TABLE 4. Detection of ETVr mutations by HBV DR v3 and direct sequencing at weeks 0, 24, 48, 72, 96, and 144

Case	INNO-LiPA detection		Direct-sequencing detection	
	Wk	ETVr	Wk	ETVr
J20	96	ILFM184 + G202	96	L184 (L184 and G202 detected at wk 144)
J27	144	SCGA184 + T184	144	S184 + T184
J30	48	G202	96	G202 (ND[b] at wk 48)
J39	72	SCGA/ILFM184 + G202	96	I184, G202 (ND at wk 72)
J40	−8	G202	0	G202 (ND at wk −8)
J33	96	G202	144	G202 (ND at wk 96)
J19	24	G202 + C202	48	G202 (ND at wk 24)
J22	144	S202 + G202	144	S202
J28	0	SCGA184, V233	0	A184, V233
J37[a]	48	S202 + G202	48	S202
U42	80	SCGA184 + T184	80	T184 + A184
U72	144	T184 + SCGA184, S202 + G202	144	T184 + A184, S202
H55	48	T184 + ILFM184	48	T184

[a] Switched from ETV to LVD plus ADV therapy at week 60.
[b] ND, ETVr was not detected.

at baseline (BT, 100%, versus non-BT, 28.6%) (Table 5). For other factors, additional analysis showed that ETVr substitutions (i.e., S202G, T184SCGA/ILFM, and M204V) were strongly associated with BT during the 3-year ETV treatment (OR, 146.67 [95% CI, 13.55 to 1,587.24], 96.25 [95% CI, 9.38 to 987.41], and 10.91 [95% CI, 4.72 to 354.28]) (Table 5).

After adjustment for age, gender, baseline HBV DNA, and reduction in HBV DNA, we found that ETVr substitutions (i.e., T184SCGA/ILFM and S202G) significantly increased the risk of BT among patients with LVDr (OR, 141.12 [95% CI, 6.94 to 2,870.20] and 201.25 [95% CI, 11.22 to 3,608.65], respectively).

Mechanism of ETVr assessed by 3D docking simulation. Modeling of the DNA binding cleft of HBV RT by docking simulation indicated that ETVr substitutions (T184L and S202G), which are located in the palm, were found to change the direction of the D205 residue (YMDD domain) and to narrow the binding pocket in comparison with the wild type and LVDr substitutions (M204V and L180M) (Fig. 3). The results of docking simulation showed that ETVr substitutions (T184L and S202G) plus LVDr substitutions (M204V and L180M) have significantly longer minimal distances between the molecular surfaces of the protein and the drug (2.2 Å and 2.1 Å) and higher potential energy (−118 and −99.8 Kcal/mol [smaller absolute values have a minus sign]) for ETV-TP than for the wild type (1.3 Å; −178 Kcal/mol) and LVDr substitutions (1.5 Å; −141 Kcal/mol) (Table 6). Since binding at higher potential energy creates a less stable structure, the deoxyribonucleotide triphosphate (dNTP)-binding domains of the ETVr substitutions plus the LVDr substitutions in HBV RT have

TABLE 5. ORs and 95% CIs of BT according to baseline characteristics among 67 patients treated with ETV for 3 years

Characteristic	Non-BT (n = 56)[a]	BT (n = 11)[a]	P for difference	Contrast	OR	95% CI
Age (mean)	45.4 ± 8.1	45.4 ± 9.4	0.932	1-yr increase	1	0.92–1.08
Male (%)	71.4	81.8	0.481	Male vs. female	1.8	0.35–9.26
ALT (mean)	115.9 ± 105.6	126.2 ± 127.3	0.832	1-U increase	1	0.99–1.00
HBeAg (%)	66.1	81.8	0.307	Positive vs. negative	2.31	0.45–11.78
HBV-DNA						
Level (mean)	6.8 ± 1.6	7.1 ± 0.8	0.959	1-U increase	1.16	0.72–1.89
2-log-unit reduction at 1 yr (%)	98.2	63.6	<0.001	With vs. without	0.03	0.00–0.33
DNA <2.6 at 1 yr (%)	58.9	0.0	<0.001	With vs. without	NA[b]	
LVD refractory (%)	32.1	100.0	<0.001	With vs. without	NA	
Amino acid substitutions at baseline						
V80 (%)	3.6	18.2	0.063	With vs. without	6	0.74–48.17
I80 (%)	17.9	36.4	0.171	With vs. without	2.63	0.64–10.72
L173 (%)	3.6	18.2	0.063	With vs. without	6	0.75–48.18
M180 (%)	28.6	100.0	<0.001	With vs. without	NA	
V204 (%)	19.6	90.9	<0.001	With vs. without	10.91	4.72–354.28
I204 (%)	23.2	36.4	0.363	With vs. without	1.89	0.48–7.49
Amino acid substitutions during ETV therapy (3 yr)						
SCGA/ILFM184 (%)	1.8	72.7	<0.001	With vs. without	96.25	9.38–987.41
G202 (%)	1.8	63.6	<0.001	With vs. without	146.67	13.55–1,587.24

[a] Values are means ± standard deviations.
[b] NA, not applicable.

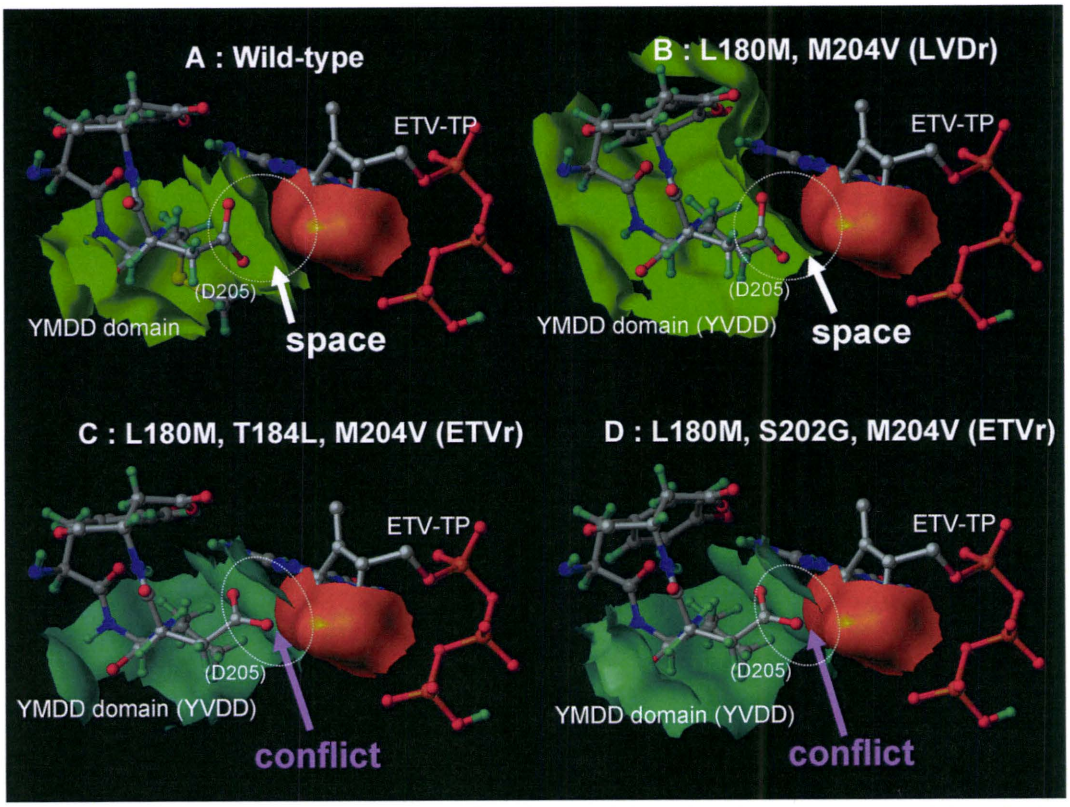

FIG. 3. 3D structures of the dNTP-binding domains of HBV RT of the wild type (A), an LVDr substitution (B), and ETVr substitutions (C and D). The molecular surfaces of the wild type and the LVDr mutant are drawn in green, those of LVDr plus ETVr mutants are drawn in blue, and that of ETV-TP is drawn in orange.

lower binding affinity for ETV-TP than the wild type. Molecular docking simulation in the present study showed that the L180M, M204V, S202G, and T184L substitutions can lessen the affinity of ETV-TP for HBV RT by heightening the potential energy between them, suggesting that S202G and T184L substitutions, in addition to M204V in the YMDD motif and L180M in domain C, could affect the initial polymerase binding of dNTP analog inhibitors.

DISCUSSION

Based on the combination of clinical observations and 3D docking simulation, this is the first report to suggest the mechanism by which ETVr substitutions (T184SCGA/ILFM and S202G, but not S202C), in addition to LVDr (L180M and M204V, but not M204I), can induce BT during ETV therapy.

TABLE 6. Minimal distances and binding potentials between ETV and the HBV RT domain in the wild type, one LVDr mutant, and 2 ETVr mutants

Strain	distance (Å)	Binding potential (GOLD score [Kcal/mol])	Reference (Fig. 3)
Wild type	1.3	−178.4	A
L180M, 204V	1.5	−141.3	B
L180M, T184L, 204V	2.2	−117.9	C
L180M, S202G, 204V	2.1	−99.8	D

First, an assessment of virological and biochemical events during a 3-year ETV treatment course showed that ETVr substitutions were absent among treatment-naïve patients but were detected in 44.8% of patients who were refractory to LVD during the preceding treatment period. Evidence of BT during ETV therapy was observed in 26.8% of LVD-refractory patients between weeks 60 and 144 of treatment. All 11 of the BT cases had both L180M and M204V/I substitutions at baseline (LVD refractory), as well as additional substitutions, such as T184 and/or S202G (not S202I/C), during the 3-year ETV treatment period.

Statistically significant risk factors for BT were the presence of LVDr (L180M and M204V) at baseline, detection of ETVr (S202G and T184SCGA/ILFM substitutions) during ETV treatment, and undetectable HBV DNA (<2.6) or more than a 2-$\log_{10}$-unit reduction in HBV DNA levels during the first year of ETV treatment. Detection of T184SCGA/ILFM and S202G was significantly associated with BT independent of age, gender, and LVDr (M204V and/or L180M) at baseline or nondetection or reduction in HBV DNA at the first year of treatment, indicating that these substitutions could be used as predictive markers for BT.

The mechanism by which combinations of ETVr (S202G and T184 SCGA/ILFM) and LVDr (L180M and M204V) can induce BT during ETV therapy is largely unknown. Note that T184L and S202G residues are located within domain B and domain C of the RT/polymerase, respectively, as well as

L180M and M204V. The modeling of HBV RT indicated that the combination changed the direction of the D205 residue (YMDD domain) and narrowed the dNTP-binding pocket in comparison with the wild type and LVDr substitutions (M204V and L180M) (Fig. 3). The results of docking simulation of HBV RT and ETV-TP showed that the ETVr (184L and S202G) plus LVDr (L180M and M204V) substitutions had significantly longer minimal distances for ETV-TP and steric conflict with the D205 residue (Fig. 3 and Table 6). These docking simulation results suggest that nucleotide analogs that have the exocyclic alkene moiety of ETV-TP replaced by a smaller atom may retain activity against ETV-resistant mutants. Differences in the mode of binding of nucleotide inhibitors to the dNTP-binding pocket of HBV polymerase, as predicted from the current modeling studies, may account for the complementary drug resistance profiles seen for different nucleotide analogs. Interestingly, a previous *in vitro* study showed that ETVr substitutions (S202I and T184G), in addition to LVDr (L180M and M204V), were associated with a >1,100-fold decrease in susceptibility to ET (20). Collectively, these data indicate that nucleoside-naïve patients treated with ETV were less likely to become resistant to ETV.

In an *in vitro* assay, the rtA181T/V clinical-isolate genome from patients refractory to LVD/ADV induced a decrease in susceptibility to LVD, ADV, and, to a lesser extent, TDF, but sensitivity to ETV remained (22). LVDr selected by LVD exposure may lead to ETV failure. Therefore, for patients refractory to LVD/ADV, a combination of emtricitabine/TDF (10) might be an effective option. Furthermore, since sequential antiviral therapy leads to the selection of multidrug-resistant HBV and fitness or maximal viral resistance (25), combination therapy using a nucleoside together with a nucleotide analog, such as emtricitabine/TDF (10), ADV/LVD, ADV/ETV, ADV/telbivudine, or TDF, would be a more appropriate treatment strategy for patients with the LVDr substitution.

Based on HBV DR v3, T184SCGA/ILMF and S202G substitutions were present at baseline in 4.8% of patients and were detected in 14.6%, 24.4%, and 44.8% during 48, 96, and 144 weeks, respectively, of ETV therapy (Fig. 2). The prevalence of ETVr in our cohort seems to be higher than that reported in previous studies, based on assessment of ETV treatment at weeks 48, 96, 144, 192, and 240 using direct sequencing, where ETVr emerged in 6%, 15%, 36%, 47%, and 51% of LVD-refractory patients, respectively (21). The differences might be attributable to the tools used to detect HBV DNA substitutions associated with drug resistance, which differed between the studies. HBV DR v.3 and v.2 performed better than direct sequencing, and monitoring of the nucleoside mutations by HBV DR v.3 and v.2 in patients before and during ETV therapy was good for selecting effective therapeutic strategies and new combination therapies.

In conclusion, the combination of clinical observations and 3D docking simulation in the present study indicated that the low binding affinity of ETV-TP for the dNTP-binding domains of HBV RT by the ETVr plus LVDr substitutions could induce BT and provides the mechanistic foundations for a mechanism of inhibition of ETV against HBV. This modeling would be useful for designing new antiviral drugs.

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Research Article

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Association between mutations in the core region of hepatitis C virus genotype 1 and hepatocellular carcinoma development

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Background & Aims: To determine whether amino acid mutations in the core region of hepatitis C virus (HCV) genotype 1 are associated with response to interferon (IFN) therapy and development of hepatocellular carcinoma (HCC).

Methods: We followed up 361 patients (median duration, 121 months), and IFN monotherapy was administered to 275 (76%) [sustained virological response (SVR) rate, 26.5%]. Using pretreatment sera, mutations at core residues 70 and 91 were analyzed [double wild (DW)-type amino acid pattern: arginine, residue 70; leucine, residue 91].

Results: A low aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio and low HCV load were independently associated with SVR, but core mutations were not. During follow-up, 12 of 81 (14.8%) patients with the DW-type pattern and 52 of 216 (24.1%) patients with non-DW-type pattern developed HCC ($p = 0.06$, Breslow–Gehan–Wilcoxon test). Multivariate analysis with the Cox proportional-hazards model revealed the following independent risk factors for HCC: male gender [$p < 0.0001$; risk ratio (RR), 3.97], older age ($p < 0.05$; RR, 2.08), advanced fibrosis ($p < 0.0001$; RR, 5.75), absence of SVR ($p < 0.01$; RR, 10.0), high AST level ($p < 0.01$; RR, 2.08), high AST/ALT ratio ($p < 0.01$; RR, 2.21), and non-DW-type pattern ($p < 0.05$; RR, 1.96). In patients with F0–F2 fibrosis at entry, non-DW-type was likely to lead to cirrhosis ($p = 0.051$).

Conclusions: In HCV genotype 1 patients, HCC risk could be predicted by studying core mutations, response to IFN, and host factors like age, gender, and liver fibrosis.

Keywords: Hepatitis C virus; Core region; Hepatocellular carcinoma; Interferon; Sustained virological response.

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Abbreviations: HCV, hepatitis C virus; IFN, interferon; HCC, hepatocellular carcinoma; SVR, sustained virological response; DW-type, double wild-type; RR, risk ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase; US, ultrasonography; CT, computed tomography; MRI, magnetic resonance imaging; PCR, polymerase chain reaction; OR, odds ratio.

Introduction

Hepatitis C virus (HCV) infection is a global health problem and the number of chronic carriers worldwide is estimated at 170 million [1]. HCV causes chronic hepatitis, which may progress to liver cirrhosis and hepatocellular carcinoma (HCC); the speed of disease progression, though, varies among patients [2,3]. Age, gender, steatosis, liver fibrosis, and response to interferon (IFN) therapy are reported to be associated with disease progression and HCC development [4–7]. HCV has six major genotypes, of which genotype 1 is most common in Japan and reported to be associated with increased severity and progression of chronic liver disease [8,9]. HCV contributes to HCC by directly modulating the pathways promoting the malignant transformation of hepatocytes [10–13]. Studies on transgenic mice revealed that the HCV core protein has oncogenic potential [14], but other studies yielded conflicting results [15,16]. Recently, mutations at amino acids 70 and 91 in the core region were shown to predict virological response to therapy with IFN plus ribavirin and also HCC development [17–19]. However, few studies support these results, and hence, the clinical impact of core mutations on HCC development is still unclear. In order to determine the viral factors associated with HCC development, we performed a retrospective cohort study on 361 patients with chronic liver disease caused by HCV genotype 1 infection and analyzed the amino acids present at core residues 70 and 91. Additionally, we evaluated whether these mutations were associated with IFN treatment, cirrhosis development, or host factors like age and gender.

Patients and methods

Study population

We enrolled 361 consecutive HCV genotype 1-infected patients who had undergone liver biopsy between August 1986 and June 1998 at Chiba University Hospital. At the enrollment time, the absence of HCC was proven by abdominal ultrasonography (US), computed tomography (CT), or magnetic resonance imaging (MRI). All the patients tested positive for anti-HCV antibody, determined by second-generation enzyme-linked immunosorbent assay. Patients with chronic hepatitis B, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, Wilson disease, or alcoholic liver disease were excluded, as were patients with a history of alcoholism, drug abuse, or IFN therapy. Written informed consent was obtained from all patients before performing liver biopsy.

Table 1. Baseline characteristics of 361 hepatitis C (HCV) genotype 1-infected patients according to hepatocellular carcinoma (HCC) development.

Patients	$n = 361$	HCC development		
		(+), $n = 82$	(−), $n = 279$	p value
Gender (male/female)	219/142	56/26	163/116	0.1
Age (years)	50.5 ± 12.2	56.8 ± 7.1	48.6 ± 12.7	<0.0001
BMI (kg/m^2)	23.1 ± 2.9	23.1 ± 2.8	23.1 ± 3.3	0.82
Staging of fibrosis (F0–1/F2/F3/F4)	197/59/52/53	13/18/23/28	184/41/29/25	<0.0001
IFN treatment and response				
SVR/non-SVR/non-IFN	73/202/86	4/55/23	69/147/63	0.0004
Laboratory data				
AST (IU/L)	87 ± 62	109 ± 59	80 ± 61	0.0001
ALT (IU/L)	125 ± 93	139 ± 80	121 ± 96	0.13
AST/ALT	0.75 ± 0.26	0.84 ± 0.28	0.73 ± 0.25	0.0003
Platelets (10^4/mm^3)	17.7 ± 6.7	13.0 ± 3.3	18.2 ± 6.9	<0.0001
Albumin (g/dL)	4.2 ± 0.36	4.1 ± 0.39	4.3 ± 0.35	<0.0001
Total bilirubin (mg/dL)	0.8 ± 0.6	0.9 ± 0.3	0.8 ± 0.6	0.39
Core protein (pg/mL)	201 ± 245	283 ± 273	177 ± 231	0.001
Amino acid pattern				
70 Wild/non-wild/ND	168/129/64	32/32/18	136/97/46	0.23*
91 Wild/non-wild/ND	139/158/64	28/36/18	111/122/46	0.58*
DW/non-DW/ND	81/216/64	12/52/18	69/164/46	0.08*

BMI, body mass index; DW, double wild (arginine at residue 70 and leucine at residue 91 in the core region); ND, not detected; ND cases were excluded.

The clinical backgrounds of the patients are shown in Table 1. The study population was predominantly male (59% men), and the mean age of the patients was 50.5 ± 12.2 years, with 15% patients having liver cirrhosis.

Laboratory examination

Serum samples were obtained and stored at −30 °C until analysis. We assumed that genotype 1 corresponds to group 1 when determining the HCV RNA genotypes by serologic grouping of serum antibodies [20]. The serum HCV load of the patients was determined at the time of liver biopsy, using the HCV core protein detection kit (Eiken Chemical, Tokyo, Japan; detection limit, 8 pg/mL) [21].

Histopathological examination

Percutaneous liver biopsy was performed, and specimens were histopathologically assessed as described previously [22]. According to the criteria of Desmet et al. [23], the staging of fibrosis was defined as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), and F4 (cirrhosis).

Core nucleotide sequences

HCV RNA was extracted from the serum samples obtained at the time of liver biopsy, and it was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Nucleic acids were amplified by PCR with the

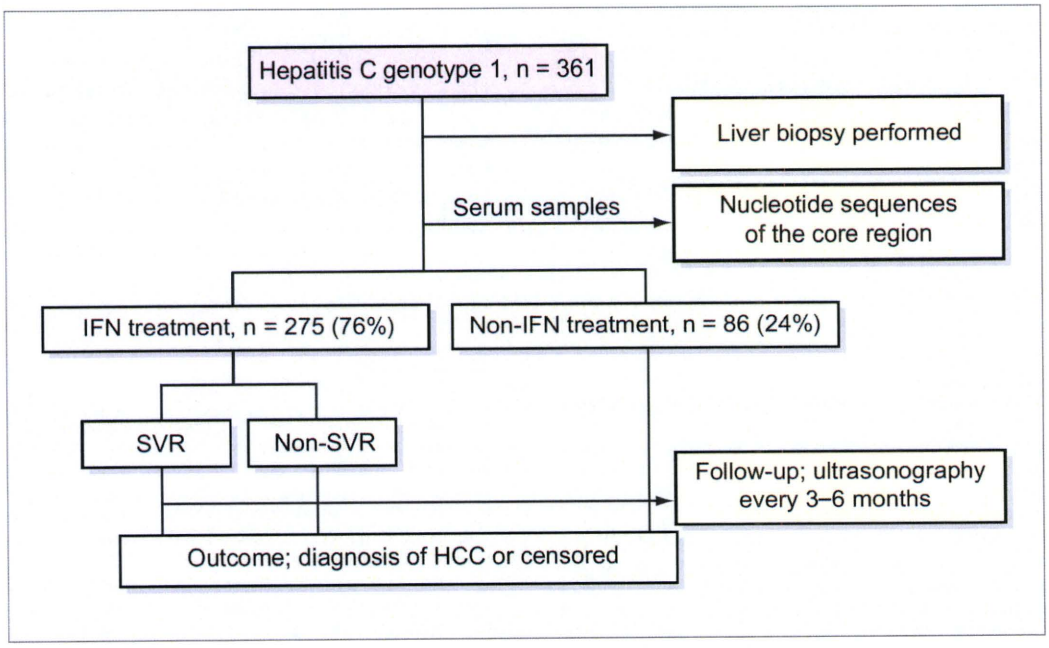

Fig. 1. Clinical courses after enrollment and the evaluation methods. IFN, interferon; SVR, sustained virological response; HCC, hepatocellular carcinoma. [This figure appears in colour on the web.]

HotStart Taq Master Mix kit (Qiagen, Hilden, Germany) and primers that have been previously described [24]. Polymerase chain reaction (PCR) was initiated with a denaturation step at 95 °C for 15 min, followed by 45 cycles at 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 3 min, and subsequent extension for 7 min. PCR products were resolved by agarose gel electrophoresis, purified using the QIA quick PCR purification kit (Qiagen), and directly sequenced using a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Tokyo, Japan). The sequences were determined using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

As described previously, the double wild-type (DW-type) amino acid pattern was defined as the presence of arginine at residue 70 (wild-type) and leucine at residue 91 (wild-type) [19].

IFN treatment

Depending on whether IFN was administered, the patients were divided into the IFN (76%) and non-IFN groups (24%) (Fig. 1). Patients who received IFN monotherapy during follow-up were divided into two subgroups: the sustained virological response (SVR) group, including patients who tested negative for HCV RNA at 24 weeks after completion of therapy, and non-SVR group (Fig. 1). Of the 275 patients in the IFN group, 73 (26.5%) achieved SVR.

Follow-up and diagnosis of cirrhosis and HCC

Clinical assessments were performed at least once every month during IFN treatment and every 3–6 months after the treatment. During follow-up, abdominal US was performed every 3–6 months to determine whether HCC had developed (Fig. 1). If necessary, additional procedures like CT, MRI, abdominal angiography, and US-guided tumor biopsy were performed to confirm HCC development. We also evaluated whether cirrhosis had developed in non-cirrhotic patients (F0–F2 stage). Cirrhosis was diagnosed according to the criteria of cirrhosis as described previously [25,26]. The follow-up period was the duration from the initial liver biopsy to HCC diagnosis or the last follow-up visit. For non-cirrhotic patients, this was the duration from the start point to cirrhosis diagnosis.

Statistical analysis

The χ^2 test was used to compare categorical variables, and Student's t test to compare continuous variables related to background characteristics among groups. Continuous variables were expressed as mean ± standard deviation. The cumulative incidence of HCC and cirrhosis was calculated using the Kaplan–Meier method and evaluated using the Breslow–Gehan–Wilcoxon test. Multivariate analysis was performed using the Cox proportional-hazards model or multiple logistic regression analysis. The Cochran–Armitage trend test was used for analyzing the association between the prevalence of mutation and subject age. Statistical significance was defined as $p < 0.05$.

Results

Cumulative HCC incidence

During follow-up (median duration, 121 months; range, 8–257 months), 82 (22.7%) patients developed HCC [HCC group; 13 of 197 (6.6%) from F0–F1, 18 of 59 (30.5%) from F2, 23 of 52 (44.2%) from F3, and 28 of 53 (52.8%) from F4 stage at entry] and 279 (77.3%) did not (non-HCC group). The cumulative HCC incidence at 5, 10, and 15 years of follow-up was 9.5%, 22.9%, and 30.9%, respectively.

Core nucleotide sequences

The core nucleotide sequence was determined for 297 of 361 (82.3%) patients. In the entire patient group, the proportions of DW-type and non-DW-type patterns were 22% and 60%, respectively (Table 1).

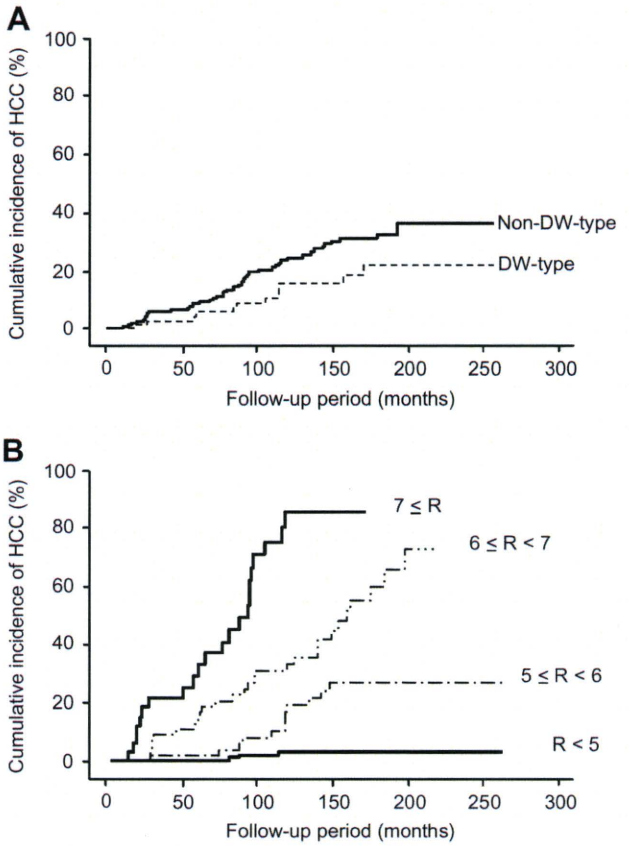

Fig. 2. Cumulative incidence of hepatocellular carcinoma (HCC) in hepatitis C genotype 1-infected patients. (A) Comparison between patients with double wild-type (DW-type: arginine, residue 70; leucine, residue 91) ($n = 81$) and non-DW-type ($n = 216$) amino acids in the core region ($p = 0.06$). (B) Comparison based on risk score (R) calculated using independent variables for HCC risk ($p < 0.0001$).

The core nucleotide sequence could not be determined for 64 patients because their samples showed significantly lower levels of the HCV core protein than those obtained from the 297 patients in whom the core sequence could be detected (119 vs. 217 pg/mL; $p = 0.0083$). There was no significant difference between the other variables shown in Table 1.

Cumulative HCC incidence according to core amino acid mutations

During follow-up, 12 of 81 (14.8%) patients with the DW-type pattern and 52 of 216 (24.1%) patients with the non-DW-type pattern developed HCC. Cumulative HCC incidence was 6.8% and 11% at 5 years, 19.1% and 27.7% at 10 years, and 26.6% and 38% at 15 years in the DW-type and non-DW-type groups, respectively. Cumulative HCC incidence in the DW-type group tended to be lower than that in the non-DW-type group ($p = 0.06$; Fig. 2A).

Predictive factors associated with HCC development

Potential predictive factors associated with HCC development are shown in Table 1. Univariate analysis revealed 10 parameters correlating with HCC development (Table 1). Multivariate analy-

Table 2. Factors associated with hepatocellular carcinoma development in hepatitis C genotype 1-infected patients, identified by multivariate analysis using the Cox proportional-hazards model.

Factor*	Category	Risk ratio (95% CI)	*p* value
Gender	Male	3.97 (2.05–7.63)	<0.0001
	Female	1.0	
Age (years)	⩾50	2.08 (1.01–4.33)	0.049
	<50	1.0	
Staging of fibrosis	⩾2	5.75 (2.68–12.35)	<0.0001
	<2	1.0	
IFN treatment and response	Absence of SVR	10.0 (2.29–43.48)	0.002
	SVR	1.0	
AST (IU/L)	>90	2.08 (1.20–3.62)	0.009
	⩽90	1.0	
AST/ALT	⩾0.8	2.21 (1.24–3.97)	0.007
	<0.8	1.0	
Amino acid pattern	Non-DW	1.96 (1.02–3.76)	0.04
	DW	1.0	

CI, confidence intervals; DW, double wild (arginine at residue 70 and leucine at residue 91 in the core region).
*Significant factors are shown.

sis with the Cox proportional-hazards model showed that the following seven independent parameters were significantly associated with HCC development: male gender ($p < 0.0001$), age ⩾ 50 years ($p = 0.049$), fibrosis ⩾ F2 ($p < 0.0001$), absence of SVR ($p = 0.002$), aspartate aminotransferase (AST) level > 90 IU/L ($p = 0.009$), AST/alanine aminotransferase (ALT) ratio ⩾ 0.8 ($p < 0.007$), and non-DW-type pattern in the core region ($p = 0.04$) (Table 2).

Prediction of HCC development based on risk score

Using the predictive variables from the previous step (Table 2), the risk score (R) for HCC development was calculated from the beta coefficients derived from the Cox proportional-hazards model as follows: $R = 0.671 \times$ (non-DW-type) $+ 2.307 \times$ (absence of SVR) $+ 0.733 \times$ (AST > 90 IU/L) $+ 0.733 \times$ (age ⩾ 50 years) $+ 1.752 \times$ (staging of fibrosis ⩾ 2) $+ 1.378 \times$ (male) $+ 0.795 \times$ (AST/ALT ⩾ 0.8) (each variable: yes = 1, no = 0). Fig. 2B shows the cumulative HCC incidence of four subgroups categorized by risk score, and the RR of each group is shown in Table 3. The cumulative HCC incidence increased with the risk score: from highest to lowest it was 84.7%, 35.1%, 18.5%, and 3.0% at 10 years.

Cumulative HCC incidence according to IFN treatment and response

During follow-up, 4 (5.5%) patients in the SVR, 55 (27.2%) in the non-SVR, and 23 (26.7%) in the non-IFN groups developed HCC; cumulative HCC incidence was 0%, 11.3%, and 13.2%, respectively, at 5 years; 7.8%, 25.6%, and 27.3%, respectively, at 10 years; and 7.8%, 36.5%, and 35.5%, respectively, at 15 years. Moreover, cumulative HCC incidence was significantly lower in the SVR group than other groups ($p < 0.001$; Fig. 3).

Table 3. Relative risk of HCC development based on risk score, using the Cox proportional-hazards model.

Score (R)	Risk ratio (95% CI)	*p* value
$R < 5$	1	
$5 \leqslant R < 6$	9.22 (2.60–32.7)	0.0006
$6 \leqslant R < 7$	26.9 (8.15–89.0)	<0.0001
$7 \leqslant R$	88.3 (25.8–302)	<0.0001

CI, confidence intervals.

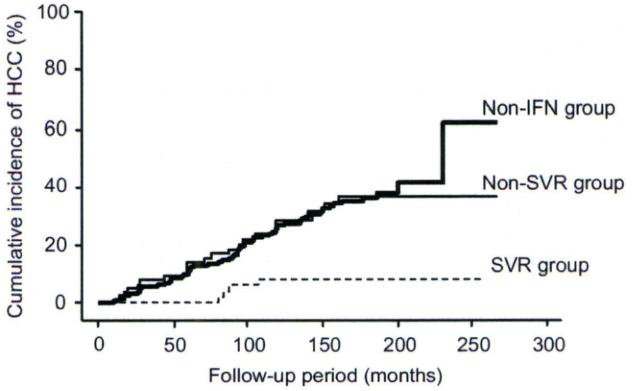

Fig. 3. Cumulative incidence of hepatocellular carcinoma (HCC). Comparison between the sustained virological response (SVR) ($n = 73$), non-SVR ($n = 202$), and non-interferon (IFN) ($n = 86$) groups ($p = 0.002$).

Analysis of SVR-associated factors

Compared to those in the non-IFN group, patients in the IFN group were younger (49 years vs. 54 years, $p = 0.003$), had higher aminotransferase levels (AST, 93 vs. 68 IU/L, $p = 0.001$; ALT, 137 vs. 87 IU/L, $p < 0.0001$) and lower core protein levels (183 vs. 263 pg/mL, $p = 0.01$). Table 4 shows baseline characteristics of patients according to interferon response. Univariate analysis revealed six SVR-associated parameters, whereas multiple logistic regression analysis revealed two independent significant predictors of SVR: AST/ALT ratio of <0.8 [$p = 0.005$; odds ratio (OR), 3.09; 95% confidence interval (CI), 1.40–6.82] and core protein level of <200 pg/mL [$p < 0.0001$; OR, 70.94; 95% CI, 9.56–526.2]. However, both univariate ($p = 0.64$) and multivariate analyses (data not shown) showed that the DW-type pattern in the core region was not associated with SVR.

Table 4. Baseline characteristics of patients according to interferon response.

Nature of the Regime	SVR $n = 73$	Non-SVR $n = 202$	*p* value
Gender (Male/Female)	47/26	126/76	0.76
Age (years)	46.6 ± 13.3	50.5 ± 11.5	0.02
BMI (kg/m^2)	22.7 ± 2.8	23.2 ± 3.0	0.24
Staging of fibrosis: (F0-1/F2/F3/F4)	45/12/9/7	104/34/34/30	0.42
Laboratory data			
AST (IU/L)	79 ± 56	97 ± 69	0.048
ALT (IU/L)	132 ± 92	139±100	0.60
AST/ALT	0.65 ± 0.22	0.75 ± 0.27	0.003
Platelets (10^4/mm^3)	18.6 ± 6.7	16.7 ± 6.1	0.03
Albumin (g/dL)	4.3 ± 0.3	4.2 ± 0.4	0.06
Total bilirubin (mg/dL)	0.7 ± 0.4	0.8 ± 0.4	0.02
Core protein (pg/mL)	31 ± 50	234 ± 226	<0.0001
Amino acid pattern			
70 Wild/Non-wild/ND	35/21/17	89/74/39	0.30
91 Wild/Non-wild/ND	24/32/17	76/87/39	0.62
DW/Non-DW/ND	14/42/17	46/117/39	0.64

BMI, body mass index; DW, double wild (arginine at residue 70 and leucine at residue 91 in the core region); ND, not detected.

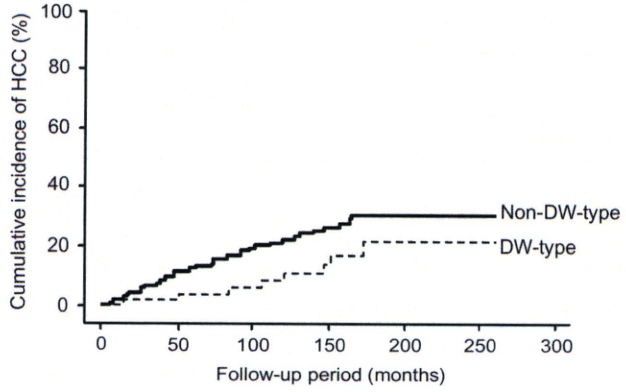

Fig. 4. Cumulative incidence of cirrhosis in non-cirrhotic patients (F0–F2). Comparison between patients with double wild-type (DW-type: arginine, residue 70; leucine, residue 91) ($n = 81$) and non-DW-type ($n = 216$) amino acids in the core region ($p = 0.051$).

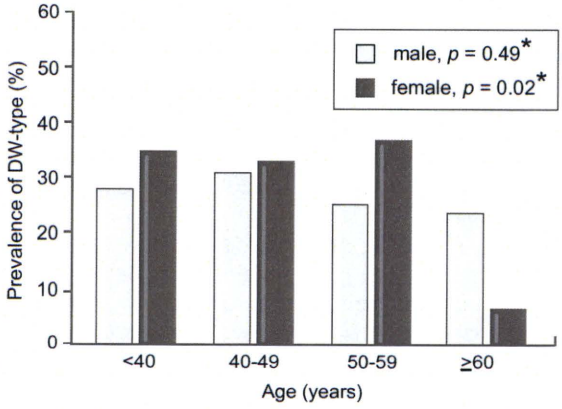

Fig. 5. Prevalence of double wild-type (DW-type: arginine, residue 70; leucine, residue 91) amino acids in the hepatitis C core region according to age and gender. *By the Cochran-Armitage trend test.

Cumulative cirrhosis incidence for non-cirrhotic patients (F0–F2)

Of the 256 non-cirrhotic patients (197 from F0–F1, 59 from F2), 50 (19.5%) developed cirrhosis (cirrhosis group) and 206 (80.5%) did not (non-cirrhosis group). The cumulative cirrhosis incidence at 5, 10, and 15 years of follow-up was 9.7%, 18.2%, and 26.4%, respectively. The HCC incidence was higher in the cirrhosis group [23/50 (46%)] than the non-cirrhosis group [8/206 (3.9%); $p < 0.0001$]. In the entire population, 71 of 82 (86.6%) patients who developed HCC had underlying cirrhosis and 11 (13.4%) did not, when HCC was detected ($p < 0.0001$).

Cumulative cirrhosis incidence according to the amino acid pattern in the core region for F0–F2 patients

The cumulative cirrhosis incidence tended to be higher in the non-DW-type group than the DW-type group (11.9% and 3.6% at 5 years, 21.5% and 10.4% at 10 years, and 29.7% and 20.7% at 15 years of follow-up, respectively; $p = 0.051$; Fig. 4).

Analysis of factors associated with cirrhosis development in F0–F2 patients

We analyzed the factors associated with cirrhosis development in patients with F0–F2 fibrosis at enrollment. Univariate analysis revealed nine parameters correlating with cirrhosis development: male gender ($p = 0.04$), older age ($p < 0.0001$), advanced fibrosis ($p < 0.0001$), absence of SVR ($p < 0.0001$), high AST level ($p < 0.0001$), high ALT level ($p = 0.01$), high AST/ALT ratio ($p = 0.001$), low platelet count ($p = 0.0009$), and high core protein level ($p = 0.02$). Multivariate analysis, including analysis of the amino acid pattern in the core region with the Cox proportional-hazards model, showed that the following three independent parameters were significantly associated with cirrhosis development: male gender ($p = 0.004$), fibrosis = F2 ($p = 0.004$), and absence of SVR ($p = 0.02$). Meanwhile, the presence of the non-DW-type pattern in the core region tended to lead to cirrhosis development (RR, 2.13; 95% CI, 0.93–4.91; $p = 0.07$).

Analysis of factors associated with mutations at core residues 70 and 91

Eighty-one patients with the DW-type pattern at core residues 70 and 91, who were at low risk for HCC, tended to be younger than the 216 patients with the non-DW-type pattern, who were at high risk for HCC (48.4 ± 11.8 years vs. 51.1 ± 11.8 years, respectively; $p = 0.08$). Separate analysis of men and women (Fig. 5) showed that the DW-type pattern was rare in women aged 60 years or above ($p = 0.02$).

Consistent with these results, HCC incidence was the same in men and women aged 60 or above (19% vs. 10% at 5 years and 32% vs. 38% at 10 years of follow-up, respectively; $p = 0.89$); however, in patients aged less than 60 years, HCC incidence was lower in women than in men (4% vs. 11% at 5 years and 15% vs. 22% at 10 years of follow-up, respectively; $p = 0.03$).

Discussion

Male gender, older age, advanced-stage fibrosis, and no IFN treatment are reported as important predictors of HCC development in chronic hepatitis C patients [4–7]. Viral factors associated with HCC development were also reported [27–29]. Several studies showed that mutations in the core protein are associated with HCC among HCV genotype 1b-infected patients, but the results varied between studies [18,30,31]. Consistent with a report by Akuta et al. [18], we showed that the presence of the non-DW-type pattern at core residues 70 and 91 is an independent risk factor for HCC development. Akuta et al. [18] studied 313 chronic hepatitis C patients who received IFN therapy (101 were excluded), and found that non-DW-type was an independent risk factor for HCC development (RR, 5.92; 95% CI, 1.58–22.2; $p = 0.008$) by using the Cox proportional-hazards model, and its correlation with HCC risk was stronger than that found in our study (RR, 1.96; 95% CI, 1.02–3.76; $p = 0.04$). We analyzed cirrhotic patients (14.7% of total population), most of whom developed HCC, and also non-cirrhotic patients, and found that the non-DW-type was still an independent risk factor for HCC development (RR, 2.90; 95% CI, 1.11–7.61; $p = 0.03$). Furthermore, we

found that the non-DW-type in patients with F0–F2 fibrosis was likely to lead to cirrhosis, diagnosed by US ($p = 0.051$). Moreover, the non-DW-type in patients with F0–F3 fibrosis was significantly associated with cirrhosis development ($p = 0.007$, data not shown). These results suggest that the non-DW-type may affect HCC development by accelerating cirrhosis development; however, prospective studies of histological findings are needed to confirm this.

It is unclear why the amino acids at residues 70 and 91 affect HCC development. The core protein cooperates with the Ras oncogene and transforms primary rat embryo fibroblasts into the tumorigenic phenotype [10]. The HCV core protein (residues 25–91) also interacts with the heterogeneous nuclear ribonucleoprotein K, which stimulates the c-myc promoter, downstream of the Wnt/beta-catenin signal [11]. Pavio et al. reported that the HCV core (residues 59–126, residues at 70 and 91 were non-wild-type) interacts with Smad3 and inhibits the TGF-beta pathway, important in apoptosis [12]. Mutations in the clustering variable regions (residues 39–76) are often seen in HCC patients [30], and mutations in the *N*-myristoylation sites (e.g., residue 91) in the core region, are associated with growth control and virus replication [31]. Delhem et al. have shown that the core protein with non-wild-type amino acids at residues 70 and 91 obtained from a HCC patient binds and activates PKR, which might cause carcinogenesis [13]. It was reported that the presence of a non-wild-type amino acid at residue 91 enhances internal initiation of HCV protein synthesis, leading to the expression of a core isoform, which may interact with viral and cellular components [32]. These results suggest that residues 70 and 91 themselves or via interactions with adjacent amino acids may be involved in HCC development; however, further studies are needed to evaluate the effect of core mutations on HCC development.

The presence of the DW-type pattern in the core region is also reportedly a predictor of the virological response to therapy with peginterferon and ribavirin [19]. With this therapy, an SVR of approximately 50% could be achieved by HCV genotype 1-infected patients having high viral load. We found the absence of an SVR and the non-DW-type pattern to be predictors of HCC development; however, the non-DW-type pattern was not a predictor of the absence of an SVR. This may be partly because we used IFN monotherapy without ribavirin, with which the SVR rate (26.5% in our study) was lower than that with peginterferon plus ribavirin [33,34]. Therefore, we believe that combination therapy, rather than IFN monotherapy, would more efficiently eradicate HCV with the DW-type pattern in the core region; however, further studies are required to test this hypothesis. Our current focus is on a prospective study to examine the association between core mutations and the outcome of combination treatment with peginterferon plus ribavirin.

Our study revealed that the DW-type pattern, associated with a low HCC risk, was rare in women aged 60 years or above. This may explain why HCC incidence in women was as high as that in men. The underlying mechanisms by which age or gender influence core-region mutations are unknown. In previous studies, a mutation at residue 70 was correlated with virological response to therapy with IFN plus ribavirin [17] and with AFP levels [35] in HCV genotype 1b-infected patients without HCC. Further follow-up studies must examine whether a mutation occurs in the wild-type amino acid.

We investigated two specific amino acid mutations in the HCV core region by direct sequencing. The HCV core sequence can be easily amplified using PCR because of its conservative nature and analysis of only two amino acid positions is timesaving; therefore, this method might be feasible for identifying predictive markers for HCC. A specific PCR method for detecting these mutations was reported [36]. Furthermore, we developed a rapid and sensitive real-time PCR method for quantitatively detecting these mutations [37]. We hope this method can be used to detect HCV sequences in case of a low viral load, and believe that it will be more useful for predicting HCC.

In conclusion, HCC risk could be predicted by studying mutations in the HCV core region, response to IFN, and host factors like age, gender, and liver fibrosis in HCV genotype 1-infected patients. These mutations might be involved in an oncogenic mechanism leading to HCC development in chronic HCV patients.

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ORIGINAL ARTICLE

Risk of Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Virus Infection

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Abstract

Objective. To determine the risk factors for the occurrence of hepatocellular carcinoma (HCC) in patients with hepatitis B virus (HBV) infection. ***Material and methods.*** A total of 620 patients who tested positive for hepatitis B surface antigen and were referred to Chiba University Hospital between February 1985 and March 2008 were included in the study and the following characteristics were analyzed: age, gender, status of hepatitis B e antigen, alanine aminotransferase level, HBV DNA level, and number of platelets (PLTs). ***Results.*** HCC was detected in 30 cases during the follow-up period (5.4 ± 5.1 years). Multivariate analysis revealed that age >40 years [compared with patients aged <40 years; odds ratio (OR) = 4.28; 95% confidence interval (CI) = 1.68–10.9] and PLT level <206,000/μl (compared with patients with a higher PLT level; OR = 8.50; 95% CI = 1.98–36.2) were predictive factors for HCC occurrence. In patients aged >40 years, the HBV DNA level (compared with <5.0 log copies/ml; OR = 4.22, 95% CI = 1.13–15.8) and PLT level (compared with patients with >196,000/μl PLTs; OR = 15.6, 95% CI = 2.06–118.3) were predictive factors for HCC occurrence. ***Conclusions.*** Advanced age and low PLT level were risk factors for HCC occurrence in patients with HBV infection. In patients aged >40 years, viral load was also a risk factor for HCC.

Key Words: *Hepatitis B virus, hepatocellular carcinoma*

Introduction

The clinical course of patients with hepatitis B virus (HBV) infection varies considerably [1]. Therefore, long-term follow-up studies of patients with HBV infection are quite complex and difficult. In most of the patients, the disease is either non-progressive or shows a slow progression and is usually accompanied by the loss of serum HBV DNA after seroconversion of hepatitis B e antigen (HBeAg) [2]. Some patients show continuous elevation of the alanine aminotransferase (ALT) level, which leads to cirrhosis [3]. HBV infection is also associated with an increased risk of developing hepatocellular carcinoma (HCC), which is one of the most common human cancers and causes of death. Although previous studies have attempted to determine factors influencing the prognosis of patients with HBV infection, the key factors remain to be identified. Recent studies have indicated that the serum level of HBV DNA correlates with the progression of liver diseases [1,4–6]. However, viral load alone cannot predict the occurrence of HCC in the future [7]. In this study, multivariate analyses of the risk factors for HCC occurrence were performed for data obtained from 620 patients with HBV infection who were referred to a single institute in Japan.

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Material and methods

Patients

This was a retrospective analysis. The study was approved by the ethical committee of Chiba University and written informed consent was obtained from all the patients. Of the hepatitis B surface antigen (HBsAg)-positive carriers ($n = 676$) who were referred to Chiba University Hospital between February 1985 and March 2008, those who tested positive for hepatitis C virus (HCV) antibody (anti-HCV) or had autoimmune liver disease and those who had another potential cause of chronic liver disease were excluded. The characteristics of the excluded HBsAg-positive carriers were as follows: anti-HCV positivity in 12, autoimmune liver disease in four and primary biliary cirrhosis in one. Five patients who had previously received lamivudine treatment were also excluded. Thirty-nine patients consulted a physician only once and were excluded from further analysis. Thus, a total of 620 patients were further analyzed. Serum samples were collected during diagnosis and stored at –20°C until analysis.

Serologic markers, HBV DNA quantitative assay, and genotyping

HBsAg, HBeAg, and anti-HBe levels were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratories, Chicago, IL) and anti-HCV was also measured by ELISA (Ortho Diagnostics, Tokyo, Japan). Serum HBV DNA levels were quantified by polymerase chain reaction (PCR) assay (Amplicor HBV Monitor; Roche Diagnostics, Basle, Switzerland); the linear range of this assay was 2.6–7.6 log copies (LC)/ml. The six major genotypes of HBV (A–F) were determined by EIA (HBV Genotype EIA; Institute of Immunology Co., Ltd., Tokyo, Japan). Aspartate aminotransferase (AST), ALT, and the number of platelets were determined and the aminotransferase to platelet ratio index (APRI) was calculated [8].

Statistical analysis

The baseline data are presented as mean ± SD. The difference in the values of clinical parameters between the two groups was analyzed by unpaired t-test, Welch's t-test, and chi-square test. The Cox proportional hazards model was used to identify factors predictive of HCC occurrence using the SPSS version 16.1 software package (SPSS Inc., Chicago, IL).

Results

Demographic characteristics of HCC and control patients

None of the study participants had HCC at entry. In total, 30 incident HCC cases (HCC group) occurred during the follow-up period. During the follow-up period, most of the patients were re-evaluated at least once a year for liver function and detection of HCC. Screening for detection of HCC was performed on the basis of typical findings of abdominal ultrasonography, dynamic CT, angiography, and/or MRI. For all patients suspected of having HCC by imaging analysis, the diagnosis of HCC was confirmed by pathological analysis. If the patient had HCC or was being treated with an antiviral drug (lamivudine or entecavir), we terminated the follow-up. At baseline, significant differences were observed in age, gender, status of HBeAg, ALT and HBV DNA levels, number of platelets (PLTs), and APRI between the HCC ($n = 30$) and control ($n = 590$) groups (Table I). The 590 patients in whom HCC was not detected during the follow-up period constituted the control group. The average follow-up period was 5.1 ± 4.1 and 5.4 ± 5.2 years in the HCC and control groups, respectively, and this difference was not significant.

Patients with HBV

The differences in age, sex, PLT and ALT levels, status of HBeAg, and HBV DNA level between the HCC and control groups were investigated. We defined threshold levels as age 40 years, HBV DNA 5.3 LC/ml, ALT 72.9 IU/l, and PLTs 206,000/μl according to the average data of all patients. Univariate analysis revealed that age, number of PLTs, and HBV DNA level at baseline were predictive factors for HCC occurrence. Multivariate analysis revealed that age >40 years [compared with patients aged <40 years; odds ratio (OR) = 4.28; 95% confidence interval (CI) = 1.68–10.9] and PLT level < 206,000/μl (compared with patients with a higher PLT level; OR = 8.50, 95% CI = 1.98–36.2) were predictive factors for HCC occurrence (Table II). Thus, these analyses revealed that age and PLT level were the most important factors influencing future occurrence of HCC. Kaplan–Meier curves were constructed for age ($P < 0.0001$; log-rank test; Figure 1a), PLT level ($P < 0.0001$; log-rank test; Figure 1b), and HBV DNA (P = NS; log-rank test; Figure 1c). Next, we categorized the HBV patients into two subgroups according to the thresholds of age and PLT level based on the average data, and performed further analysis. Because there was only one HCC patient aged <40 years and

Table I. Characteristics of study subjects and their association with HCC.

Parameter	Group			*P*
	Total	HCC	Controls	
No. of patients	620	30	590	
Gender; *n* (%)				<0.001[a]
Male	364 (59)	20 (67)	344 (58)	
Female	256 (41)	10 (33)	246 (42)	
Age (years); mean ± SD	40.0 ± 14.2	50.0 ± 11.6	40.0 ± 14.2	<0.001[b]
HBeAg status; *n* (%)				<0.001[a]
Positive	269 (43)	17 (57)	252 (43)	
Negative	351 (57)	13 (43)	338 (57)	
HBV DNA (LC/mL); mean ± SD	5.3 ± 2.0	6.4 ± 1.3	5.3 ± 2.0	0.002[b]
ALT (IU/l); mean ± SD	72.9 ± 89.3	105.0 ± 129.3	71.0 ± 86.6	0.041[c]
PLTs (/μl); mean ± SD	206,000 ± 66,000	130,000 ± 51,160	210,000 ± 64,410	<0.001[c]
APRI > 0.5; *n* (%)	294 (47.4)	27 (90)	267 (45.3)	<0.001[a]
Interval between two consecutive visits (years); mean ± SD	5.4 ± 5.1	5.1 ± 4.1	5.4 ± 5.2	NS[c]
Genotype A/B/C/D/not determined; *n*	7/38/333/0/242	1/0/24/0/5	6/38/309/0/237	NS[a]

[a]Chi-square test.
[b]Welch's *t*-test.
[c]Unpaired *t*-test.

only two cases had a PLT level >206,000/μl, we did not analyze these groups.

Analysis of the subgroup of HBV patients aged > 40 years

HCC was detected in 29 patients in the group aged >40 years (n = 372). Significant differences were observed in the status of HBeAg, HBV DNA, and PLT levels at baseline between the HCC (n = 29) and control groups (n = 343). The average follow-up period was 5.1 ± 4.1 and 5.0 ± 4.7 years in the HCC and control groups, respectively, and this difference was not significant. We defined thresholds as age 49 years, HBV DNA 5.0 LC/ml, ALT 66.0 IU/l, and PLTs 196,000/μl, according to the average data for the patients aged >40 years. The risk factors for HCC occurrence in patients aged >40 years were analyzed by Cox regression analysis. Univariate analysis revealed that ALT, PLT, and HBV DNA levels at baseline were predictive factors for HCC occurrence. Multivariate analysis revealed that the HBV DNA

Table II. Multivariate analysis of risk factors associated with HCC in patients with HBV infection.

Risk factor	All patients[a]		Patients aged >40 years[b]		Patients with PLTs <206,000 /μl[c]	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Age	4.28 (1.68–10.9)	0.002	2.16 (0.88–5.29)	NS	1.75 (0.71–4.34)	NS
Male gender	1.48 (0.67–3.26)	NS	2.25 (0.86–5.90)	NS	1.43 (0.61–3.35)	NS
HBeAg-positive	1.34 (0.59–3.06)	NS	0.98 (0.41–2.33)	NS	1.06 (0.45–2.51)	NS
HBV-DNA	1.59 (0.62–4.13)	NS	4.22 (1.13–15.8)	0.032	1.20 (0.49–2.94)	NS
ALT	0.86 (0.40–1.87)	NS	1.44 (0.61–3.44)	NS	0.923 (0.40–2.11)	NS
PLTs	8.50 (1.98–36.2)	0.004	15.6 (2.06–118.3)	0.008	4.49 (1.62–12.5)	0.004

[a]The thresholds of age, HBV-DNA, ALT, and PLTs were defined as 40 years, 5.3 LC/ml, 72.9 IU/l, and 206,000 /μl, respectively.
[b]The thresholds of age, HBV-DNA, ALT, and PLTs were defined as 49 years, 5.0 LC /ml, 66.0 IU/l, and 196,000 /μl, respectively.
[c]The thresholds of age, HBV-DNA, ALT, and PLTs were defined as 42 years, 5.8 LC /ml, 84 IU/l, and 159,000 /μl, respectively.
HR = hazard ratio.

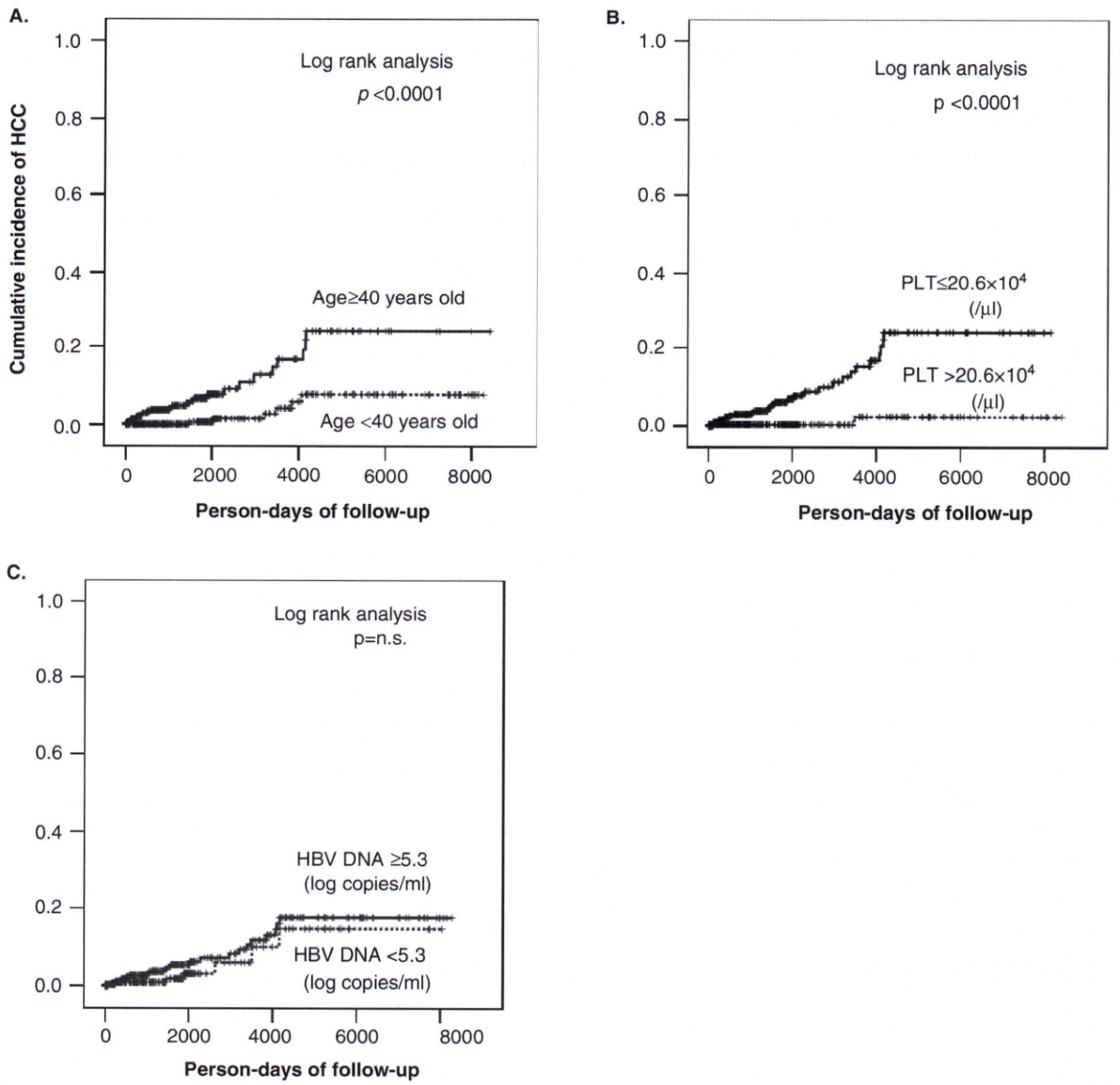

Figure 1. Cumulative occurrence of HCC based on (a) number of PLTs, (b) age, and (c) HBV DNA level. Thresholds for age, number of PLTs, and HBV DNA level were defined according to the average data for all patients. Dotted lines indicate the control group (high number of PLTs, younger age, and low HBV DNA level).

level (compared with < 5.0 LC/ml; OR = 4.22; 95% CI = 1.13–15.8) and PLT level (compared with > 196,000/μl; OR = 15.6; 95% CI = 2.06–118.3) were predictive factors for HCC occurrence (Table II). Kaplan–Meier curves were constructed for HBV DNA ($P = 0.001$; log-rank test; Figure 2).

Analysis of the subgroup of HBV patients with PLTs < 206,000/μl

HCC was detected in 28 patients in the group with PLTs < 206,000/μl ($n = 329$). The risk factors for HCC occurrence in the group with < 206,000/μl PLTs were analyzed by Cox regression analysis. Univariate analysis revealed that age and PLT level at baseline were predictive factors for HCC occurrence. Multivariate analysis revealed that PLT level (compared with patients with > 159,000/μl; OR = 4.49; 95% CI = 1.62–12.5) was the only predictive factor for HCC occurrence (Table II).

Discussion

In Japan, HBV infection is one of the most important factors determining HCC occurrence [9]. Moreover, HCC is one of the most important determinants for

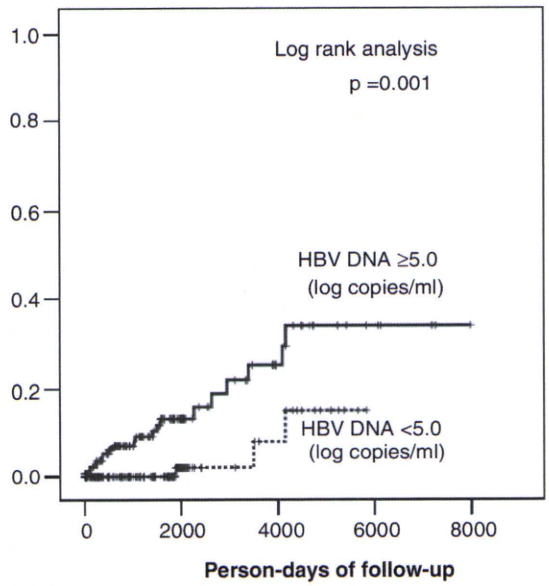

Figure 2. Cumulative occurrence of HCC based on the HBV DNA level in patients aged >40 years. The threshold for the HBV DNA level was defined according to the average data for the patients aged >40 years. A significant difference was observed by log-rank test. The dotted line indicates the control group (low HBV DNA level).

the prognosis of patients with HBV infection. In previous studies, factors associated with an increased risk of HCC among people with chronic HBV infection included demographic characteristics, lifestyle, and environmental, viral and clinical factors. Among these, male gender, older age, HBV genotype, cirrhosis, elevated ALT, and high viral load were found to be factors associated with HCC [6,10–19]. We focused on clinical factors which may be tested easily and for which tests are available all over the world. This report clarifies the relative risk for HCC in all patients with HBV who were referred to a single institute in Japan and provides important information for physicians.

In this study, the relative risk of HCC was found to be increased to 4.28 (95% CI 1.68–10.9) times higher for patients aged >40 years compared with those aged <40 years. In addition, a low PLT level, which indicates advanced fibrosis in the liver, including cirrhosis, was a risk factor for HCC: the relative risk was found to be increased to 8.50 (95% CI 1.98–36.2) times higher for patients with a PLT level <206,000/μl compared with higher levels. The HBV DNA level was not selected as a risk factor for HCC occurrence in all patients with HBV infection by multivariate analysis. Previous follow-up studies have shown that viral load is an important and independent factor for HCC occurrence [4,5,20]. However, in the present study, although various thresholds of HBV DNA level were used for analysis, none of the thresholds showed statistical significance in multivariate analysis (data not shown). In contrast, the analysis intended for patients aged >40 years revealed that high HBV viral load was added as a risk factor for HCC. By changing the threshold of HBV DNA from 4.5 to 5.3 LC/ml in 0.1-log increments, 5.0 or 5.1 LC/ml were found to be the best (data not shown); therefore we designated the threshold of HBV DNA level as >5.0 LC/ml. In our study, HBV carriers aged >40 years with HBV DNA levels >5.0 LC/ml had a 4.22-times higher risk of HCC compared to HBV carriers with lower viral loads. In previous studies in Japan regarding predictive factors for HCC, Ohata et al. [5] reported that age, HBV DNA, and staging of fibrosis were the important factors, while Murata et al. [21] reported that the number of PLTs was the only factor after HBeAg seroconversion. On the other hand, in an analysis of patients with liver cirrhosis in Japan, levels of HBV DNA and/or ALT were the predictive factors for HCC [12,19]. Taken together with the present study, these reports suggest that the HBV DNA level may not be an absolute factor for predicting HCC in the analysis, irrespective of the age of the patients and the number of PLTs, but that in patients with advanced age or low numbers of PLTs, indicating advanced fibrosis of the liver, HBV DNA could be a predictive factor for the occurrence of HCC. The PLT level negatively reflects the extent of liver fibrosis [22], therefore it is very difficult to achieve an improvement in liver fibrosis and to recover the PLT level concomitantly, but a high viral load can be lowered by antiviral drug treatment. Therefore, in patients aged >40 years, lowering the viral load using an antiviral drug might be an important way to avoid the occurrence of HCC but, in younger patients, lowering the HBV DNA level may not result in direct inhibition of HCC occurrence, although the activity of hepatitis could be suppressed.

The decrease in the number of PLTs in patients with liver disease reflects advanced fibrosis of the liver, which is strongly related to HCC occurrence. In fact, the patients in the HCC group of our study were suggested to show advanced fibrosis because they had higher values of APRI than the controls. In addition to being a marker of liver fibrosis, the influence of PLTs on cytotoxic T lymphocytes (CTLs) has been studied with keen interest. Chronic HBV infection is characterized by an inefficient CTL response, which often results in continuous destruction of hepatocytes. A recent study indicated that PLTs are required for virus-specific CTLs to accumulate within the liver and perform pathogenetic and/or antiviral roles [23]. In our study, low PLT number was a strong risk factor for HCC in all the HBV carriers, irrespective of age or PLT number at baseline. Especially in the HBV

carriers aged >40 years, low PLT number has the strongest association with HCC occurrence. Therefore, older HBV carriers with low PLT levels should be followed closely because of a high possibility of HCC occurrence, as for HCV carriers with low PLT levels [24].

The presence of HBeAg is often associated with active liver disease, whereas HBeAg seroconversion often coincides with loss of HBV DNA in serum, normalization of the ALT level, and clinical remission [25]. Spontaneous HBeAg seroconversion confers a good long-term outcome on most patients. In this study, the status of HBeAg at baseline differed significantly between the HCC and control groups; however, the status of HBeAg was not identified by univariate analysis as a predictive factor for HCC occurrence. From these results, we speculated that the HBe protein was not the direct precursor of HCC, although the HBe antigen status often reflects the replication of HBV DNA.

In this study, we evaluated parameters for predicting HCC only at first admission. A previous study reported that changes in ALT or HBV DNA levels during the follow-up period were important for predicting advanced liver disease and HCC [26]. We need to evaluate the importance of following changes in these parameters.

There was only one HCC patient aged <40 years. This patient was male and was followed up from the age of 27 years; his ALT, HBV DNA, and PLT levels and the status of HBeAg at baseline were 34 IU/l, 7.7 LC/ml, 203,000/μl, and positive, respectively. It was difficult to predict the occurrence of HCC in this case only on the basis of the risk factors for HCC indicated in this study. Hence, we need to find an adequate risk factor to predict HCC in such a case.

In conclusion, advanced age and low PLT level were the risk factors for HCC in patients with HBV infection, irrespective of the PLT level at baseline. In patients aged >40 years, viral load was added as a risk factor for HCC.

Declaration of interests: The authors indicated no potential conflict of interest.

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Ⅳ. 研究成果の刊行物・別冊

慢性肝炎の治療(C型)

treatment of chronic hepatitis C

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【概念】

C型肝炎ウイルス(HCV)は，感染すると自然に排除される可能性は低く，その多くが持続感染する．宿主の免疫応答は，多くの場合不完全でHCVは排除されることなく，壊死炎症反応が持続する．この結果が慢性肝炎であり，壊死炎症の継続の結果，線維化は進展し肝硬変へと移行する．特に，肝線維化の程度と肝発癌率は密接に関連しており，肝線維化の進展に伴い発癌率が急速に増加することから，慢性肝炎の段階での診断と適切な治療がきわめて重要である．

治療方針

治療の第1の目標はHCVの排除である．現在，HCVを排除することができるのはインターフェロン(IFN)を用いる抗ウイルス療法のみである．かつてのIFN単独療法では全体の1/3の患者でウイルス排除が可能であるにすぎなかったが，リバビリン(RBV)を併用することで格段に治療効果が高まっている．また，IFNにポリエチレングリコール(Peg)を結合させたPeg-IFNは週1回の投与で持続的な効果を発揮し，治療効果・認容性に優れ副作用も軽減されている．Peg-IFN + RBV併用療法はきわめて強力であるが，その一方で副作用が多く，なかには重篤なものも起こりうることから慎重な適応判断が望まれる．一方，ウイルス排除が困難な場合やIFNが無効の場合は，肝炎を沈静化し肝発癌抑止を目指した治療を行うことが必要である．

治療法

❶インターフェロン療法

a) **初回治療**：IFNをウイルス排除の目的で用いる場合には，予測される治療成績・副作用を考慮し，最も治療効果が得られる治療法を第1選択とし，年齢・性別・肝線維化の程度・合併症などの感染者(宿主)の条件を加味して決定する．治療成績を規定するウイルス側の因子としては，HCVのgenotype，ウイルス量が知られており，genotype 1b(セログループ1)では，さらにHCVの遺伝子変異(ISDR，コア領域70番，91番のアミノ酸置換)などが知られている．すなわちセログループ2型は1型よりIFN感受性が高く，ウイルス量は少ないと治療効果が高い．また，ISDR(インターフェロン感受性領域)内のアミノ酸変異数は多いほどIFN感受性が高く，コア領域70番・91番のアミノ酸置換があるとIFN感受性が劣る．

このような背景のもと，厚生労働省の研究班により，初回治療のガイドライン(表11-12)が示されている．ウイルス量は，現在一般的に用いられているreal time PCR法で5.0 log IU/mL以上は高ウイルス量とされ，genotype 1かつ高ウイルス量症例は最も難治であり，最も強力なPeg-IFNα-2b + RBVないしはPeg-IFNα-2b + RBV併用の48週間投与が推奨されるが，この治療法のウイルス排除率は40～50%程度にすぎない．一方，genotype 2では，この治療法を24週間行えば80～90%の高い治療効果が得られるほか，低ウイルス量症例では，RBV併用療法とIFN単独療法ではいずれも80～90%の高いSVR率で，差を認めないため，初回治療ではIFN単独療法が推奨される．また，治療効果を高めるために，治療開始12週後にHCV-RNA量が治療開始前の1/100