Table 4. Factors associated with SVR in patients who achieved ETR response to 48-week IFN + ribavirin combination therapy in patients infected with HCV-1b, identified by multivariate analysis

Factor	Category	OR (95% CI)	p
Gender	1: Female 2: Male	1 4.27 (2.15–8.55)	<0.001
Ribavirin dose, mg/kg	1: <11.0 2: ≥11.0	1 2.95 (1.48–5.86)	0.002
ISDR of NS5A	1: WT 2: Non-WT	1 4.00 (1.35–11.8)	0.012
Core aa 70 and 91	1: Gln70 (His70) and Met91 2: Arg70 and/or Leu91	1 2.96 (1.16–7.52)	0.023
Platelet count $\times 10^4/mm^3$	1: <15.0 2: ≥15.0	1 2.19 (1.07–4.50)	0.033
α-Fetoprotein μg/l	1: ≥10 2: <10	1 2.66 (1.04–6.80)	0.042

Only variables that achieved statistical significance ($p < 0.05$) on multivariate logistic regression are shown.

Table 5. Comparison of factors associated with efficacy of 48-week IFN + ribavirin combination therapy in patients infected with HCV-1b, identified by multivariate analysis

Factor	ETR response (at 48 weeks)	SVR after ETR response	SVR
IL28B	rs8099917 p < 0.001, 18.2 (6.29–52.6)[a]		rs8099917 p < 0.001, 16.7 (4.54–61.3)[a]
Virus	Core aa 70 p = 0.004, 4.68 (1.65–13.3)[a]	Core aa 70 and 91 p = 0.023, 2.96 (1.16–7.52)[a]	
	Level of viremia p = 0.001, 9.20 (2.59–32.6)[a]	ISDR p = 0.012, 4.00 (1.35–11.8)[a]	ISDR p = 0.027, 5.68 (1.22–26.3)[a]
Others	Albumin p = 0.030, 3.08 (1.11–8.47)[a]	α-Fetoprotein p = 0.042, 2.66 (1.04–6.80)[a]	
		Platelet count p = 0.033, 2.19 (1.07–4.50)[a]	
		Gender p < 0.001, 4.27 (2.15–8.55)[a]	Gender p < 0.001, 10.5 (3.47–32.3)[a]
		Ribavirin dose p = 0.002, 2.95 (1.48–5.86)[a]	

Only variables that achieved statistical significance ($p < 0.05$) on multivariate logistic regression are shown.
[a] OR (95% CI).

nificantly lower in patients with non-WT of ISDR than in those with WT [8]. The present study indicated that substitution of IRRDR and core aa 91, in addition to substitution of ISDR, also significantly influenced levels of viremia. Furthermore, there was a significant positive correlation between the number of aa substitutions in ISDR and those in IRRDR, and the number of aa substitutions in ISDR/IRRDR of patients with Leu91 was significantly higher than that of patients with Met91. To our knowledge, this is the first report of the relationship between viremia levels and aa substitutions in core region/ISDR/IRRDR. This result might be interpreted to mean

that core aa 91/ISDR/IRRDR might be associated with viremia levels involved in resistance to combination therapy. Further studies that examine the functional impact of aa substitutions to combination therapy should be conducted to confirm the above finding.

The present results showed that α-fetoprotein, albumin, platelet count, and gender were predictors of virological response to IFN/ribavirin combination therapy. Previous data indicated that absence of advanced liver fibrosis was a positive predictor of SVR to IFN monotherapy and IFN/ribavirin combination therapy [2, 3, 13, 27–29], and that advanced liver fibrosis was usually associated with higher levels of α-fetoprotein, and lower levels of albumin and platelet count [1, 3, 30–32]. Furthermore, gender is also a predictor of treatment response to IFN/ribavirin combination therapy [2, 3, 14]. In the present study based on a large number of patients, histopathological changes in the liver and gender were identified as independent predictors of virological response, in addition to genetic variation near *IL28B* and viral factors (core region, ISDR, and level of viremia).

In a previous study, multivariate analysis identified core region, gender, and stage of liver fibrosis as parameters that independently influenced the SVR of patients who achieved early virological response, but ISDR was not entered into uni- and multivariate analysis [3]. To our knowledge, the present study based on multivariate analysis is the first report to identify ISDR as pretreatment predictor of SVR after ETR to combination therapy. Interestingly, ISDR was not a predictor of ETR, but was a significant predictor of SVR to combination therapy. Thus, the underlying mechanisms of failure to develop SVR in those patients who achieve HCV-RNA negativity remain unclear. Further studies that examine the impact of aa substitutions of ISDR to combination therapy should be conducted to confirm the above finding.

One limitation of the present study was that aa substitutions in areas other than the core region and NS5A-ISDR/IRRDR of the HCV genome were not examined. Other limitations were differences in host factors including race [24, 33, 34] and differences in viral factors, such as the distribution of HCV-1a or -1b, and geographic diversities of HCV-1b [35]. Further large-scale prospective studies are necessary to investigate whether the present results relate to the efficacy of 48-week IFN/ribavirin combination therapy, and further understanding of the complex interaction between virus- and host- related factors should facilitate the development of more effective therapeutic regimens.

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Complicated Relationships of Amino Acid Substitution in Hepatitis C Virus Core Region and *IL28B* Genotype Influencing Hepatocarcinogenesis

Norio Akuta,[1] Fumitaka Suzuki,[1] Yuya Seko,[1] Yusuke Kawamura,[1] Hitomi Sezaki,[1] Yoshiyuki Suzuki,[1] Tetsuya Hosaka,[1] Masahiro Kobayashi,[1] Tasuku Hara,[1] Mariko Kobayashi,[2] Satoshi Saitoh,[1] Yasuji Arase,[1] Kenji Ikeda,[1] and Hiromitsu Kumada[1]

The impact of amino acid (aa) 70 substitution in the core region on hepatocarcinogenesis and survival for liver-related death in patients of hepatitis C virus (HCV) genotype 1b (HCV-1b), who had not received antiviral therapy, is unknown. The relationships among aa 70 substitution, *IL28B* genotype, and hepatocarcinogenesis are also not clear. A total of 1,181 consecutive HCV-infected patients, who had not received antiviral therapy, were included in a follow-up study to determine predictive factors of hepatocarcinogenesis and survival for liver-related death. The cumulative hepatocarcinogenesis rates in HCV-1b of Gln70(His70) (glutamine (histidine) at aa 70) were significantly higher than those in HCV-1b of Arg70 (arginine at aa 70) and HCV-2a/2b. The cumulative survival rates for liver-related death in HCV-1b of Gln70(His70) were significantly lower than those in HCV-1b of Arg70 and HCV-2a/2b. Multivariate analysis identified gender (male), age (≥60 years), albumin (<3.9 g/dL), platelet count (<15.0 × 10^4/mm^3), aspartate aminotransferase (≥67 IU/L), and HCV subgroup (HCV-1b of Gln70(His70)) as determinants of both hepatocarcinogenesis and survival rates for liver-related death. In HCV-1b patients, the cumulative change rates from Arg70 to Gln70(His70) by direct sequencing were significantly higher than those from Gln70(His70) to Arg70. In patients of Arg70 at the initial visit, the cumulative change rates from Arg70 to Gln70(His70) in *IL28B* rs8099917 non-TT genotype were significantly higher than those in the TT genotype. *Conclusion:* Substitution of aa 70 in the core region of HCV-1b is an important predictor of hepatocarcinogenesis and survival for liver-related death in HCV patients who had not received antiviral therapy. The *IL28B* genotype might partly affect changes over time of dominant amino acid in core aa 70 of HCV-1b. (HEPATOLOGY 2012;56:2134-2141)

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC).[1,2] At present, treatments based on interferon (IFN), in combination with ribavirin, are the mainstay for combating HCV infection. In Japan, HCV genotype 1b (HCV-1b) and high viral loads account for more than 70% of HCV infections, making it difficult to treat patients with chronic hepatitis C.[3]

Despite numerous lines of epidemiologic evidence connecting HCV infection and the development of HCC, it remains controversial whether HCV itself plays a direct role or an indirect role in the pathogenesis of HCC.[4] It has become evident that HCV core region has oncogenic potential through the use of transgenic mice, but the clinical impact of the core region on hepatocarcinogenesis is still unclear.[5] Previous reports indicated that amino acid (aa) substitutions at position 70 in the HCV core region of patients infected with HCV-1b are pretreatment predictors of poor virological response to pegylated IFN (PEG-IFN)/ribavirin combination therapy and triple therapy

Abbreviations: aa, amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PEG/IFN, pegylated interferon.

From the [1]Department of Hepatology, Toranomon Hospital, and Okinaka Memorial Institute for Medical Research, Tokyo, Japan; [2]Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan.

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of telaprevir/PEG-IFN/ribavirin,[6-9] and also affects hepatocarcinogenesis.[10-13] These reports support the findings of oncogenic potential by core region from the clinical aspect. However, its impact on hepatocarcinogenesis and survival for liver-related death in patients of HCV-1b who had not received antiviral therapy is still unknown.

The *IL28B* genotype is a poor predictor of virological response to PEG-IFN/ribavirin combination therapy and triple therapy of telaprevir/PEG-IFN/ribavirin[9,14-17] and is reported to be associated with HCC, although its impact on HCC is controversial.[18-21] Furthermore, treatment-resistant substitution of core aa 70 (glutamine/histidine at aa 70 (Gln70/His70)), which might affect hepatocarcinogenesis, was significantly more frequent in patients with treatment-resistant genotype (non-TT) than -sensitive genotype (TT) at *IL28B* rs8099917.[21-23] Thus, the significant linkage between substitution of aa 70 and *IL28B* genotype had been shown, but it is not clarified whether the existence of a complex interaction between the virus and host might affect hepatocarcinogenesis.

The present study included 1,181 consecutive HCV-infected patients who had not received antiviral therapy. The aims of the study were: (1) To evaluate the impact of aa substitutions in the core region of HCV-1b on hepatocarcinogenesis and survival for liver-related death; and (2) To investigate the association of *IL28B* genotype and time-dependent aa changes in the core region of HCV-1b.

Patients and Methods

Patients. Among 2,799 consecutive HCV-infected patients in whom antiviral therapy (IFN monotherapy or IFN plus ribavirin combination therapy) was not induced between December 1962 and November 2010 at Toranomon Hospital, 1,181 were selected in the present study based on the following criteria. (1) Positive for anti-HCV (third-generation enzyme immunoassay, Chiron, Emerville, CA) and positive for HCV RNA (nested polymerase chain reaction [PCR]), at the initial visit. (2) Patients without HCC at the initial visit. (3) Patients infected with single genotype of HCV-1b, 2a, or 2b. (4) In HCV-1b, patients analyzed aa substitutions of the core region by direct sequencing, one or more times from the initial visit. (5) Patients negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan). (6) Patients free of coinfection with human immunodeficiency virus. (7) Patients free of other types of chronic liver disease, including hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, autoimmune liver disease, inherited liver disease including alpha-1 antitrypsin deficiency, and hepatic venous outflow block. (8) Patients who consented to the study.

Table 1. Profiles and laboratory data at the initial visit of 1,181 patients infected with HCV, who had not received antiviral therapy

Demographic data	
Number of patients	1,181
Sex (male/female)	608/573
Age (years)*	60 (20-93)
History of blood transfusion	526 (49.2%)
Family history of liver disease	201 (20.3%)
Lifetime cumulative alcohol intake (>500 kg)	110 (10.8%)
Laboratory data*	
Total bilirubin (mg/dl)	0.7(0.1-20.0)
Aspartate aminotransferase (IU/l)	71 (13-1,052)
Alanine aminotransferase (IU/l)	88 (4-1,210)
Albumin (g/dl)	4.1 (1.0-5.5)
Hemoglobin (g/dl)	14.0 (7.8-18.0)
Platelet count ($\times 10^4/mm^3$)	15.3 (2.6-52.9)
HCV genotype (Ib / 2a or 2b)	750/431
Levels of viremia (high viral load)	757 (74.4%)
Amino acid substitutions in the HCV genotype Ib	
Core aa 70 (arginine / glutamine (histidine))	431/319
Core aa 91 (leucine / methionine)	482/268

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.

Table 1 summarizes the profiles and laboratory data at the initial visit of 1,181 patients infected with HCV who had not received antiviral therapy. They did not receive antiviral therapy based on concerns about adverse effects, lack of time for treatment, physician recommendation based on the appearance of depression and cardiopulmonary disease, lower levels of aspartate aminotransferase (AST) / alanine aminotransferase (ALT), or elderly patients. They included 608 males and 573 females, aged 20 to 93 years (median, 60 years). The median follow-up time from the initial visit until death or until the last visit was 9.0 years (range, 0.0-37.7

Address reprint requests to: Norio Akuta, M.D., Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo, 105-0001, Japan. E-mail: akuta-gi@umin.ac.jp; fax: +81-44-860-1623.

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Potential conflict of interest: Norio Akuta has received speakers' bureau from MSD K.K., and holds a right to get some loyalty from SRL. Inc.. Hiromitsu Kumada has received speakers' bureau from MSD K.K., Mitsubishi Tanabe Pharma, Dainippon Sumitomo Pharma, Bristol-Myers Squibb, and holds a right to get some loyalty from SRL. Inc.. Fumitaka Suzuki has received speakers' bureau from Bristol-Myers Squibb. The other authors have nothing to disclose.

years). The study protocol was approved by the Human Ethics Review Committee of the institution.

Laboratory Investigations. Blood samples were frozen at −80°C within 4 hours of collection and were not thawed until used for testing. Anti-HCV, HCV RNA, HCV genotype, and aa substitutions of the HCV-1b core region were assayed using stored frozen sera. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of the NS5 region.[24] HCV RNA quantitative analysis was measured by branched DNA assay v. 2.0 (Chiron), AMPLICOR GT HCV Monitor v. 2.0 using the 10-fold dilution method (Roche Molecular Systems, Pleasanton, CA), or COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). High viral load of viremia levels was defined as branched DNA assay ≥1.0 Meq/mL, AMPLICOR GT HCV Monitor $\geq 100 \times 10^3$ IU/mL, or COBAS TaqMan HCV test ≥5.0 log IU/mL. Low viral load was defined as branched DNA assay <1.0 Meq/mL, AMPLICOR GT HCV Monitor $< 100 \times 10^3$ IU/mL, or COBAS TaqMan HCV test <5.0 log IU/mL.

Detection of Amino Acid Substitutions in Core Regions of HCV-1b. In the present study, aa substitutions of the core region of HCV-1b were analyzed by direct sequencing. HCV RNA was extracted from serum samples and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). Nucleic acids of the core region were amplified by nested PCR using the following primers. The first-round PCR was performed with CE1 (sense, 5′-GTC TGC GGA ACC GGT GAG TA-3′, nucleotides: 134-153) and CE2 (antisense, 5′-GAC GTG GCG TCG TAT TGT CG-3′, nucleotides: 1096-1115) primers, and the second-round PCR with CC9 (sense, 5′-ACT GCT AGC CGA GTA GTG TT-3′, nucleotides: 234-253) and CE6 (antisense, 5′-GGA GCA GTC GTT CGT GAC AT-3′, nucleotides: 934-953) primers. All samples were initially denatured at 95°C for 2 minutes. The 35 cycles of amplification were set as follows: denaturation for 30 seconds at 95°C, annealing of primers for 30 seconds at 55°C, and extension for 1 minute at 72°C with an additional 7 minutes for extension. Then 1 μL of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan).

With the use of HCV-J (accession no. D90208) as a reference,[25] the dominant sequence of 1-191 aa in the core protein of HCV-1b was determined by direct sequencing and then compared with the consensus sequence constructed on 50 clinical samples to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91).[6] Especially, patients were classified into three HCV subgroups according to HCV genotype in combination with aa substitutions in HCV-1b core region (HCV-1b of Arg70, HCV-1b of Gln70(His70), and HCV-2a/2b).

Determination of IL28B Genotype. IL28B rs8099917 was genotyped by the Invader assay, TaqMan assay, or direct sequencing, as described.[26,27]

Follow-Up and Diagnosis of HCC. Follow-up of patients was made on a monthly to trimonthly basis after the initial visit. Imaging diagnosis was made one or more times per year with ultrasonography, computed tomography, or magnetic resonance imaging. During this time, liver-related death, which included HCC, cholangiocellular carcinoma, liver failure, or esophageal variceal bleeding, was also evaluated.

Statistical Analysis. The cumulative rates of hepatocarcinogenesis, survival for liver-related death, and amino acid changes in the core region were calculated using the Kaplan-Meier technique; differences between the curves were tested using the log-rank test. Statistical analyses of hepatocarcinogenesis, survival, and amino acid changes, according to groups, were calculated using the period from the initial visit. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with hepatocarcinogenesis and survival for liver-related death. The hazard ratio (HR) and 95% confidence interval (95% CI) was also calculated. Potential predictive factors associated with hepatocarcinogenesis and survival for liver-related death included the variables: sex, age, history of blood transfusion, family history of liver disease, lifetime cumulative alcohol intake, total bilirubin, AST, ALT, albumin, hemoglobin, platelet count, levels of viremia, and HCV subgroup according to HCV genotype in combination with aa substitution in core region. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were tested by multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (Chicago, IL). $P < 0.05$ by the two-tailed test were considered significant.

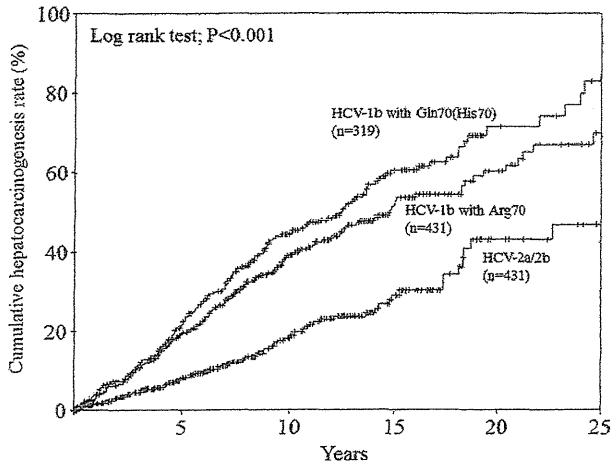

Fig. 1. Cumulative hepatocarcinogenesis rates according to HCV genotype in combination with amino acid substitutions in core region of HCV-1b. The rates were significantly different among the three HCV subgroups ($P < 0.001$; log-rank test). Especially, the rates in HCV-1b of Gln70(His70) were significantly higher than those in HCV-1b of Arg70 ($P = 0.028$; log-rank test) and HCV-2a/2b ($P < 0.001$; log-rank test), and the rates in HCV-1b of Arg70 were also significantly higher than those in HCV-2a/2b ($P < 0.001$; log-rank test).

Results

Hepatocarcinogenesis Rates and Survival Rates for Liver-Related Death in Patients Infected With HCV Who Had Not Received Antiviral Therapy. During the follow-up, 413 patients (35.0%) developed HCC. The cumulative hepatocarcinogenesis rates were 16.3, 34.3, 48.3, 58.7, and 69.1% at the end of 5, 10, 15, 20, and 25 years, respectively. The median interval between the initial visit and detection of HCC was 6.2 years (range, 0.1-31.7 years).

During the follow-up period, 243 patients (20.6%) died due to liver-related causes, and 97 of 243 (90.5%) developed HCC. The cumulative survival rates for liver-related death were 96.2, 84.8, 68.9, 55.0, and 46.1% at the end of 5, 10, 15, 20, and 25 years, respectively. The median interval between the initial visit and liver-related death was 10.1 years (range, 0.4-35.8 years).

Hepatocarcinogenesis Rates and Survival Rates for Liver-Related Death According to HCV Genotype in Combination with Amino Acid Substitutions in Core Region of HCV-1b. During the follow-up, 163 patients (51.3%), 175 (41.2%), and 75 (17.6%) developed HCC in HCV-1b of Gln70(His70), HCV-1b of Arg70, and HCV-2a/2b, respectively. In HCV-1b of Gln70(His70), HCV-1b of Arg70, and HCV-2a/2b, the cumulative hepatocarcinogenesis rates were 21.7, 19.3, 8.0% at the end of 5 years; 44.4, 39.4, 18.2% at the end of 10 years; 60.4, 52.7, 29.1% at the end of 15 years; 71.6, 60.3, 43.1% at the end of 20 years; and 87.1, 69.8, 46.9% at the end of 25 years, respectively. The rates were significantly different among the three HCV subgroups ($P < 0.001$) (Fig. 1). Especially, the rates in HCV-1b of Gln70(His70) were significantly higher than those in HCV-1b of Arg70 ($P = 0.028$) and HCV-2a/2b ($P < 0.001$), and the rates in HCV-1b of Arg70 were also significantly higher than those in HCV-2a/2b ($P < 0.001$).

During the follow-up, 104 patients (34.4%), 97 (23.4%), and 42 (10.0%) died due to liver-related causes in HCV-1b of Gln70(His70), HCV-1b of Arg70, and HCV-2a/2b, respectively. In HCV-1b of Gln70(His70), HCV-1b of Arg70, and HCV-2a/2b, the cumulative survival rates for liver-related death were 95.2, 95.4, 97.9% at the end of 5 years; 77.7, 83.3, 93.9% at the end of 10 years; 58.4, 68.4, 81.2% at the end of 15 years; 39.3, 58.4, 69.0% at the end of 20 years; and 33.8, 47.5, 59.5% at the end of 25 years, respectively. The rates were significantly different among the three HCV subgroups ($P < 0.001$) (Fig. 2). Especially, the rates in HCV-1b of Gln70(His70) were significantly lower than those in HCV-1b of Arg70 ($P = 0.016$) and HCV-2a/2b ($P < 0.001$), and the rates in HCV-1b of Arg70 were also significantly lower than those in HCV-2a/2b ($P < 0.001$).

Predictive Factors Associated with Hepatocarcinogenesis and Survival for Liver-Related Death in Patients Infected with HCV Who Had Not Received Antiviral Therapy. The data for the whole population

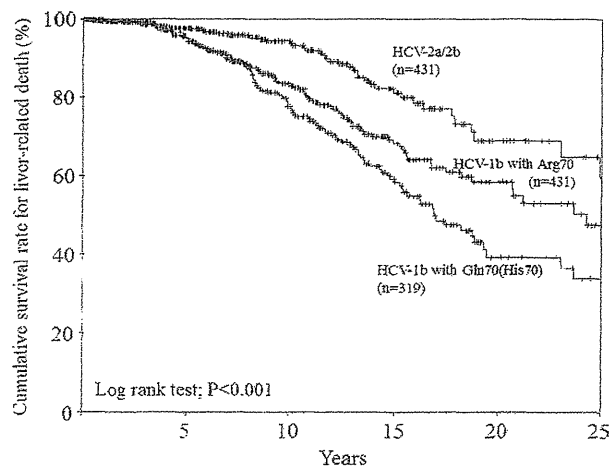

Fig. 2. Cumulative survival rates for liver-related death according to HCV genotype in combination with amino acid substitutions in the core region of HCV-1b. The rates were significantly different among the three HCV subgroups ($P < 0.001$; log-rank test). Especially, the rates in HCV-1b of Gln70(His70) were significantly lower than those in HCV-1b of Arg70 ($P = 0.016$; log-rank test) and HCV-2a/2b ($P < 0.001$; log-rank test), and the rates in HCV-1b of Arg70 were also significantly lower than those in HCV-2a/2b ($P < 0.001$; log-rank test).

Table 2. Factors associated with hepatocarcinogenesis in patients infected with HCV, who had not received antiviral therapy, identified bymultivariate analysis

[Factors]	[Category]	Hazard ratio (95% confidence interval)	P
Gender	1: female	1	
	2: male	1.78 (1.44-2.21)	<0.001
Age (years)	l:<60	1	
	2:≥60	1.68 (1.35-2.09)	<0.001
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	1.94 (1.55-2.42)	<0.001
Platelet count ($\times 10^4$/mm^3)	1: ≥15.0	1	
	2:<15.0	2.89 (2.25-3.72)	<0.001
Aspartate aminotransferase (IU/l)	1:<67	1	
	2:≥67	1.92 (1.47-2.52)	<0.001
HCV subgroup	1: HCV-2a/2b	1	
	2: HCV-1b with Arg70	1.91 (1.42-2.55)	<0.001
	3: HCV-1b with Gln70(His70)	1.94 (1.45-2.61)	<0.001

Cox proportional hazard model

sample were analyzed to determine those factors that could predict hepatocarcinogenesis and survival for liver-related death.

Univariate analysis identified eight parameters that significantly correlated with hepatocarcinogenesis. These included gender (male; $P < 0.001$), age (≥60 years; $P < 0.001$), total bilirubin (≥1.2 mg/dL; $P < 0.001$), AST (≥67 IU/L; $P < 0.001$), ALT (≥85 IU/L; $P < 0.001$), platelet count (<15.0 $\times$ 10^4/mm^3; $P < 0.001$), albumin (<3.9 g/dL; $P < 0.001$), and lifetime cumulative alcohol intake (≥500 kg; $P = 0.025$). Furthermore, the rates in HCV-1b of Gln70(His70) were significantly higher than those in HCV-1b of Arg70 ($P = 0.028$) and HCV-2a/2b ($P < 0.001$). These factors were entered into multivariate analysis, which then identified six parameters that significantly influenced hepatocarcinogenesis independently: gender (male; HR 1.78, $P < 0.001$), age (≥60 years; HR 1.68, $P < 0.001$), albumin (<3.9 g/dL; HR 1.94, $P < 0.001$), platelet count (<15.0 $\times$ 10^4/mm^3; HR 2.89, $P < 0.001$), AST (≥67 IU/L; HR 1.92, $P < 0.001$), and HCV subgroup (HCV-1b of Gln70(His70); HR 1.94, $P = 0.001$) (Table 2).

Univariate analysis identified seven parameters that significantly correlated with survival for liver-related death. These included gender (male; $P < 0.001$), age (≥60 years; $P < 0.001$), total bilirubin (≥1.2 mg/dL; $P < 0.001$), AST (≥67 IU/L; $P < 0.001$), ALT (≥85 IU/L; $P < 0.001$), platelet count (<15.0 $\times$ 10^4/mm^3; $P < 0.001$), and albumin (<3.9 g/dL; $P < 0.001$). Furthermore, the rates in HCV-1b of Gln70(His70) were significantly lower than those in HCV-1b of Arg70 ($P = 0.016$) and HCV-2a/2b ($P < 0.001$). These factors were entered into multivariate analysis, which then identified six parameters that significantly influenced survival for liver-related death independently: gender (male; HR 1.91, $P < 0.001$), age (≥60 years; HR 1.61, $P = 0.001$), albumin (<3.9 g/dL; HR 2.49, $P < 0.001$), platelet count (<15.0 $\times$ 10^4/mm^3; HR 3.69, $P < 0.001$), AST (≥67 IU/L; HR 4.16, $P < 0.001$), and HCV subgroup (HCV-1b of Gln70(His70); HR 2.16, $P < 0.001$) (Table 3).

IL28B Genotype and Time-Dependent Amino Acid Changes in Core Region of HCV-1b. Among 1,181 patients, 359 could be evaluated for changes over time of dominant amino acid by direct sequencing in core aa 70 of HCV-1b. Furthermore, among 359 patients, 142 could also be analyzed for the relationship between *IL28B* rs8099917 genotype and time-dependent changes of core aa 70.

In 199 patients of Arg70 at the initial visit, 34 patients (17.1%) changed from Arg70 to Gln70(His70) during the follow-up. Inversely, in 160 patients of Gln70(His70) at the initial visit, eight patients (5.0%) changed from Gln70(His70) to Arg70 during the follow-up. In change from Arg70 to Gln70(His70), and change from Gln70(His70) to Arg70, the cumulative change rates were 3.0, 0% at the end of 5 years; 16.8, 5.8% at the end of 10 years; 27.4, 11.5% at the end of 15 years; and 38.9, 16.7% at the end of 20 years, respectively. The cumulative change rates from Arg70 to Gln70(His70) were significantly higher than those from Gln70(His70) to Arg70 ($P = 0.002$).

In 78 patients of Arg70 and TT genotype at the initial visit, nine (11.5%) changed from Arg70 to Gln70(His70) during the follow-up. In 11 patients of Arg70 and non-TT genotype at the initial visit, seven (63.6%) changed from Arg70 to Gln70(His70) during the follow-up. In TT and non-TT genotype, the cumulative change rates were 0, 9.1% at the end of 5 years; 3.2, 65.4% at the end of 10 years; 14.8, 65.4% at the end of 15 years; and 29.0, 65.4% at the end of 20 years, respectively. The cumulative change rates in non-TT genotype were significantly higher than those in TT genotype ($P < 0.001$) (Fig. 3A).

In 30 patients of Gln70(His70) and TT genotype at the initial visit, three patients (10.0%) changed from Gln70(His70) to Arg70 during the follow-up. In 23 patients of Gln70(His70) and non-TT genotype at the initial visit, no patients changed from Gln70(His70) to Arg70 during the follow-up. In TT and non-TT genotype, the cumulative change rates were 0, 0% at

Table 3. Factors associated with survival for liver-related death in patients infected with HCV, who had not received antiviral therapy, identified by multivariate analysis

[Factors]	[Category]	Hazard ratio (95% confidence interval)	P
Gender	1: female	1	
	2: male	1.91 (1.45-2.52)	<0.001
Age (years)	1:<60	1	
	2:≥60	1.61 (1.21-2.12)	0.001
Albumin (g/dl)	1:≥3,9	1	
	2:<3.9	2.49 (1.87-3.31)	<0.001
Platelet count ($\times 10^4/mm^3$)	1:≥15.0	1	
	2:<15.0	3.69 (2.65-5.13)	<0.001
Aspartate aminotransferase (IU/l)	1:<67	1	
	2:≥67	4.16 (2.43-7.11)	<0.001
HCV subgroup	1: HCV-2a/2b	1	
	2: HCV-1b with Arg70	1.83 (1.25-2.68)	0.002
	3: HCV-1b with Gln70(His70)	2.16 (1.48-3.16)	<0.001

Cox proportional hazard model

the end of 5 years; 9.1, 0% at the end of 10 years; 20.5, 0% at the end of 15 years; and 20.5, 0% at the end of 20 years, respectively. The cumulative change rates in TT genotype were not significantly higher than those in non-TT genotype ($P = 0.114$) (Fig. 3B).

Discussion

This is the first report to indicate that aa substitution in the core region might affect hepatocarcinogenesis and survival for liver-related death in HCV patients who had not received antiviral therapy. The treatment-resistant mechanism and oncogenic potential of HCV core region are still unclear. Moriishi et al.[28,29] showed that a knockout of the PA28γ gene induces the accumulation of HCV core protein in the nucleus of hepatocytes of HCV core gene transgenic mice and disrupts development of both hepatic steatosis and HCC. Hu et al.[13] indicated that the point-mutations of the core gene, including core aa 70 and aa 91, might change the secondary structure of not only RNA but also protein. As a result, the functions of both RNA and protein of the core region, such as an interaction with other DNA/RNA or proteins, might change and lead to hepatocarcinogenesis. Funaoka et al.[30] recently reported that treatment-resistant substitutions of core aa 70 and aa 91 (Gln70/His70 and Met91) were resistant to interferon *in vitro*, and the resistance might be induced by interleukin 6-induced upregulation of SOCS3. Further studies should be performed to investigate the treatment-resistant mechanism and oncogenic potential of aa substitution in the core region.

The association between HCV genotype and the risk of HCC is not clear. A previous report indicated that hepatocarcinogenesis rates in patients infected with HCV-1b were significantly higher than those in patients infected with HCV-2a/2c, based on an Italian cohort,[31] and this finding might be partly explained by distribution of HCV-1b of Arg70 or Gln70(His70). In fact, the hepatocarcinogenesis rates in HCV-1b of Gln70(His70) were significantly higher than those in HCV-1b of Arg70 and HCV-2a/2b in the present study based on a Japanese cohort. The present study is the first report to indicate that substitution of aa 70 in

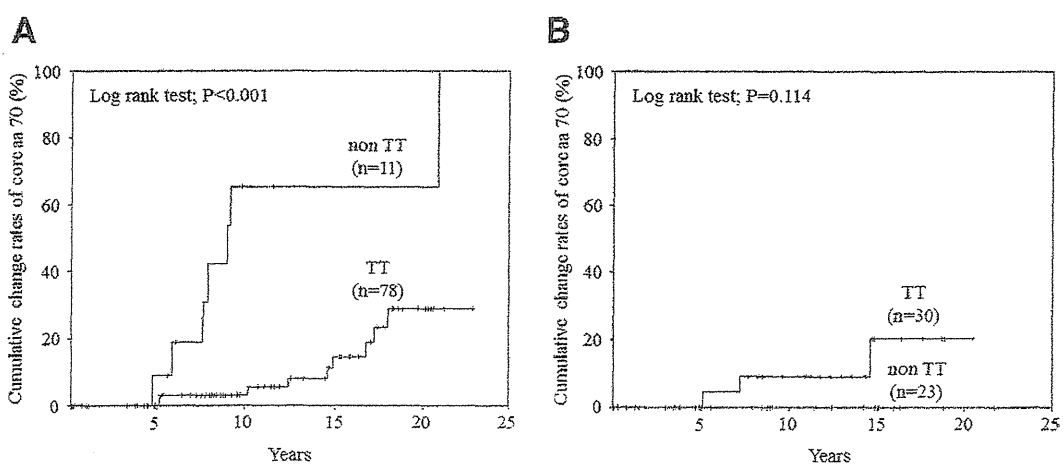

Fig. 3. Changes over time of dominant amino acid by direct sequencing in core aa 70 of HCV-1b, according to *IL28B* rs8099917 genotype. (A) In HCV-1b patients of Arg70 at the initial visit, cumulative change rates from Arg70 to Gln70(His70) during follow-up. The rates in non-TT genotype were significantly higher than those in TT genotype ($P < 0.001$; log-rank test). (B) In HCV-1b patients of Gln70(His70) at the initial visit, cumulative change rates from Gln70(His70) to Arg70 during follow-up. The rates in TT genotype were not significantly higher than those in non-TT genotype ($P = 0.114$; log-rank test).

the core region of HCV-1b is not only an important predictor of hepatocarcinogenesis, but also of survival for liver-related death in HCV patients who had not received antiviral therapy. The reason for the higher rates of liver-related death in HCV-1b of Gln70(His70) might be due to the higher rates of HCC. In conclusion, reducing the risk of hepatocarcinogenesis by HCV RNA eradication and/or ALT normalization by antiviral therapy should be recommended, especially in HCV-1b of Gln70(His70) as a high-risk group for hepatocarcinogenesis.[32]

The significant linkage between substitution of aa 70 and *IL28B* genotype had been shown,[21-23] but the mechanism of complex interaction between the virus and host is not clear. In the present study, the cumulative change rates from Arg70 to Gln70(His70) were significantly higher than those from Gln70(His70) to Arg70. Especially, the rates from Arg70 to Gln70(His70) in *IL28B* rs8099917 non-TT genotype were significantly higher than those in TT genotype. Although the molecular mechanisms of their relationship remain unknown, it could be speculated that *IL28B* genotype has an influence on the time-dependent changes of core aa 70, and refractory factors for treatment might accumulate in HCV-1b patients with non-TT. Hence, elucidating the relationship between substitution of aa 70 and *IL28B* genotype is an important step in understanding the mechanism of HCV treatment-resistance and disease progression.

The impact of *IL28B* genotype on hepatocarcinogenesis is controversial.[18-21] In this study, the effect of *IL28B* rs8099917 genotype on HCC was assessed in 515 of 2,799 consecutive HCV-infected patients who had not received antiviral therapy. Interestingly, the cumulative hepatocarcinogenesis rates in TT of the treatment-sensitive genotype was not significantly lower than those in non-TT of the treatment-resistant genotype ($P = 0.930$; log-rank test) in a preliminary study based on a small numbers of patients (Fig. 4). This result suggests that core aa 70 as a predictor of hepatocarcinogenesis might not only be influenced by *IL28B* genotype, but also by other factors strongly related to hepatocarcinogenesis independent of *IL28B* genotype. As a whole, it is regrettable that its impact on hepatocarcinogenesis in HCV patients who had not received antiviral therapy could not be investigated in this study. Further comprehensive studies should be performed to disclose the molecular mechanisms for the complicated relationships among core aa 70, *IL28B* genotype, and hepatocarcinogenesis.

The limitations of the present study are that patients who had received treatment besides IFN-related therapy (such as ursodeoxycholic acid, branched chain amino acid, and phlebotomy) could not be excluded. Furthermore, the clinical impact of metabolic factors (such as diabetes, insulin resistance, hepatocyte steatosis, and obesity) on hepatocarcinogenesis could also not be investigated. Further studies should be performed to investigate the clinical impact of treatment besides IFN-related therapy and metabolic factors on hepatocarcinogenesis.[33-37]

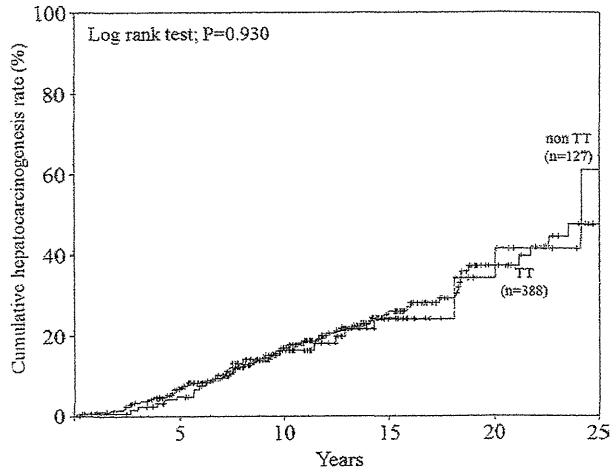

Fig. 4. Cumulative hepatocarcinogenesis rates according to *IL28B* rs8099917 genotype. The rates in TT genotype were not significantly lower than those in non-TT genotype ($P = 0.930$; log-rank test) in a preliminary study based on a small number of 515 patients.

In conclusion, substitution of aa 70 in the core region of HCV-1b is the important predictor of hepatocarcinogenesis and survival for liver-related death in HCV patients who had not received antiviral therapy. This study emphasizes the importance of antiviral therapy to reduce the risk of hepatocarcinogenesis, especially in HCV-1b of Gln70(His70) as a high-risk group for hepatocarcinogenesis. Furthermore, *IL28B* genotype might partly affect changes over time of dominant amino acid in core aa 70. This result should be interpreted with caution because races other than Japanese populations and patients infected with HCV-1a were not included. Any generalization of the results should await confirmation by studies of patients of other races and HCV-1a. Further prospective studies of a larger number of patients matched for race and HCV genotype are required to explore the relationship between core aa 70, *IL28B* genotype, and hepatocarcinogenesis.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Long-term efficacy of interferon therapy in patients with chronic hepatitis B virus infection in Japan

Fumitaka Suzuki · Yasuji Arase · Yoshiyuki Suzuki · Norio Akuta · Hitomi Sezaki · Yuya Seko · Yusuke Kawamura · Tetsuya Hosaka · Masahiro Kobayashi · Satoshi Saito · Kenji Ikeda · Mariko Kobayashi · Hiromitsu Kumada

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Abstract
Background Few studies have investigated the long-term effects of interferon (IFN) therapy for chronic hepatitis B (CHB). In this retrospective study, we investigated the efficacy of and predictors of response to IFN therapy in CHB patients.
Methods We analyzed data for 615 Japanese CHB patients (hepatitis B e antigen [HBeAg]-positive 414, HBeAg-negative 201) treated with IFN, and conducted follow up for a median duration of 8.1 years (range 0.5–23.2). Responders were defined as patients who showed continuously normalized alanine transaminase (ALT) levels, HBeAg clearance, and low hepatitis B virus (HBV) DNA levels at 6 months post-treatment or for a span of more than 6 months until each test point at 1, 3, 5, and 10 years.
Results The IFN response rates of all patients were 21, 18, 21, 23, and 25% at 6 months and 1, 3, 5, and 10 years, respectively. On multivariate analysis, significant determinants of the outcome of IFN therapy were as follows: at 6 months and 1 year, young age, low HBV DNA levels, and long duration of treatment; at 3 years, long duration of treatment, young age, and high level of albumin; at 5 years, high level of albumin, female, and pretreated with IFN; and at 10 years, HBeAg-negative. Sixty-nine of the 615 patients (11%) achieved seroclearance of hepatitis B surface antigen (HBsAg). On multivariate analysis, age ≥30 years, HBV genotype A, and male were all independent factors predicting the achievement of HBsAg seroclearance.
Conclusion HBeAg, HBV DNA level, age, sex, albumin, duration of treatment, pretreatment with IFN, and HBV genotype were important factors in determining long-term response to IFN therapy.

Keywords Interferon · Hepatitis B virus · Chronic hepatitis B · Genotype · Hepatitis B surface antigen

Abbreviations

CHB	Chronic hepatitis B
HBV	Hepatitis B virus
IFN	Interferon
HBeAg	Hepatitis B e antigen
ALT	Alanine transaminase
MU	Million units
HBsAg	Hepatitis B surface antigen
CLEIA	Chemiluminescent enzyme immunoassay
bDNA	Branched-chain DNA probe assay
TMA-HPA	Transcription-mediated amplification and hybridization protection assay
PCR	Polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
AST	Aspartate transaminase
AFP	α Fetoprotein
OR	Odds ratio
CI	Confidence interval
HCC	Hepatocellular carcinoma

F. Suzuki (✉) Y. Arase Y Suzuki N. Akuta H. Sezaki Y Seko Y Kawamura T Hosaka M. Kobayashi · S. Saito K. Ikeda H. Kumada
Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan
e-mail: fumitakas@toranomon.gr.jp

M. Kobayashi
Research Institute for Hepatology, Toranomon Hospital, Tokyo, Japan

Introduction

Hepatitis B virus (HBV) infection is a common disease that can induce a chronic carrier state and is associated with the risk of developing progressive disease and hepatocellular carcinoma [1]. Interferon (IFN) and several nucleoside/nucleotide analogues such as lamivudine, adefovir dipivoxil, entecavir, and tenofovir disoproxil fumarate are currently approved as treatments for chronic hepatitis B (CHB) in most countries [2–5]. Successful treatment of CHB with clearance of hepatitis B e antigen (HBeAg), reduction in serum HBV DNA levels, and normalization of alanine transaminase (ALT) levels is associated with a favorable long-term outcome, independent of the antiviral drug used [6, 7].

A meta-analysis of IFN therapy published in 1993 reviewed 15 randomized controlled studies involving 837 adult patients who received IFN-α at doses of 5–10 million units (MU) administered at intervals ranging from daily to three times weekly for 4–6 months [8]. Clearance of HBeAg was noted in 33% of the treated patients compared with 12% of controls. Elimination of detectable HBV DNA and normalization of ALT levels were also more common in the treated patients than in the controls. The major pretreatment factors that correlated with a response were high ALT levels [9–11], low HBV DNA levels [9, 10], female sex, and elevated liver activity and fibrosis on liver biopsy [8]. Another recent meta-analysis of 24 randomized controlled trials concluded that the rates of persistent ALT normalization, clearance of HBeAg, and sustained elimination of HBV DNA (determined by hybridization) induced by IFN therapy were approximately 25% greater than the rates for controls. A more recent meta-analysis report showed that IFN increased the incidence of HBeAg and hepatitis B surface antigen (HBsAg) seroclearance after long-term follow up of 3–7 years [12].

However, specific data on the long-term effects of IFN therapy (median follow-up duration of 8.1 years), particularly among the Japanese, are limited. Moreover, few reports have investigated factors predicting the achievement of HBsAg seroclearance. To further evaluate factors influencing clinical outcome, we performed a retrospective cohort study on CHB patients treated with IFN in our hospital.

Patients and methods

Patients

We retrospectively examined 615 Japanese patients (151 females and 464 males) who commenced IFN treatment between June 1984 and April 2008 in the Department of Hepatology at Toranomon Hospital (Table 1). Several of the patients have been included in previous reports [13–15].

Table 1 Characteristics of patients at commencement of interferon therapy

Demographic data	
Total number	615
Sex, female/male	151/464
Age, years (range)	35 (15–68)
Previously treated with interferon	123 (20%)
Duration of treatment, weeks (range)	26 (4–981)
Follow-up period, years (range)	8.1 (0.5–23.2)
Laboratory data	
Aspartate transaminase, IU/L (range)	72 (18–990)
Alanine transaminase, IU/L (range)	138 (12–1578)
Bilirubin, mg/dL (range)	0.7 (0.2–8.8)
Albumin, g/dL (range)	3.9 (2.6–5.3)
Platelets, $\times 10^3/\mu L$ (range)	174 (48–500)
Staging of liver histology (F0/1/2/3/4/ND)	8/77/185/162/72/111
Serum HBV DNA, log copies/mL (range)	>7.6 (<2.6 to >7.6)
HBeAg (positive/negative)	414/201
HBV genotype (A/B/C/D/H/B + C/unknown)	24/37/504/1/1/1/47

Values are expressed as medians and ranges (in parentheses) or as numbers and percentages (in parentheses)

HBV hepatitis B virus, *HBeAg* hepatitis B e antigen, *ND* not done

All enrolled patients were followed up for a range of 0.5–23.2 years from completion of IFN treatment, with a median follow-up duration of 8.1 years. Before the commencement of IFN treatment, all patients had been positive for HBsAg in the serum for more than 6 months, and all were confirmed to have hepatitis caused by HBV and not by another vector, such as infection with hepatitis C virus or autoimmune hepatitis. None had a history of drug abuse or alcoholic hepatitis, and none had received nucleoside/nucleotide analogue therapy. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethics Committee. Informed consent was obtained from each patient.

Interferon therapy and assessment of response to therapy

Patients received 3–12 MU of IFN-α or IFN-β (Sumiferon: Dainippon Sumitomo Pharma, Osaka, Japan; Canferon A. Takeda Chemical Industries, Osaka, Japan; Intron A. Schering-Plough MSD KK, Osaka, Japan; and Feron: Toray, Tokyo, Japan). The durations and regimens of treatment were as follows: 4 weeks (89 patients; daily for

4 weeks), 26 weeks (270 patients; daily for 4 weeks followed by 2 or 3 times a week), 52 weeks (103 patients; 2 or 3 times a week), 104 weeks (80 patients; 2 or 3 times a week), and more than 104 weeks (73 patients; 2 or 3 times a week). The median duration of treatment was 26 weeks (range 4–981).

The numbers of responders were evaluated at 6 months and 1, 3, 5, and 10 years after the completion of IFN therapy. In the baseline HBeAg-positive patients, responders were defined as patients who showed normalization of serum ALT level (normal level 6–30 IU/L), HBeAg clearance, and low HBV DNA level (<5 log copies/mL) at 6 months after completion of IFN therapy. In addition, baseline HBeAg-positive patients who showed continuous normalization of ALT levels, HBeAg clearance, and low HBV DNA level for more than 6 months until each test point at 1, 3, 5, and 10 years after completion of IFN therapy were also classified as "responders." In the baseline HBeAg-negative patients, responders were defined as those who showed sustained normalization of ALT level and low HBV DNA level (<4 log copies/mL) for more than 6 months until each test point after completion of IFN therapy.

All patients not considered to be responders were termed "non-responders." Patients receiving other therapies (IFN or nucleoside/nucleotide analogues) after the completion of IFN therapy were also termed non-responders.

Blood tests and serum viral markers

Routine biochemical tests were performed monthly via standard procedures during and for the first 12 months following the completion of IFN treatment and at least every 2 months thereafter. Levels of HBsAg, HBeAg, and anti-HBe were determined using radioimmunoassay kits (Abbott Diagnostics, Chicago, IL, USA) or a chemiluminescent enzyme immunoassay (CLEIA; Lumipulse System; Fujirebio, Tokyo, Japan). HBV DNA levels were measured using a branched-chain DNA probe assay (bDNA) (Chiron Laboratory Service, Van Nuys, CA, USA), a transcription-mediated amplification and hybridization protection assay (TMA-HPA) (Chugai Diagnostics Science, Tokyo, Japan), or a polymerase chain reaction (PCR)-based assay (COBAS Amplicor HBV Monitor Test or COBAS TaqMan HBV Test; Roche Diagnostics, Indianapolis, IN, USA).

HBV genotype

The major genotypes of HBV were determined using an enzyme-linked immunosorbent assay (ELISA; Institute of Immunology, Tokyo, Japan) or a PCR-invader assay (BML, Tokyo, Japan) according to the methods described by Usuda et al. [16] or Tadokoro et al. [17].

Statistical analysis

Differences between groups were examined for statistical significance using the χ^2 or Fisher's exact test and Mann–Whitney *U*-test where appropriate. Independent predictive factors associated with response to IFN treatment were determined using multivariate multiple logistic regression. The following 14 potential predictors of response to IFN treatment were assessed in this study: age, sex, pretreatment with IFN, duration of IFN treatment, severity of liver disease (CH or liver cirrhosis), HBV genotype, and levels of aspartate transaminase (AST), ALT, bilirubin, albumin, platelets, α fetoprotein (AFP), HBeAg, and HBV DNA. All factors found to be at least marginally associated with response to IFN therapy ($P < 0.10$) were entered into the multivariate multiple logistic regression analysis. The above calculations were performed using the Windows SPSS software package version 11.0.1 J (SPSS, Chicago, IL, USA).

The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to assess the relative risk. Independent risk factors predicting the achievement of HBsAg seroclearance were studied using stepwise Cox regression analysis. Potential factors predicting the achievement of HBsAg seroclearance assessed here were the above 14 variables, each transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All factors found to be at least marginally associated with HBsAg seroclearance ($P < 0.10$) were tested in the multivariate Cox proportional hazard model. A Kaplan–Meier estimate was performed using the SPSS software, and *P* values were calculated using the Cox-Mantel log-rank test. A two-tailed *P* value of <0.05 was considered statistically significant.

Results

Study population

Twenty-four (4%), 37 (6%), 504 (82%), 1 (0.2%), 1 (0.2%), and 1 (0.2%) patients were infected with HBV genotypes A, B, C, D, H, and B + C, respectively. Genotype could not be measured in the remaining 47 patients. The baseline characteristics of the patients are shown in Table 1. Although few patients had genotypes A and B, the distribution of HBV genotype was similar to that in patients with CHB who had received care in our hospital, with a follow-up period of more than 2 years [18]. Twenty-two of 24 patients with genotype A, 14 of 37 with

genotype B, 342 of 504 with genotype C, 1 of 1 with genotype H, and 34 of 47 with unknown genotype were HBeAg-positive at the commencement of treatment. While we were able to measure HBV DNA levels in 254 patients at the commencement of IFN therapy, levels in the remaining 361 could not be measured owing to a lack of commercial kits before the bDNA assay was available. The numbers of patients receiving other additional therapies after the completion of IFN therapy were 111 (HBeAg-positive/-negative, 90/21), 92 (67/25), 34 (25/9), and 61 (39/22) at the 1-, 3-, 5-, and 10-year time points, respectively.

Response to interferon therapy in all patients

The IFN response rates in all patients were 21% (105/497), 18% (86/491), 21% (90/428), 23% (82/359), and 25% (59/235) at 6 months and 1, 3, 5, and 10 years, respectively, after completion of the IFN therapy (Fig. 1). In patients with genotype A, the response rate was highest at 6 months post-treatment and gradually decreased at subsequent time points from 1 to 10 years thence. In patients with genotype B, response rates were over 20% at all time points except for 6 months post-treatment, whereas rates in patients with genotype C were under 25% at all time points (Fig. 2a).

Evaluation of efficacy of IFN in relation to clinical factors in all patients

The data of all patients were subjected to univariate analyses to determine the clinical factors contributing to the efficacy of IFN at each time point. We then investigated the significance of response to IFN therapy using multivariate logistic regression analysis.

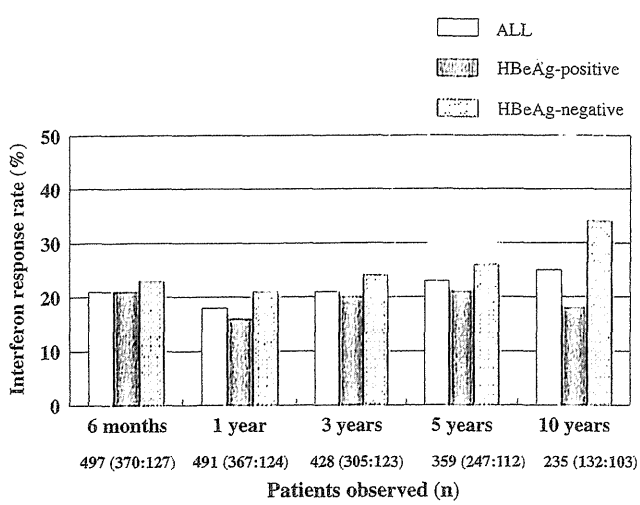

Fig. 1 Interferon response rates of all patients and hepatitis B e antigen (*HBeAg*)-positive and HBeAg-negative patients at 6 months and 1, 3, 5, and 10 years

Multivariate analyses including the variables noted above revealed several parameters that independently influenced the outcome of IFN therapy; namely, at 6 months: age ($P = 0.013$), HBV DNA level ($P = 0.019$), and duration of treatment ($P = 0.034$); at 1 year: HBV DNA level ($P < 0.001$) and age ($P = 0.001$); at 3 years: duration of treatment ($P < 0.001$), age ($P = 0.013$) and albumin level ($P = 0.013$); at 5 years: albumin level ($P = 0.004$), sex ($P = 0.005$), and pretreatment with IFN ($P = 0.039$); and at 10 years: HBeAg ($P < 0.001$) (Table 2).

Response to interferon therapy and evaluation of efficacy of IFN in relation to clinical factors in HBeAg-positive patients

Response rates in baseline HBeAg-positive patients were 21% (76/370), 16% (60/367), 20% (61/305), 21% (53/247), and 18% (24/132) at 6 months and 1, 3, 5, and 10 years, respectively (Fig. 1). In patients with genotype A, the response rate was highest at 6 months post-treatment and the rate was roughly equivalent to the 6 months post-treatment rate at subsequent time points from 1 to 10 years. Response rates in patients with genotype B in particular were above 40% at all time points except at 6 months, although few patients had genotype B. On the other hand, response rates in patients with genotype C were under 20% at all time points (Fig. 2a).

In addition, multivariate analyses in HBeAg-positive patients also revealed several parameters that independently influenced the outcome of IFN therapy–at 6 months: duration of treatment ($P = 0.001$) and age ($P = 0.014$); at 1 year: age ($P = 0.011$) and HBV DNA level ($P = 0.027$); at 3 years: sex ($P = 0.008$), duration of treatment ($P = 0.019$), age ($P = 0.020$), pretreatment with IFN ($P = 0.029$), and albumin level ($P = 0.043$); at 5 years: sex ($P = 0.002$) and pretreatment with IFN ($P = 0.005$); and at 10 years, genotype ($P = 0.019$) and AST ($P = 0.035$) (Table 3).

Response to interferon therapy and evaluation of efficacy of IFN in relation to clinical factors in HBeAg-negative patients

Response rates in baseline HBeAg-negative patients were 23% (29/127), 21% (26/124), 24% (29/123), 26% (29/112), and 34% (35/103) at 6 months and 1, 3, 5, and 10 years, respectively (Fig. 1). Rates in patients with genotype C were gradually increased at subsequent time points, whereas those in patients with genotype B remained under 30% at all time points (Fig. 2b).

In addition, univariate and multivariate analyses in HBeAg-negative patients revealed that duration of treatment (≥1 year) independently influenced the outcome of

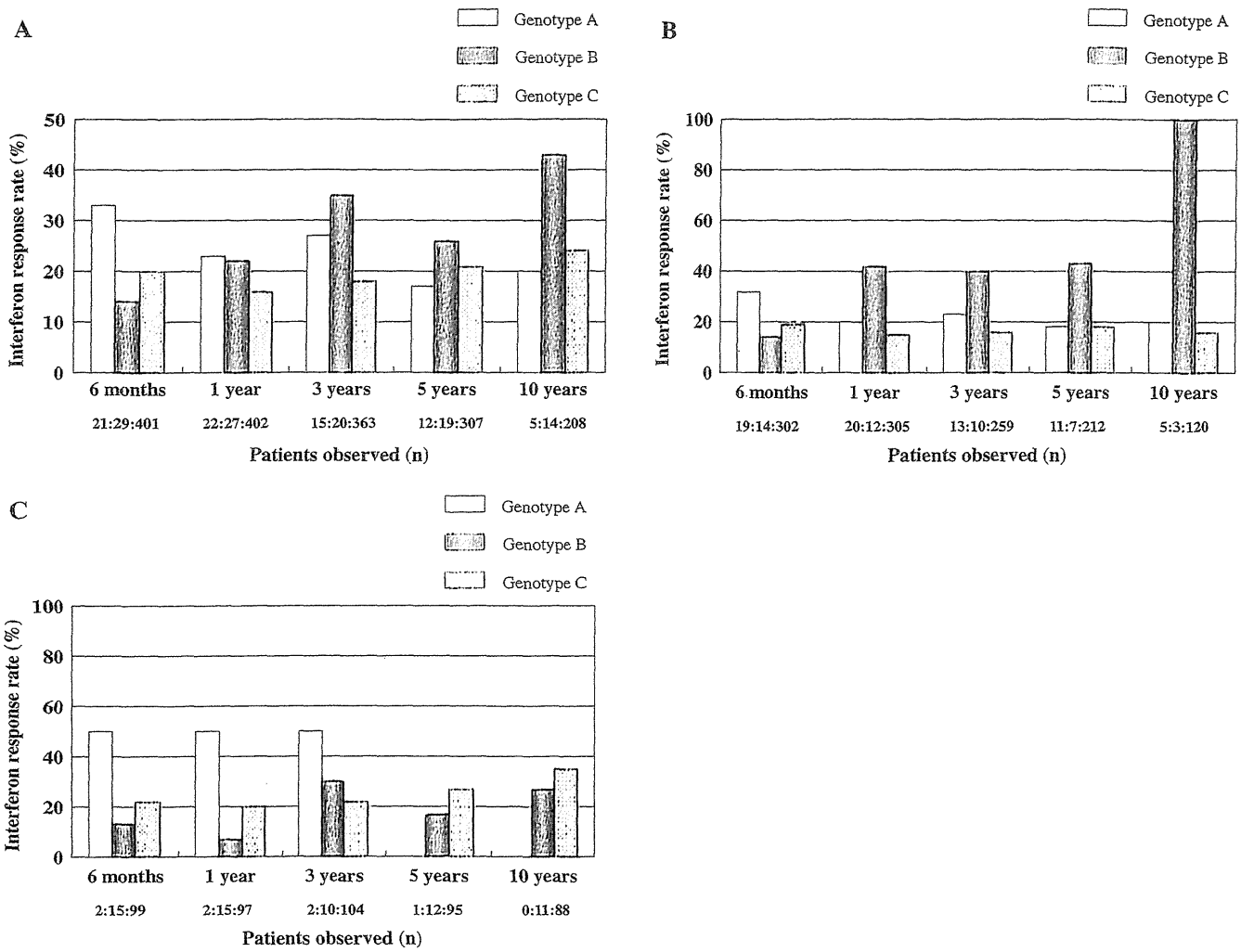

Fig. 2 Interferon response rates of patients with genotypes A, B, and C at 6 months and 1, 3, 5, and 10 years. **a** All patients, **b** HBeAg-positive patients, **c** HBeAg-negative patients

IFN therapy at 6 months, and at 1 and 3 years. No parameters independently influenced the outcome of IFN therapy at 5 or 10 years.

Evaluation of efficacy of IFN in relation to HBs antigen seroclearance

The HBsAg seroclearance rate in this study was obtained from patients who received IFN therapy alone; 69 of 615 patients (11%) achieved seroclearance of HBsAg. The cumulative HBsAg seroclearance rates in all patients from the commencement date of IFN therapy were 6.5% at 5 years, 15% at 10 years, 35% at 15 years, and 44% at 20 years (Kaplan–Meier method; Fig. 3a). No patients experienced the reappearance of HBsAg after seroclearance. Five factors found to be associated with achievement of HBsAg seroclearance on univariate analysis were: male sex ($P = 0.002$), age $\geq$30 years ($P = 0.011$), genotype A ($P = 0.038$), HBeAg-negativity ($P = 0.045$), and bilirubin $\leq$1.0 mg/dL ($P = 0.064$). On multivariate analysis, independent factors predicting the achievement of HBsAg seroclearance were: age $\geq$30 years, genotype A, and male sex (Table 4). The cumulative HBsAg seroclearance rate for genotype A patients was significantly higher than the rate for those with genotypes B or C ($P = 0.0116$) (Fig. 3b).

Relationship between the response to IFN and the development of hepatocellular carcinoma

Twenty-nine patients developed hepatocellular carcinoma (HCC) during the observation period, excluding 17 patients who received other additional therapies after the completion of IFN therapy and developed HCC thereafter. IFN response rates in the 29 patients who developed HCC were 5% (1/22), 5% (1/20), 10% (2/20), 13% (2/15), and 13% (2/16), respectively, at 6 months and 1, 3, 5, and 10 years after the completion of IFN. No patient developed HCC after HBsAg seroclearance.

Table 2 Factors associated with response to interferon therapy for all patients at 6 months and 1, 3, 5, and 10 years

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
6 Months after completion of IFN therapy ($n = 229$)				
Duration of treatment (≥1 year)	2.680 (1.724–4.166)	<0.001	2.107 (1.058–4.198)	0.034
HBV DNA level (≤7.0 log copies/mL)	2.165 (1.107–4.219)	0.026	2.309 (1.148–4.630)	0.019
Age (<30 years)		0.057	2.451 (1.209–4.950)	0.013
1 year after completion of IFN therapy ($n = 231$)				
Duration of treatment (≥1 year)	2.553 (1.588–4.104)	<0.001		
HBV DNA level (≤7.0 log copies/mL)	3.268 (1.597–6.667)	0.001	4.464 (2.058–9.709)	<0.001
Age (<35 years)	1.799 (1.125–2.874)	0.014	3.831 (1.718–8.547)	0.001
3 years after completion of IFN therapy ($n = 397$)				
Duration of treatment (≥1 year)	2.410 (1.495–3.885)	<0.001	2.739 (1.618–4.634)	<0.001
Age (<30 years)	2.070 (1.215–3.521)	0.009	2.110 (1.171–3.802)	0.013
Albumin (≥3.9 g/dL)	1.697 (1.045–2.757)	0.030	2.009 (1.158–3.486)	0.013
Genotype (non-C)	2.155 (1.033–4.504)	0.041		
5 years after completion of IFN therapy ($n = 356$)				
Albumin (≥3.9 g/dL)	1.869 (1.108–3.153)	0.017	2.321 (1.316–4.093)	0.004
Pretreatment with IFN (positive)	1.770 (1.016–3.084)	0.048	1.821 (1.029–3.222)	0.039
Sex (female)		0.060	2.381 (1.297–4.367)	0.005
Duration of treatment (≥1 year)		0.080		
10 years after completion of IFN therapy ($n = 234$)				
HBeAg (negative)	2.315 (1.269–4.219)	0.006	2.252 (1.230–4.115)	0.009
ALT (≥100 IU/L)	1.972 (1.053–3.690)	0.036		
Pretreatment with IFN (positive)		0.058		

ALT alanine transaminase, *IFN* interferon, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *CI* confidence interval, *OR* odds ratio, *n* number submitted to multivariate analysis, including all factors found to be associated with response to IFN therapy

Discussion

Although IFN has been reported to exert beneficial effects in CHB patients, the response rate is not high. A meta-analysis published in 1993 reviewed 15 randomized controlled studies involving 837 adult patients who received IFN-α for 4–6 months, and elimination of HBeAg occurred in 33% of the treated patients [8]. In previous studies, we found the response rates among HBeAg-positive patients at 6 months after the completion of therapy to be 20 and 31% for 6 months and 1 year of IFN therapy, respectively [13, 15]. Although a recent meta-analysis reported that IFN increased the incidence of HBeAg and HBsAg seroclearance after long-term follow up of 3–7 years [12], the factors that influenced the clinical outcome were unclear.

In Japan, from 1988, 4-week IFN treatment was reimbursed by the healthcare system, and since 2002, 24-week IFN treatment has been conducted. In the present study, these two regimens were the major ones, and other regimens were used in clinical studies at our hospital (including previously reported studies [14, 15]). Although the durations of treatment differed, we analyzed the factors associated with long-term response to IFN therapy, including the factor of duration of treatment.

In the present study, response rates were low among HBeAg-positive patients and relatively high among HBeAg-negative patients at all time points. Approximately 20% of the HBeAg-positive patients had sustained a response at 6 months to 10 years of follow up. Long-term follow-up studies after a four- to six-month course of IFN therapy in HBeAg-positive patients in European and Taiwanese studies showed higher (33–75%) response rates (HBeAg loss) than our study [7, 19, 20]. The difference in response rates between our present study and previous studies in other countries may be due to differences in ethnicity or HBV genotype (mainly genotype C in Japan). Moreover, the low IFN response rates at 1, 3, 5, and 10 years in the HBeAg-positive patients in our study were likely due to the change in treatments (IFN or nucleoside/nucleotide analogues). On the other hand, the response rates of HBeAg-negative patients in the present study were about 20% at 6 months and gradually increased thereafter. The sustained response rate in HBeAg-negative patients was usually <30% in European studies [21–23]. The response

Table 3 Factors associated with response to interferon therapy for HBeAg-positive patients at 6 months and 1, 3, 5, and 10 years

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
6 months after completion of IFN therapy ($n = 279$)				
Duration of treatment (≥1 year)	2.449 (1.457–4.114)	0.001	2.801 (1.540–5.096)	0.001
Age (<35 years)	1.855 (1.112–3.096)	0.017	2.128 (1.164–3.891)	0.014
1 year after completion of IFN therapy ($n = 172$)				
Duration of treatment (≥1 year)	2.483 (1.407–4.380)	0.002		
HBV DNA level (≤7.0 log copies/mL)	3.509 (1.495–8.264)	0.005	3.003 (1.130–7.937)	0.027
Age (<35 years)	1.996 (1.133–3.521)	0.015	3.610 (1.351–9.615)	0.011
3 years after completion of IFN therapy ($n = 283$)				
Age (<35 years)	2.041 (1.155–3.597)	0.013	2.083 (1.122–3.861)	0.020
Duration of treatment (≥1 year)	2.055 (1.153–3.661)	0.016	2.130 (1.132–4.008)	0.019
Pretreatment with IFN (positive)	2.054 (1.050–4.019)	0.041	2.336 (1.091–4.998)	0.029
Albumin (≥3.9 g/dL)		0.055	1.974 (1.020–3.820)	0.043
Sex (female)		0.089	2.646 (1.284–5.464)	0.008
5 years after completion of IFN therapy ($n = 247$)				
Sex (female)	2.571 (1.328–4.975)	0.006	2.924 (1.477–5.814)	0.002
Pretreatment with IFN (positive)	2.460 (1.213–4.988)	0.015	2.870 (1.377–5.980)	0.005
10 years after completion of IFN therapy ($n = 122$)				
Genotype (non-C)	5.319 (1.222–23.26)	0.032	6.410 (1.364–30.30)	0.019
AST (≥100 IU/L)		0.081	2.932 (1.078–7.972)	0.035

AST aspartate transaminase, *IFN* interferon, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *CI* confidence interval, *OR* odds ratio, *n* number submitted to multivariate analysis, including all factors found to be associated with response to IFN therapy

rates of HBeAg-negative patients in our present study and the studies in other countries [21–23] were similar.

Few reports have identified the factors associated with long-term virological response to IFN therapy. In our present study, HBeAg-negativity was the most important factor for predicting a long-term response (10 years). While the HBV DNA level was important for predicting the response at 6 months and 1 year for all patients and the response at 1 year for HBeAg-positive patients, other factors (age, sex, albumin level, AST, IFN pretreatment, and duration of treatment) were found to be important at some time points for all patients and for HBeAg-positive patients. The HBV DNA level may not have been associated with long-term response to IFN therapy because the follow-up period (median 5.7 years) in patients with an HBV DNA level measurable with commercial kits was significantly shorter than that in the other patients (median 11.2 years; $P < 0.001$).

Previous studies have reported that high ALT levels, low HBV DNA level, female sex, and elevated liver activity and level of fibrosis on liver biopsy were major pretreatment factors correlated with a response to IFN [8–11, 24]. However, in these studies the follow-up times for judging the response were short (typically 6 months to 1 year). Our present study has clarified that HBeAg, HBV DNA level, age, sex, IFN pretreatment, duration of treatment, and levels of albumin and AST are important factors in the long-term response to IFN. Further, non-C genotype was an important factor for long-term response in HBeAg-positive patients. Kao et al. [25] and Lin et al. [20] reported that HBV genotype B was associated with a higher response rate to IFN-α therapy than genotype C among CHB patients positive for HBeAg. Similarly, response rates among HBeAg-positive patients with genotype B in the present study were also higher than the response rates in those with genotype C in terms of long-term response (Fig. 2b). The long-term response rate among HBeAg-negative patients was relatively higher than that in HBeAg-positive patients. Previous reports have shown that response rates to a 6- to 12-month course of IFN-α in HBeAg-negative CHB patients range from 10 to 47% (average 24%) [26–29]. In addition, our previous report showed that 9 of 12 (75%) patients who received IFN-β twice per week for 24 weeks responded to the therapy [14]. However, the follow-up periods of these studies were short, and the long-term efficacy has not been clarified. While the efficacy of IFN in HBeAg-negative patients was high in the present study, the factors that might be useful in predicting a sustained response were less well-defined than those in HBeAg-positive patients, as previously reported [5].

A meta-analysis of IFN therapy published in 2010 reviewed 6 clinical controlled studies including 828 patients who received IFN [12]. The duration of follow-up

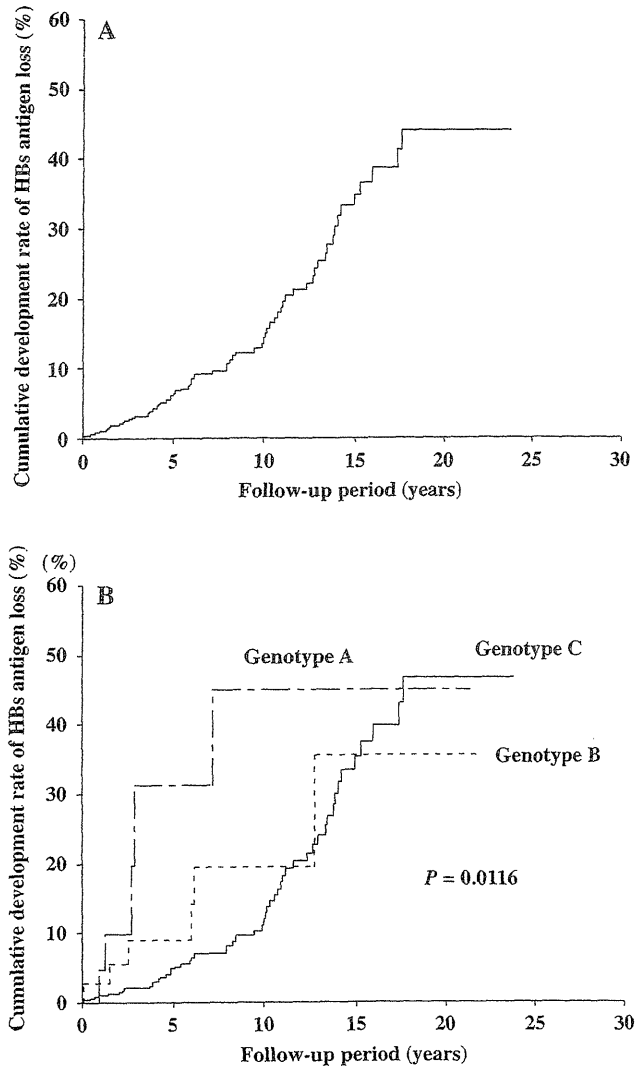

Fig. 3 Cumulative clearance of hepatitis B surface (*HBs*) antigen in patients treated with interferon (Kaplan–Meier method). **a** All patients, **b** patients stratified by genotypes A, B, and C

Table 4 Factors associated with HBsAg seroclearance by interferon therapy, determined by multivariate analysis

Parameter	Category	Hazard ratio	95% CI	*P*
Age	<30 years	1		0.002
	≥30 years	4.433	1.703–11.538	
Genotype	A	1		0.004
	B	0.296	0.087–1.005	
	C	0.199	0.075–0.528	
Sex	Female	1		0.005
	Male	2.962	1.387–6.327	

HBsAg hepatitis B surface antigen, *CI* confidence interval

ranged from 35.8 months to 7 years, and HBsAg seroclearance occurred in 9.5% (79/828). In the present study, we observed HBsAg seroclearance in 69 of 615 (11%) patients, with a median follow-up duration of 8.1 years. However, few reports have investigated factors predicting the achievement of HBsAg seroclearance. In our study, important factors for achieving HBsAg seroclearance were age ≥30 years, genotype A, and male sex. Patients with genotype A had primarily been infected during adulthood via sexual contact, and the average duration of infection was relatively short. In contrast, most Japanese carriers are infected perinatally and possess HBV genotype C, and therefore the efficacy of IFN therapy for patients with genotype C may be low. Male sex was also an important factor in determining potential to achieve HBsAg seroclearance, although female sex was an important factor in determining long-term response to IFN therapy. In our previous study of HBsAg seroclearance (mainly spontaneous seroclearance), we found that response rates were low among females (19%; 45/231) [30]. These present and previous findings indicate that male patients tended to achieve HBsAg seroclearance more frequently than females, although the reason is unclear. We previously reported that Kaplan–Meier analysis in 486 patients who received lamivudine therapy for 5 and 10 years showed an estimated loss of HBsAg in 3 and 13% of the patients, respectively, [31]. The cumulative clearance rates of HBsAg, also determined by Kaplan–Meier analysis, in patients treated with IFN were higher than those in the patients treated with lamivudine, albeit that there were differences in the baseline characteristics of the patients at the commencement of the respective therapies. The effects of IFN therapy in modulating the host immune response might induce HBsAg clearance.

In conclusion, we investigated the long-term efficacy of IFN therapy in Japanese CHB patients. Response rates were low among HBeAg-positive patients and relatively high among HBeAg-negative patients at all time points examined. HBeAg-negative status, HBV DNA level, age, sex, pretreatment with IFN, duration of treatment, and levels of albumin and AST were important factors in predicting long-term response for all patients and for HBeAg-positive patients. Age, genotype, and sex were important factors in predicting ability to achieve HBsAg seroclearance. Further studies exploring the efficacy of therapy over a longer duration may be necessary to confirm these findings and establish true response rates to IFN therapy, including treatment with pegylated IFN.

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