rank test. **d** Serum IFN-λ_3 levels in acute hepatitis patients of various causes were quantified by CLEIA as described in "Materials and methods." All samples were collected from patients whose alanine aminotransferase levels were two times higher than the upper limit of the normal range. *HV* healthy volunteers ($N = 23$), *A-AH* acute hepatitis A patients ($N = 34$), *B-AH* acute hepatitis B patients ($N = 2$), *E-AH* acute hepatitis E patients ($N = 9$), *two asterisks* $p < 0.0001$ by the Mann–Whitney *U* test

revealed that AST, *IFNL3* genotype, fibrosis score, and MIP-1α were associated with the SVR (Table 3). However, serum IFN-λ_3 or IP-10 levels were not different between the SVR and non-SVR groups (Table 3). Subsequently, multivariate analysis including such factors of significance ($p < 0.05$ by univariate analysis) showed that *IFNL3* SNPs, fibrosis score, and MIP-1α were involved in the SVR (Table 3). These results suggest that serum IFN-λ_3 fails to be a predictive marker for SVR in PEG-IFN-α plus RBV therapy.

Discussion

In this study, we demonstrated that serum IFN-λ_3 levels were higher in patients with C-CH than in uninfected or HBV-positive patients, the levels in whom did not differ regardless of the *IFNL3* genotype. Serum IFN-λ_3 levels were correlated with clinical and immunological markers of liver inflammation and fibrosis, suggesting that the production of IFN-λ_3 may be regulated by not only the presence or absence of HCV but also by the status of liver

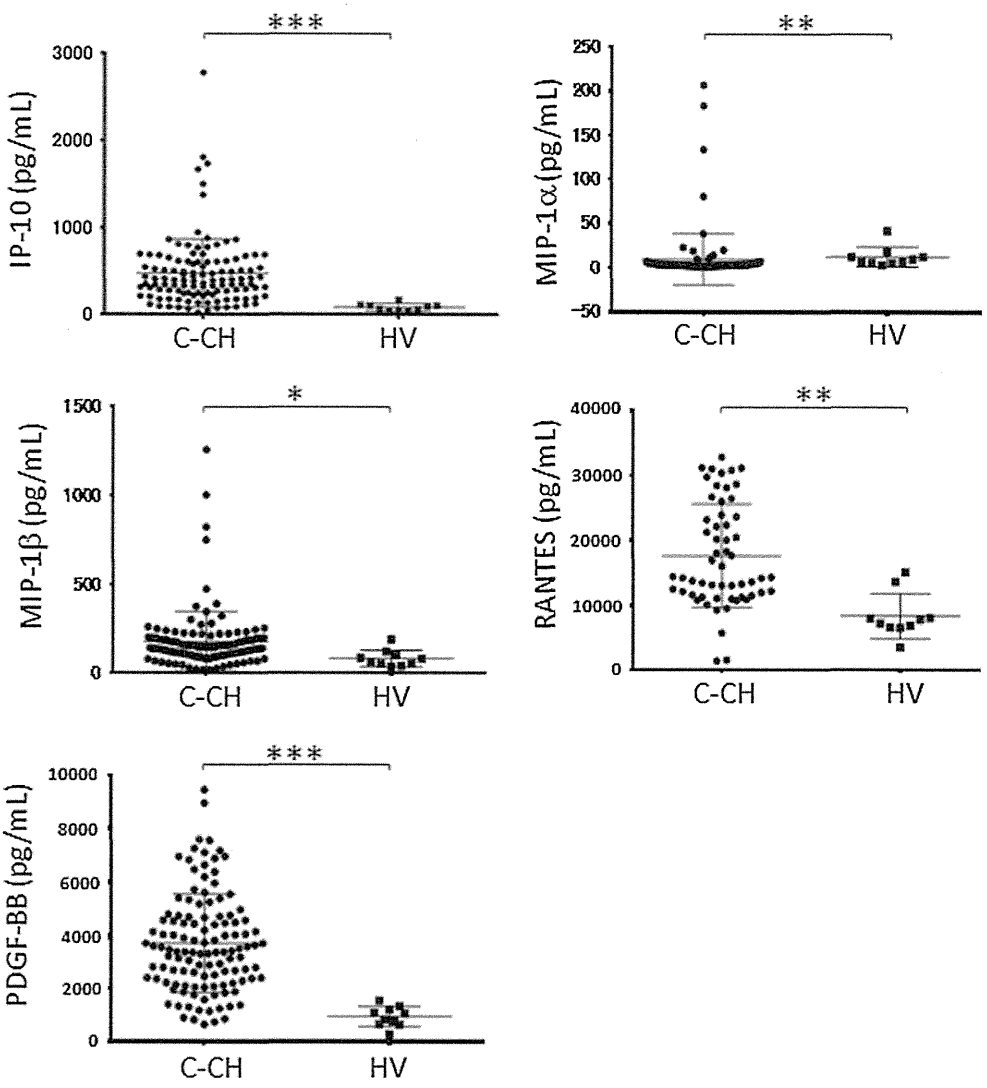

Fig. 2 The levels of several chemokines are increased in patients with chronic hepatitis C virus infection. Twenty-seven chemokines and cytokines in serum from chronic hepatitis C patients (*C-CH*) and healthy volunteers (*HV*) were assayed by means of the BioPlex method. Interferon-γ-inducible protein 10 (*IP-10*) was measured by ELISA. Representative results for chemokines that showed statistical significance between the groups are shown, such as IP-10, macrophage inflammatory protein 1α (*MIP-1α*), macrophage inflammatory protein 1β (*MIP-1β*), regulated on activation, normally T cell expressed, and secreted (*RANTES*), and platelet-derived growth factor BB (*PDGF-BB*). * $p < 0.005$, ** $p < 0.001$, *** $p < 0.0001$ by the Mann–Whitney *U* test

disease. It is well acknowledged that *IFNL3* genotype is a strong predictor of SVR in PEG-IFN-α plus RBV therapy for C-CH [7–9]. However, serum IFN-λ_3 fails to be a surrogate marker for *IFNL3* genotype in the combination therapy.

On primary HCV infection, IFN-λ is produced by hepatocytes that subsequently induce antiviral IFN-stimulated genes [23]. Parallel reduction of serum IFN-λ_3 levels in C-CH patients who attained SVR by PEG-IFN-α plus RBV treatment indicates that the presence of HCV is involved in the production of IFN-λ_3. In addition to hepatocytes, dendritic cells or macrophages are capable of producing IFN-λ in response to HCV [24]. For sensing HCV, hepatocytes and BDCA3$^+$ dendritic cells mainly utilize Toll-like receptor 3 and retinoic acid inducible gene I, and plasmacytoid dendritic cells utilize Toll-like receptor 7 [24, 25]. It is yet to be clarified which cells—hepatocytes or dendritic cells—have stronger potential to secrete IFN-λ at the single-cell level. However, it is rational to consider that serum IFN-λ_3 levels in patients are determined by the sum of IFN-λ_3 sporadically released from both types of cells. Therefore, it is plausible that the amount of IFN-λ released from hepatocytes or dendritic cells is influenced by the environment of the producers, such as inflammation and fibrosis. A positive correlation observed between serum IFN-λ_3 levels and AST levels, FIB-4 score, and APRI in B-CH patients may support such a possibility. In this study, serum IFN-λ_3 levels in the B-CH group were higher than those in HVs. However, this difference was slim compared with the difference between the C-CH group and HVs, suggesting that the difference in their genome structure, either RNA or DNA virus, may influence IFN-λ_3 production by infected cells. Of interest is the finding that serum IFN-λ_3 levels were higher in patients with acute hepatitis E than in patients with acute hepatitis A. It is reported that dendritic cells localized in the

Table 2 Correlation of interferon-λ_3 (*IFN-λ_3*) with clinical or immunological parameters in patients with chronic hepatitis C

Factors	CC with IFN-λ_3	*p*
Age (years)	−0.10	–
WBC (/mm^3)	−0.05	–
Hb (g/dL)	0.07	–
Plt ($\times 10^4$/mm^3)	−0.09	–
TP (g/dL)	0.07	–
Alb (g/dL)	−0.01	–
AST (U/L)	0.34	<0.0001
ALT (U/L)	0.34	<0.0001
T-bil (mg/dL)	0.03	–
T-chol (mg/dL)	−0.22	0.02
AFP (ng/mL)	0.30	0.001
HCV RNA (log IU/mL)	−0.05	–
Fibrosis score	0.07	–
Histological activity score	0.25	0.01
FIB-4 score	0.10	–
APRI	0.29	0.001
IP-10 (pg/mL)	0.53	<0.0001
MIP-1α (pg/mL)	−0.08	–
MIP-1β (pg/mL)	−0.18	–
RANTES (pg/mL)	0.26	–
PDGF-BB (pg/mL)	0.40	<0.0001

Alb albumin, *AFP* α-fetoprotein, *ALT* alanine aminotransferase, *APRI* aspartate aminotransferase platelet ratio index, *AST* aspartate aminotransferase, *CC* correlation coefficient by Spearman's analysis, *FIB-4* fibrosis-4, *Hb* hemoglobin, *HCV* hepatitis C virus, *IP-10* interferon-γ-inducible protein 10, *MIP-1α* macrophage inflammatory protein 1α, *MIP-1β* macrophage inflammatory protein 1β, *PDGF-BB* platelet-derived growth factor BB, *Plt* platelets, *RANTES* regulated on activation, normally T cell expressed, and secreted, *T-bil* total bilirubin, *T-chol* total cholesterol, *TP* total protein, *WBC* white blood cells

intestine are capable of producing IFN-λ in response to rotavirus to protect the host from infection [26]. Although both hepatitis E virus and hepatitis A virus are RNA viruses that are transmissible by the enterofecal route, the difference in serum IFN-λ_3 levels suggests that there are distinct mechanisms of recognition of hepatitis E virus and hepatitis A virus by the hosts. Further investigation is needed to disclose which pattern recognition receptors are utilized in hepatocytes or immune cells for the recognition of such viruses to produce IFN-λ.

The regulatory mechanisms of transcription and translation of IFN-λ_3 have not been well documented. The *IFNL3* SNPs (rs8099917) are located 8.9 kb upstream of the promoter region of the *IFNL3* gene [8, 9, 11]. Because of such localization, it is less likely that the genetic variation has some impact on the transcriptional level of *IFNL3*. With regard to the relationship between the *IFNL3* genotype and its transcripts, controversial results have been reported thus far. Some groups reported that IFN-λ_3 messenger RNA levels in peripheral blood mononuclear cells were higher in patients with the *IFNL3* major genotype than in those with the minor genotype [9]. In contrast, others showed that in hepatocytes such levels were comparable regardless of *IFNL3* SNPs. In the search for some genetic factors influencing *IFNL3* transcription, Sugiyama et al. [27] reported the existence of variable-length TA repeats in the promoter of the *IFNL3* gene. Other investigators showed that a certain structure of the 3′ untranslated region in the *IFNL3* gene is involved in the durability/stability of the gene [28]. Nevertheless, the contribution of such factors is not enough to fill in the gap, suggesting that certain other regulatory factors for *IFNL3* are still to be revealed.

Reports concerning serum IFN-λ in C-CH patients are limited. Langhans et al. [29] showed that serum levels of IFN-λ, which includes IFN-λ_2 and IFN-λ_3, were higher in patients with the *IFNL3* major genotype than in those with the minor genotype. One of the limitations of their study seems to be the lack of specificity for the measurement of IFN-λ_3. Since the homology of *IFNL2* (which encodes IFN-λ_2) and *IFNL3* is quite high, it is difficult to quantify specifically IFN-λ_3 by excluding contamination by IFN-λ_2. To exclude such a possibility, we used the newly developed chemiluminescence enzyme immunoassay for IFN-λ_3, which enables one to quantify IFN-λ_3 without any influence from IFN-λ_2 in the range from 0 to 1,000 pg/mL. By means of this system, we found that serum levels of IFN-λ_3 are not statistically different between patients with the *IFNL3* major genotype and those with the minor genotype.

On primary HCV exposure, the significance of IFN-λ family members as an antiviral protein is evident. However, such impact of IFN-λ_3 in chronically HCV-infected patients is still elusive. Langhans et al. [29] reported that serum IFN-λ levels in patients who had spontaneously cleared HCV were higher than in patients with chronic HCV infection, implying that a higher level of IFN-λ somewhat contributed to HCV eradication. In this study, we aimed to clarify the significance of IFN-λ_3 in patients with chronic HCV infection with different approaches. Firstly, we searched for the factors influencing serum IFN-λ_3 quantity by correlation analysis with clinical markers and multiple cytokines/chemokines. We found that AST, ALT, and α-fetoprotein levels and histological activity were positively correlated with serum IFN-λ_3 levels. In addition, one of the noninvasive fibrosis markers, APRI, was weakly correlated with serum IFN-λ_3 levels. Among the chemokines examined in this study, serum IFN-λ_3 was positively correlated with IP-10 and PDGF-BB. IP-10 (CXCL10) is induced in HCV-infected hepatocytes as one of the IFN-stimulated genes, and attracts CXCR3-positive T cells and natural killer cells and subsequently activates inflammation. IP-10 is also reported to be involved in the early stage of liver fibrosis [30, 31]. A similar fibrotic