Determination of *IL28B* and *ITPA* genotypes. The *IL28B* rs8099917 and *ITPA* rs112735 genotypes have been reported as predictors of treatment efficacy and side effects to PEG-IFN–ribavirin dual therapy, and they were genotyped by using the Invader assay, TaqMan assay, or direct sequencing, as described previously (9–13).

Detection of amino acid substitutions in core and NS5A regions of HCV-1b. With the use of HCV-J (GenBank accession no. D90208) as a reference type (14), the sequence of amino acids (aa) 1 to 191 in the core protein of HCV-1b was determined and then compared with the consensus sequence constructed in a previous study to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and at aa 91 of leucine (Leu91) or methionine (Met91) (15). The sequence of aa 2209 to 2248 in the NS5A of HCV-1b (the interferon sensitivity-determining region [ISDR]) reported by Enomoto and coworkers (16) was determined, and the number of amino acid substitutions in the ISDR was defined as wild type (≤1) or non-wild type (≥2) compared to that of HCV-J. Furthermore, the sequence of aa 2334 to 2379 in the NS5A region of HCV-1b (the IFN/ribavirin resistance-determining region [IRRDR]) reported by El-Shamy and coworkers (17), including the sequence of aa 2356 to 2379 referred to as the variable region 3 (V3), was determined and then compared with the consensus sequence constructed in a previous study. The numbers of amino acid substitutions in the IRRDR and V3 regions were divided into two groups for analysis (those with ≤5 and ≥6 aa substitutions in the IRRDR, and those with ≤2 and ≥3 aa substitutions in the V3). In the present study, the amino acid substitutions of the core region and the NS5A-ISDR/IRRDR/V3 of HCV-1b were analyzed by direct sequencing.

Assessment of NS3/4A protease inhibitor-resistant variants. The genome sequence of 609 nucleotides (203 amino acids) in the N terminal of the NS3 region of HCV isolates from the patients was examined. HCV RNA was extracted from 100 μl of blood serum sample, and the nucleotide sequences were determined by direct sequencing and deep sequencing. The primers used to amplify the NS3 region were NS3-F1 (5′-ACA CCG CGG CGT GTG GGG ACA T-3′, nucleotides 3295 to 3316) and NS3-AS2 (5′-GCT CTT GCC GCT GCC AGT GGG A-3′, nucleotides 4040 to 4019) as the first (outer) primer pair and NS3-F3 (5′-CAG GGG TGG CGG CTC CTT-3′, nucleotides 3390 to 3407) and NS3-AS2 as the second (inner) primer pair (18). Thirty-five cycles of first and second amplifications were performed as follows: denaturation for 30 s at 95°C, annealing of primers for 1 min at 63°C, extension for 1 min at 72°C, and final extension at 72°C for 7 min. The PCR-amplified DNA was purified after agarose gel electrophoresis and then used for direct sequencing and ultradeep sequencing.

Patients were examined for NS3/4A protease inhibitor-resistant variants by direct sequencing at baseline and at the time of reelevation of viral loads. Furthermore, patients who did not have an SVR with the first course of triple therapy with telaprevir and received the second course of the triple therapy with telaprevir were analyzed for telaprevir-resistant variants by ultradeep sequencing at baseline and at the time of reelevation of viral loads. NS3/4A protease inhibitor-resistant variants included V36A/C/M/L/G, T54A/S, Q80K/R/H/G/L, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, D168A/V/E/G/N/T/Y/H/I, and V170A. Telaprevir-resistant variants (at aa 36, aa 54, aa 155, aa 156, and aa 170) and TMC435-resistant variants (at aa 80, aa 155, and aa 168) were evaluated (19, 20).

Direct sequencing was analyzed by the Dye Terminator method. Dideoxynucleotide termination sequencing was performed with the BigDye deoxy terminator version 1.1 cycle sequencing kit (Life Technologies, Carlsbad, CA) (18). The sequence data were deposited in GenBank. Also, ultradeep sequencing was performed using the Ion Personal Genome Machine (PGM) sequencer (Life Technologies). An Ion Torrent adapter-ligated library was prepared using an Ion Xpress Plus fragment library kit (Life Technologies). Briefly, 100 ng of fragmented genomic DNA was ligated to the Ion Torrent adapters P1 and A. The adapter-ligated products were nick translated and PCR amplified for a total of 8 cycles. Subsequently, the library was purified using AMPure beads (Beckman Coulter, Brea, CA) and the concentration determined using the StepOnePlus real-time PCR (Life Technologies) and Ion Library quantitation kit, according to the instructions provided by the manufacturers. Emulsion PCR was performed using the Ion OneTouch (Life Technologies) in conjunction with the Ion OneTouch 200 template kit version 2 (Life Technologies). Enrichment for templated Ion Sphere particles (ISPs) was performed using the Ion OneTouch enrichment system (Life Technologies) according to the instructions provided by the manufacturer. Templated ISPs were loaded onto an Ion 314 chip and subsequently sequenced using 130 sequencing cycles according to the Ion PGM 200 sequencing kit user guide. The total output read length per run was >10 Mb (0.5 million tags, 200-base read) (21). The results were analyzed with the CLC Genomics Workbench software (CLC bio, Aarhus, Denmark) (22).

We also included a control experiment to validate the error rates in ultradeep sequencing of the viral genome. In this study, the amplification products of the second-round PCR were ligated with a plasmid and transformed in *Escherichia coli* by using a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). A plasmid-derived NS3 sequence was determined as the template, in a control experiment. The fold coverages evaluated per position for aa 36, aa 54, aa 155, aa 156, and aa 170 in the NS3 region were 359,379×, 473,716×, 106,435×, 105,979×, and 49,058×, respectively. Thus, using the control experiment based on a plasmid carrying the HCV NS3 sequence, amino acid mutations were defined as amino acid substitutions at a frequency of >0.2% among the total coverage. This frequency ruled out putative errors caused by the ultradeep sequence platform used in this study (23).

Statistical analysis. Nonparametric variables were compared between the groups by the chi-square and Fisher's exact probability tests. Univariate and multivariate analyses for factors affecting the presence of telaprevir-resistant variants by direct sequencing at the reelevation of viral load were performed by the chi-square test and logistic regression, respectively. Patients who achieved an SVR were said to have no detection of resistant variants at the reelevation of viral load. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated to determine the reliability of the predictors of the response to therapy.

Nucleotide sequence accession numbers. The N-terminal sequences of the NS3 regions of the telaprevir-resistant variant isolates were deposited in GenBank under accession numbers AB709241, AB709263, AB709264, AB709276, AB709279, AB709283, AB709286, AB709289, AB709295, AB709296, AB709300, AB709303, AB709307, AB709310, AB709311, AB709312, AB709317, AB709319, AB709321, AB709322, AB709345, AB709348, AB709352, AB709353, AB709354, AB709356, AB709357, AB709358, AB709360, AB709370, AB709377, AB709382, AB709383, AB709384, AB709388, AB709390, AB709392, AB709396, AB709398, AB709399, AB709401, AB709405, AB709409, AB709410, AB709414, AB709418, AB709422, AB709426, AB709437, AB709444, AB709445, AB709451, AB709456, AB709461, AB709474, AB709476, AB709481, AB709484, AB709485, AB709486, AB709488, AB709489, AB709490, AB709491, AB709492, AB709493, AB709502, AB709507, AB709508, AB709514, AB709515, AB709525, AB709526, AB709527, and AB826566 to AB826684.

RESULTS

Virological response to therapy. An analysis of the entire group showed that 76% (192 of 252 patients) achieved an SVR. According to the treatment regimen, an SVR was achieved by 45% (9 of 20 patients) and 79% (183 of 232 patients) of the T12PR12 and T12PR24 groups, respectively. Taking into consideration the response to prior treatment, an SVR was achieved by 86% (68 of 79 patients), 84% (91 of 109 patients), and 35% (32 of 63 patients) of the treatment-naive patients, patients who showed relapse following prior treatment, and nonresponders to prior treatment, respectively. In the 231 patients of the T12PR24 group, an SVR was achieved by 88% (61 of 69 patients), 85% (89 of 105 patients), and

TABLE 2 Frequencies of the subjects in whom NS3/4A protease inhibitor-resistant variants were detected by direct sequencing at baseline and at the time of reelevation of viral loads[a]

Time of variant detection	% (*n*) by aa position[b]:						
	36	54	80	155	156	168	170
Baseline	0.4 (1)	3 (7)	22 (55)	0.4 (1)	0.8 (2)	10 (26)	0 (0)
Reelevation of viral load	7 (18)	12 (30)	5 (11)	0.4 (1)	4 (10)	1.2 (3)	0.4 (1)

[a] NS3/4A protease inhibitor-resistant variants included V36A/C/M/L/G, T54A/S, Q80K/R/H/G/L, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, D168A/V/E/G/N/T/Y/H/I, and V170A (19, 20).

[b] The data represent the percentages (*n*) of patients in whom NS3/4A protease inhibitor-resistant variants were detected by direct sequencing. Patients who achieved a sustained virological response were said to have no detection of resistant variants by direct sequencing at the time of reelevation of the viral load.

56% (32 of 57 patients) of the treatment-naive patients, patients who showed relapse following prior treatment, and nonresponders to prior treatment, respectively. Furthermore, an SVR was achieved by 86% (12 of 14 patients) and 47% (20 of 43 patients) of the nonresponders to prior IFN monotherapy and ribavirin combination therapy, respectively.

NS3/4A protease inhibitor-resistant variants detected by direct sequencing at baseline and at the time of reelevation of viral loads. All of the 252 patients were evaluated for resistant variants by direct sequencing at baseline. Sixty patients who did not achieve an SVR were also analyzed for resistant variants by direct sequencing at the time of reelevation of viral load. One hundred ninety-two patients who achieved SVR were said to have no detection of resistant variants as determined by direct sequencing at the reelevation of viral load.

As a whole, the frequency of the subjects in whom telaprevir-resistant variants were detected increased from 5% (12 of 252 patients) at baseline to 18% (45 of 252 patients) at the time of reelevation of viral load. On the other hand, the frequency of the subjects in whom TMC435-resistant variants were detected decreased from 31% (78 of 252 patients) at baseline to 6% (14 of 252 patients) at the time of reelevation of viral load. Table 2 shows the frequencies of subjects in whom resistant variants were detected at baseline and at the time of reelevation of viral load per position for aa 36, aa 54, aa 80, aa 155, aa 156, aa 168, and aa 170 in the NS3 region.

Pretreatment factors associated with detection of telaprevir-resistant variants by direct sequencing at the time of reelevation of viral load. Univariate analysis of the data of the entire group identified eight pretreatment factors that were significantly associated with the detection of telaprevir-resistant variants by direct sequencing at the time of reelevation of viral load: *IL28B* rs8099917 genotype (genotype non-TT) ($P < 0.001$), nonresponse to prior treatment ($P < 0.001$), PEG-IFN dose of <1.3 μg/kg ($P = 0.001$), detection of variants at aa 54 at baseline ($P = 0.002$), Gln70/His70 substitution of aa 70 ($P = 0.003$), gamma-glutamyl transpeptidase (GGT) level of ≥50 IU/liter ($P = 0.006$), leukocyte count of <5,000/mm^3 ($P = 0.026$), and ribavirin dose of <8.0 mg/kg ($P = 0.026$). Multivariate analysis that included the above variables identified five pretreatment factors that were independently associated with the detection of telaprevir-resistant variants at the time of reelevation of viral load: PEG-IFN dose of <1.3 μg/kg (odds ratio [OR], 9.71; $P < 0.001$), *IL28B* rs8099917 genotype (genotype non-TT) (OR, 8.61; $P < 0.001$), detection of variants at aa 54 at baseline (OR, 33.4; $P = 0.002$), nonresponse to prior treatment (OR, 2.66, $P = 0.018$), and leukocyte count of <5,000/mm^3 (OR, 2.46; $P = 0.042$) (Table 3).

Prediction of treatment efficacy by the combination of response to prior treatment and presence of telaprevir-resistant variants by direct sequencing at baseline. The SVR rates based on the combination of response to prior treatment and the presence of telaprevir-resistant variants by direct sequencing at baseline are shown in Fig. 1. In 79 treatment-naive patients, the SVR rates were not different between those patients in whom there were no detected telaprevir-resistant variants (86% [65 of 76 patients]) and those in whom variants were detected (67% [2 of 3 patients]). In 109 patients who showed relapse following prior treatment, the SVR rates were not different between those patients in whom there were no detected variants (83% [86 of 104 patients]) and those in whom variants were detected (100% [5 of 5 patients]). In contrast, in 63 patients who showed nonresponse to prior treatment, a higher proportion of patients with undetected telaprevir-resistant variants (54% [32 of 59 patients]) achieved an SVR than did patients in whom telaprevir-resistant variants were detected (0% [0 of 4 patients]) ($P = 0.053$). Thus, with the combination of nonresponse to prior treatment and detection of telaprevir-resistant variants, the sensitivity, specificity, PPV, and NPV for those with non-SVR were 7% (4 of 60 patients), 100% (191 of 191 patients), 100% (4 of 4 patients), and 77% (191 of 247 patients), respectively. These results indicated that the use of the combination of the above two factors has high specificity and PPV for the prediction of a non-SVR.

TABLE 3 Multivariate analysis of factors associated with detection of telaprevir-resistant variants by direct sequencing at the reelevation of viral load, to telaprevir, peginterferon, and ribavirin triple therapy in patients infected with HCV genotype 1b

Detection factors	Category	Odds ratio (95% CI[a])	*P*[b]
PEG-IFN-α2b dose (μg/kg)	≥1.3	1	
	<1.3	9.71 (3.23–29.4)	<0.001
IL28B rs8099917 genotype	TT genotype	1	
	Non-TT genotype	8.61 (3.48–21.3)	<0.001
Variants of aa 54 at baseline	No detection	1	
	Detection	33.4 (3.77–295)	0.002
Response to treatment	Naive or relapse	1	
	Nonresponse	2.66 (1.18–5.96)	0.018
Leukocyte count (/mm^3)	≥5,000	1	
	<5,000	2.46 (1.03–5.85)	0.042

[a] CI, confidence interval.

[b] Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression analysis are shown.

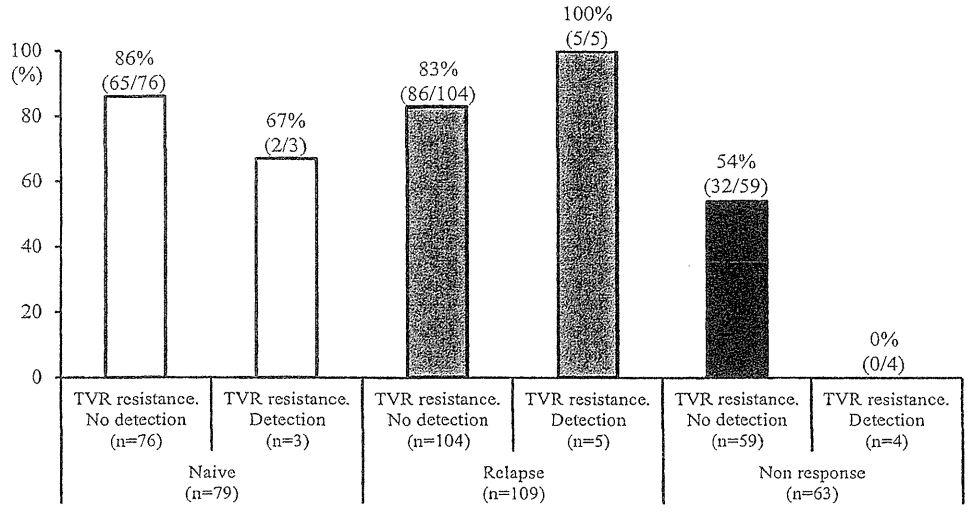

FIG 1 The rates of sustained virological response by the combination of response to prior treatment and presence of telaprevir (TVR)-resistant variants by direct sequencing at baseline are shown. Of those who showed nonresponse to prior treatment, a higher proportion of patients with undetected TVR-resistant variants (54%) achieved a sustained virological response than patients with detected TVR-resistant variants (0%) ($P = 0.053$).

Table 4 summarizes the profiles of 4 patients with nonresponse to prior treatment and in whom telaprevir-resistant variants were detected by direct sequencing at baseline. All of these 4 patients did not achieve an SVR with triple therapy. Interestingly, both T54S as a telaprevir-resistant variant and Q80L as a TMC435-resistant variant (19) were detected by direct sequencing at baseline.

Evolution of telaprevir-resistant variants over time as investigated by ultradeep sequencing in patients who received the second course of triple therapy. Two of 60 patients who did not achieve an SVR with the first course of triple therapy with telaprevir received the second course of triple therapy with telaprevir. They were analyzed for telaprevir-resistant variants by ultradeep sequencing at baseline and at the time of reelevation of viral loads.

Figure 2A shows the clinical course of case 1. In the first course of triple therapy with telaprevir (T12PR24) in a 57-year-old, V36C (0% of 32,413× coverage) was not detected by ultradeep sequencing at baseline of the first course, but very-high-frequency variants of V36C (97.2% of 36,757× coverage) were detected at the time of reelevation of viral loads. In the second course of triple therapy with telaprevir (T12PR54) when the patient was 59 years old, very-high-frequency variants of V36C (98.1% of 94,547× coverage) persisted at baseline of the second course, despite the passing of 2 years after cessation of the first therapy course. Case 1 achieved HCV RNA-negative status at 20 weeks after the start of the second course (late virological response), so PEG-IFN and ribavirin therapy was extended to 54 weeks. In conclusion, case 1 achieved an SVR after the second course of triple therapy with telaprevir, despite the persistence of very-high-frequency variants.

Figure 2B shows the clinical course of case 2. In the first course of triple therapy with telaprevir (T12PR24) in a 61-year-old patient, R155Q (0% of 23,751× coverage) and A156T (0% of 16,040× coverage) were not detected by ultradeep sequencing at baseline of the first course, but very-low-frequency variants of R155Q (0.2% of 11,572× coverage) and A156T (0.2% of 16,040× coverage) were detected at the time of reelevation of viral loads. In the second course of triple therapy with telaprevir (T12PR20) when the patient was 64 years old, R155Q (0% of 80,572× coverage) and A156T (0% of 87,686× coverage) were not detected by ultradeep sequencing at baseline of the second course, which was 2 years after cessation of the first course. In conclusion, case 2 achieved an SVR by the second course of triple therapy with telaprevir, despite the history of the emergence of variants.

TABLE 4 Profiles of 4 patients with nonresponse to prior treatment and detection of telaprevir-resistant variants by direct sequencing at baseline

Case no.	Sex	Age (yr)	Response to prior treatment[a]	Amino acid detected at aa position: 36	54	80	155	156	168	170	Time of HCV RNA-negative result during treatment (wks)	Efficacy of triple therapy
1	Male	70	Nonresponse to IFN monotherapy	V	S	L	R	A	D	I	2	Non-SVR
2	Male	47	Nonresponse to IFN monotherapy	V	S	L	R	A	D	I	4	Non-SVR
3	Male	61	Nonresponse to RBV combination therapy	V	S	L	R	A	D	I	3	Non-SVR
4	Female	60	Nonresponse to RBV combination therapy	V	S	L	R	A	D	I	4	Non-SVR

[a] RBV, ribavirin.

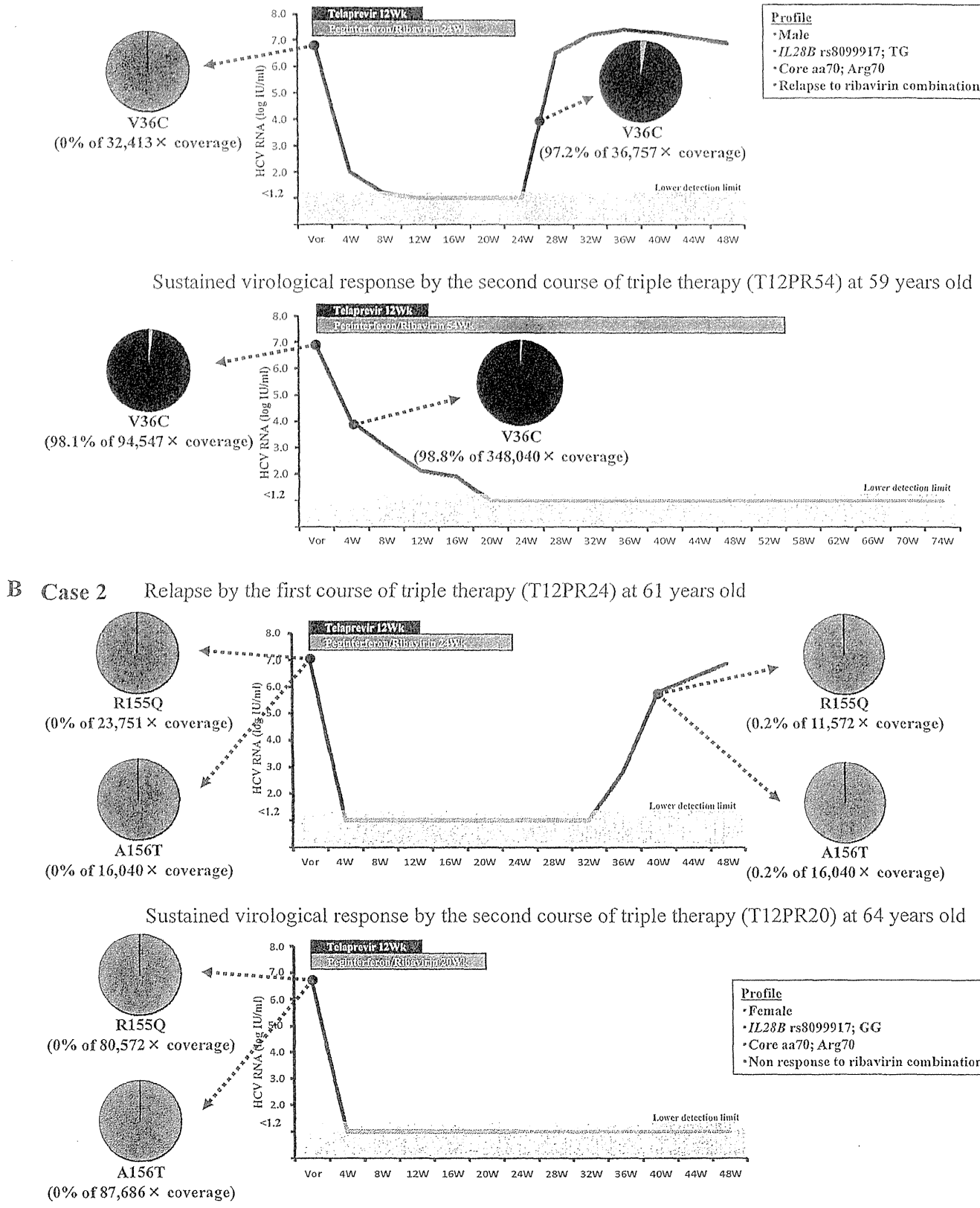

FIG 2 Two patients who did not achieve a sustained virological response with the first course of triple therapy with telaprevir received the second course of the triple therapy with telaprevir. They were analyzed for telaprevir-resistant variants by ultradeep sequencing at baseline and at the time of reelevation of viral loads. (A) Case 1 achieved a sustained virological response with the second course of therapy despite the persistence of very-high-frequency variants. (B) Case 2 achieved a sustained virological response with the second course of therapy despite the history of the emergence of variants.

DISCUSSION

Patients who fail to achieve an SVR to triple therapy need to be identified to avoid unnecessary side effects, high costs, and the emergence of telaprevir-resistant variants. Host genetic factors (e.g., *IL28B* genotype), and viral factors (e.g., amino acid substitutions in the core/NS5A region) have often been used as pretreatment predictors of poor virological response to PEG-IFN–ribavirin dual therapy (9–11, 15, 17) and telaprevir–PEG-IFN–ribavirin triple therapy (24–26). However, the pretreatment factors associated with the detection of telaprevir-resistant variants at the time of reelevation of viral load are still unknown. The present study identified that the detection of telaprevir-resistant variants at the time of reelevation of viral load can be predicted by a combination of host (*IL28B* rs8099917 genotype and leukocyte count), viral (variants of aa 54 at baseline), and treatment factors (PEG-IFN dose). All of the 4 patients with nonresponse to prior treatment and in whom telaprevir-resistant variants were detected at baseline did not achieve an SVR with triple therapy, and the use of the combination of nonresponse to prior treatment and the detection of telaprevir-resistant variants at baseline had high specificity and PPV for the prediction of a non-SVR. This finding suggests that there is a complex relationship between host susceptibility to IFN and viral sensitivity to NS3/4A protease inhibitors in determining treatment efficacy. Interestingly, in all of the 4 patients, both T54S as a telaprevir-resistant variant and Q80L as a TMC435-resistant variant (19) were detected by direct sequencing at baseline. This result suggests that patients with the above two factors should be carefully introduced to NS3/4A protease inhibitors besides telaprevir because of the high risk of the emergence of resistant variants. However, the present study was performed with a small number of patients, so further studies based on a larger number of patients should be performed.

In the present study employing ultradeep sequencing technology, 2 patients who did not achieve an SVR with the first course of triple therapy with telaprevir received the second course of the triple therapy with telaprevir. They achieved an SVR with the second course, despite the persistence of very-high-frequency variants (case 1, 98.1% for V36C) or a history of the emergence of variants (case 2, 0.2% for R155Q and 0.2% for A156T) as determined by ultradeep sequencing. This finding may be due to one or more reasons. One reason is probably related to the high susceptibility of telaprevir-resistant variants to IFN. One previous study indicated that mice infected with the resistant strain (A156F [99.9%]) developed only low-level viremia, and the virus was successfully eliminated with IFN therapy (27). In the other clinical report, telaprevir-resistant variants that emerged during 24-week telaprevir monotherapy were eliminated by the combination therapy of PEG-IFN plus ribavirin (28). Furthermore, this finding probably suggests that a small number of mutant-type viral RNAs may be incomplete or defective, since a large proportion of viral genomes are thought to be defective due to their high replication and mutation rates (29). Further studies employing ultradeep sequencing should be performed to evaluate whether a history of the emergence of NS3/4A protease inhibitor-resistant variants, besides telaprevir-resistant variants, affects the efficacy of a second course of NS3/4A protease inhibitor-based treatment.

The results of the present study should be interpreted with caution, since the study was performed with a small number of Japanese patients infected with HCV-1b. Any generalization of the results should await confirmation by a multicenter randomized trial based on a larger number of patients, including patients of other races and those infected with HCV-1a. Furthermore, the other limitation of the present study is that the loss of telaprevir-resistant variants was not investigated long after the cessation of therapy. Further large-scale studies should be performed to investigate the impacts of telaprevir-resistant variants on the response to treatment using new drugs, including direct-acting antiviral agents.

In conclusion, this study based on Japanese patients infected with HCV-1b indicates that telaprevir-resistant variants at the time of reelevation of viral load can be predicted by a combination of host, viral, and treatment factors. In those patients with no response to prior treatment, the present results suggest that telaprevir-resistant variants at baseline might partly affect the efficacy of triple therapy treatment. This finding indicates the clinical utility of detecting telaprevir-resistant variants to predict treatment efficacy, and it suggests a complex relationship between host susceptibility to IFN and viral sensitivity to NS3/4A protease inhibitors in determining treatment efficacy. Further large-scale prospective studies are needed to investigate the clinical usefulness of telaprevir-resistant variants and to develop more effective therapeutic regimens in patients infected with HCV-1.

ACKNOWLEDGMENTS

This study was supported in part by a grant-in-aid from the Ministry of Health, Labor, and Welfare, Japan.

Norio Akuta received honoraria from MSD K.K. and Mitsubishi Tanabe Pharma and holds a right for royalty from SRL, Inc. Hiromitsu Kumada received honoraria from MSD K.K., Mitsubishi Tanabe Pharma, Dainippon Sumitomo Pharma, and Bristol-Myers Squibb and holds a right for royalty from SRL, Inc. The other authors declare no conflicts of interest.

REFERENCES

1. Lin C, Kwong AD, Perni RB. 2006. Discovery and development of VX-950, a novel, covalent, and reversible inhibitor of hepatitis C virus NS3.4A serine protease. Infect. Disord. Drug Targets 6:3–16. http://dx.doi.org/10.2174/187152606776056706.
2. McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, McNair L, Alam J, Muir AJ, PROVE1 Study Team. 2009. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N. Engl. J. Med. 360:1827–1838. http://dx.doi.org/10.1056/NEJMoa0806104.
3. Hézode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, Bronowicki JP, Bourlière M, Gharakhanian S, Bengtsson L, McNair L, George S, Kieffer T, Kwong A, Kauffman RS, Alam J, Pawlotsky JM, Zeuzem S, PROVE2 Study Team. 2009. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. N. Engl. J. Med. 360:1839–1850. http://dx.doi.org/10.1056/NEJMoa0807650.
4. Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. 2012. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. J. Hepatol. 56:78–84. http://dx.doi.org/10.1016/j.jhep.2011.07.016.
5. McHutchison JG, Manns MP, Muir AJ, Terrault NA, Jacobson IM, Afdhal NH, Heathcote EJ, Zeuzem S, Reesink HW, Garg J, Bsharat M, George S, Kauffman RS, Adda N, Di Bisceglie AM, PROVE3 Study Team. 2010. Telaprevir for previously treated chronic HCV infection. N. Engl. J. Med. 362:1292–1303. http://dx.doi.org/10.1056/NEJMoa0908014.
6. Lin C, Gates CA, Rao BG, Brennan DL, Fulghum JR, Luong YP, Frantz JD, Lin K, Ma S, Wei YY, Perni RB, Kwong AD. 2005. *In vitro* studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. J. Biol. Chem. 280:36784–36791. http://dx.doi.org/10.1074/jbc.M506462200.
7. Kieffer TL, Sarrazin C, Miller JS, Welker MW, Forestier N, Reesink HW, Kwong AD, Zeuzem S. 2007. Telaprevir and pegylated interferon-

alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. Hepatology 46:631–639. http://dx.doi.org/10.1002/hep.21781.
8. Sullivan JC, De Meyer S, Bartels DJ, Dierynck I, Zhang EZ, Spanks J, Tigges AM, Ghys A, Dorrian J, Adda N, Martin EC, Beumont M, Jacobson IM, Sherman KE, Zeuzem S, Picchio G, Kieffer TL. 2013. Evolution of treatment-emergent resistant variants in telaprevir phase 3 clinical trials. Clin. Infect. Dis. 57:221–229. http://dx.doi.org/10.1093/cid/cit226.
9. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461:399–401. http://dx.doi.org/10.1038/nature08309.
10. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Müller T, Bahlo M, Stewart GJ, Booth DR, George J. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat. Genet. 41:1100–1104. http://dx.doi.org/10.1038/ng.447.
11. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of *IL28B* with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat. Genet. 41:1105–1109. http://dx.doi.org/10.1038/ng.449.
12. Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. 2001. A high-throughput SNP typing system for genome-wide association studies. J. Hum. Genet. 46:471–477. http://dx.doi.org/10.1007/s100380170047.
13. Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, Nagasaki M, Nakayama-Hamada M, Kawaida R, Ono M, Ohtsuki M, Furukawa H, Yoshino S, Yukioka M, Tohma S, Matsubara T, Wakitani S, Teshima R, Nishioka Y, Sekine A, Iida A, Takahashi A, Tsunoda T, Nakamura Y, Yamamoto K. 2003. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat. Genet. 34:395–402. http://dx.doi.org/10.1038/ng1206.
14. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K. 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. U. S. A. 87:9524–9528. http://dx.doi.org/10.1073/pnas.87.24.9524.
15. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Matsuda M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2005. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 48:372–380. http://dx.doi.org/10.1159/000086064.
16. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N. Engl. J. Med. 334:77–81. http://dx.doi.org/10.1056/NEJM199601113340203.
17. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2008. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 48:38–47. http://dx.doi.org/10.1002/hep.22339.
18. Suzuki F, Sezaki H, Akuta N, Suzuki Y, Seko Y, Kawamura Y, Hosaka T, Kobayashi M, Saito S, Arase Y, Ikeda K, Kobayashi M, Mineta R, Watahiki S, Miyakawa Y, Kumada H. 2012. Prevalence of hepatitis C virus variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients with genotype 1b. J. Clin. Virol. 54:352–354. http://dx.doi.org/10.1016/j.jcv.2012.04.024.
19. Romano KP, Ali A, Royer WE, Schiffer CA. 2010. Drug resistance against HCV NS3/4A inhibitors is defined by the balance of substrate recognition versus inhibitor binding. Proc. Natl. Acad. Sci. U. S. A. 107:20986–20991. http://dx.doi.org/10.1073/pnas.1006370107.
20. Barbotte L, Ahmed-Belkacem A, Chevaliez S, Soulier A, Hézode C, Wajcman H, Bartels DJ, Zhou Y, Ardzinski A, Mani N, Rao BG, George S, Kwong A, Pawlotsky JM. 2010. Characterization of V36C, a novel amino acid substitution conferring hepatitis C virus (HCV) resistance to telaprevir, a potent peptidomimetic inhibitor of HCV protease. Antimicrob. Agents Chemother. 54:2681–2683. http://dx.doi.org/10.1128/AAC.01796-09.
21. Elliott AM, Radecki J, Moghis B, Li X, Kammesheidt A. 2012. Rapid detection of the ACMG/ACOG-recommended 23 CFTR disease-causing mutations using ion torrent semiconductor sequencing. J. Biomol. Tech. 23:24–30. http://dx.doi.org/10.7171/jbt.12-2301-003.
22. Vogel U, Szczepanowski R, Claus H, Jünemann S, Prior K, Harmsen D. 2012. Ion torrent personal genome machine sequencing for genomic typing of *Neisseria meningitidis* for rapid determination of multiple layers of typing information. J. Clin. Microbiol. 50:1889–1894. http://dx.doi.org/10.1128/JCM.00038-12.
23. Akuta N, Suzuki F, Seko Y, Kawamura Y, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Hara T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2013. Emergence of telaprevir-resistant variants detected by ultradeep sequencing after triple therapy in patients infected with HCV genotype 1. J. Med. Virol. 85:1028–1036. http://dx.doi.org/10.1002/jmv.23579.
24. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Chayama K, Nakamura Y, Kumada H. 2010. Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. Hepatology 52:421–429. http://dx.doi.org/10.1002/hep.23690.
25. Chayama K, Hayes CN, Abe H, Miki D, Ochi H, Karino Y, Toyota J, Nakamura Y, Kamatani N, Sezaki H, Kobayashi M, Akuta N, Suzuki F, Kumada H. 2011. IL28B but not ITPA polymorphism is predictive of response to pegylated interferon, ribavirin, and telaprevir triple therapy in patients with genotype 1 hepatitis C. J. Infect. Dis. 204:84–93. http://dx.doi.org/10.1093/infdis/jir210.
26. Akuta N, Suzuki F, Fukushima T, Kawamura Y, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Hara T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2013. Prediction of treatment efficacy and telaprevir-resistant variants after triple therapy in patients infected with hepatitis C virus genotype 1. J. Clin. Microbiol. 51:2862–2868. http://dx.doi.org/10.1128/JCM.01129-13.
27. Hiraga N, Imamura M, Abe H, Hayes CN, Kono T, Onishi M, Tsuge M, Takahashi S, Ochi H, Iwao E, Kamiya N, Yamada I, Tateno C, Yoshizato K, Matsui H, Kanai A, Inaba T, Tanaka S, Chayama K. 2011. Rapid emergence of telaprevir resistant hepatitis C virus strain from wild type clone *in vivo*. Hepatology 54:781–788. http://dx.doi.org/10.1002/hep.24460.
28. Ozeki I, Akaike J, Karino Y, Arakawa T, Kuwata Y, Ohmura T, Sato T, Kamiya N, Yamada I, Chayama K, Kumada H, Toyota J. 2011. Antiviral effects of peginterferon alpha-2b and ribavirin following 24-week monotherapy of telaprevir in Japanese hepatitis C patients. J. Gastroenterol. 46:929–937. http://dx.doi.org/10.1007/s00535-011-0411-0.
29. Bartenschlager R, Lohmann V. 2000. Replication of hepatitis C virus. J. Gen. Virol. 81:1631–1648.

Journal of Medical Virology 86:131–138 (2014)

Correlation Between Hepatitis B Virus Surface Antigen Level and Alpha-Fetoprotein in Patients Free of Hepatocellular Carcinoma or Severe Hepatitis

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Alfa-fetoprotein (AFP) is used as a marker of early hepatocarcinogenesis. However, the impact of hepatitis B virus surface antigen (HBsAg) on this relationship in patients with HBV infection is not clear. The present study evaluated the relation between HBsAg and AFP levels at the initial visit in 1,610 untreated HBV patients, free of hepatocellular carcinoma (HCC) or severe hepatitis. The cumulative rate of HCC was significantly lower in patients with a low AFP level (≤10 μg/L; below the upper limit of normal) than in those with a high AFP level (≥11 μg/L) at the initial visit. In patients with HBsAg levels more than 500 IU/ml, HBsAg levels correlated significantly and negatively with AFP levels, and significantly with platelet count. Multivariate analysis of data of patients with HBsAg more than 500 IU/ml identified HBsAg (<7,000 IU/ml), albumin (<3.9 g/dl), platelet count ($<20.0 \times 10^4/mm^3$), gamma-glutamyl transpeptidase (≥50 IU/L), aspartate aminotransferase (≥34 IU/L), HBeAg (positive), and HBV core-related antigen (≥3.0 log U/ml) as determinants of a high AFP. Especially, in patients with HBsAg more than 500 IU/ml and low transaminase levels (below the upper limit of normal), HBsAg was identified as significant determinant of a high AFP. On the other hand, in patients with HBsAg less than 500 IU/ml, multivariate analysis identified albumin, gamma-glutamyl transpeptidase, and HBV core-related antigen as determinants of a high AFP. The results indicated that HBsAg level seems to affect, at least in part, the AFP levels, and that it can be used as a surrogate marker of early hepatocarcinogenesis. ***J. Med. Virol. 86:131–138, 2014.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: HBV; AFP; HBsAg; HBcrAg; genotype; hepatocellular carcinoma

INTRODUCTION

Hepatitis B virus (HBV) is a small, enveloped DNA virus known to cause chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma (HCC) [Viola et al., 1981; Kobayashi et al., 2002; Yao, 2003]. Evidence suggests that the use of elevated alpha-fetoprotein (AFP) for the prediction of early hepatocarcinogenesis in non-HCC patients could be clinically useful. AFP is a fetal glycoprotein produced by the yolk sac and fetal liver [Bergstrand and Czar, 1956] and has been widely used as a serum marker for the diagnosis of HCC [Sato et al., 1993; Johnson, 2001]. Furthermore, high serum AFP levels are also associated with various chronic liver diseases and hepatic regeneration [Kew et al., 1973; Silver et al., 1974; Elftherious et al., 1977; Alpert and Feller, 1978]. Many patients with chronic hepatitis B who are free of HCC have high AFP levels [Chen and Sung, 1979; Di Bisceglie and Hoofnagle, 1989], and some patients with cirrhosis and concomitant high

Grant sponsor: Ministry of Health, Labor and Welfare, Japan

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Accepted 21 August 2013

DOI 10.1002/jmv.23790
Published online 12 October 2013 in Wiley Online Library (wileyonlinelibrary.com).

inflammatory activity have very high AFP levels [Yao, 2003; Cheema et al., 2004]. On the other hand, some patients with small HCC lesions have only moderately elevated levels of AFP [Shinagawa et al., 1984; Ebara et al., 1986; Bruix and Sherman, 2005]. At present, however, there are no cutoff levels for serum AFP used to predict HCC in patients with HBV infection.

There is growing interest in the use of hepatitis B surface antigen (HBsAg) level as a prognostic marker in chronic hepatitis B patients [Chan et al., 2010]. The HBsAg levels are useful for identifying the stage of disease [Jaroszewicz et al., 2010; Nguyen et al., 2010], to distinguish true inactive carriers from patients with HBe antigen-negative disease [Brunetto et al., 2010; Martinot-Peignoux et al., 2010; Chan et al., 2011; Liaw, 2011], and to predict the response to interferon therapy [Brunetto et al., 2009; Moucari et al., 2009]. Recent studies has also demonstrated that the HBsAg levels are associated with the risk of progression to HCC, especially in patients with low HBV DNA levels [Chan, 2012; Tseng et al., 2012], and that there is a potential correlation between the HBsAg levels and the stage of liver fibrosis [Seto et al., 2012; Martinot-Peignoux et al., 2013]. However, the impact of viral factors, such as the HBsAg level, on serum AFP level as a predictor of early HCC is not clear at present.

The present study included 1,610 untreated patients with HBV infection, free of HCC or severe hepatitis. The present study was designed to provide answers to the following questions: (1) what is the relation between a high serum AFP level at the initial outpatient visit and subsequent development of hepatocarcinogenesis in antiviral-therapy-naive patients with hepatitis B viral infection? (2) What is the impact of viral factors, such as the HBsAg level, on serum AFP level in such patients, and (3) What is a good surrogate marker for a high serum AFP at the initial visit.

PATIENTS AND METHODS

Patients

Among 6,466 consecutive patients who were diagnosed with HBV infection between March 1972 and December 2012 at Toranomon Hospital, 1,610 were selected in the present study based on the following criteria: (1) They were positive for HBsAg (radioimmunoassay, Dainabot, Tokyo, Japan) and negative for anti-HCV (third-generation enzyme immunoassay, Chiron, CA). (2) They were free of HCC at the initial visit. (3) HBV hepatitis was assessed as less than severe at the initial visit, in order to minimize the potential effects of high inflammatory activity. Severe hepatitis was defined as serum transaminase level of ≥300 IU/L, and/or total bilirubin level of ≥3.0 mg/dl. (4) They had not received antiviral therapy in the past (e.g., interferon and/or nucleot(s)ide analogs) at the initial visit. (5) They underwent examination of the AFP level (upper limit of normal, 10 μg/L) at the initial visit. Furthermore, the HBsAg level, HBV core-related antigen (HBcrAg) level, and HBV DNA were also assayed using stored frozen sera obtained at the initial visit. (6) They were free of coinfection with human immunodeficiency virus. (7) They were free of other types of chronic liver disease, including hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, autoimmune liver disease, inherited liver disease including alpha-1 antitrypsin deficiency, and hepatic venous outflow block. (8) They consented to the study.

Table I summarizes the profile and laboratory data at the initial visit of the 1,610 patients included in the present study. They included 1,047 males and 563 females, with a median age of 40 years (range: 18–83 years). The median AFP level was 4 μg/L (range, 1–1,770 μg/L) and the median follow-up time (from the initial visit until the last visit) was 6.0 years (range, 0.0–34.6 years). The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital.

Laboratory Tests

HBsAg, HBcrAg, and HBV DNA levels were assayed using stored frozen sera obtained at the initial visit. Blood samples were frozen at −80°C within 4 hr of collection and were not thawed until used for testing. Serum HBsAg level was measured using Architect HBsAg QT assay kit (Abbott Laboratories, Tokyo, Japan), which has a lower limit of detection of

TABLE I. Profiles and Laboratory Data at the Initial Visit of 1,610 Patients Infected With HBV

Demographic data	
Number of patients	1,610
Sex (male/female)	1,047/563
Age (years)*	40 (18–83)
Family history of liver disease[a]	836 (51.9%)
Lifetime cumulative alcohol intake (≥500 kg)	112 (7.0%)
Laboratory data*	
Total bilirubin (mg/dl)	0.6 (0.1–2.9)
Aspartate aminotransferase (IU/L)	37 (5–220)
Alanine aminotransferase (IU/L)	48 (5–297)
Albumin (g/dl)	4.2 (1.0–5.6)
Gamma-glutamyl transpeptidase (IU/L)	37 (2–2,370)
Hemoglobin (g/dl)	14.5 (6.9–18.2)
Platelet count (×104/mm^3)	19.1 (2.7–44.7)
Alpha-fetoprotein (μg/L)	4 (1–1,770)
Virological data	
HBeAg (No. of positive)	690 (42.9%)
HBsAg (IU/ml)*	2,845 (0.09 to >125,000)
HBcrAg (log U/ml)*	4.9 (<3.0 to >6.8)
HBV DNA (log copies/ml)*	5.7 (<2.1 to >9.1)
HBV genotype (A/B/C/others/ND)	65/218/1,119/6/202

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.
[a]Family history of positivity for hepatitis B surface antigen including third-degree relatives.

0.05 IU/ml and upper limit of detection of 250 IU/ml. To expand the upper range from 250 to 125,000 IU/ml, serum samples with the HBsAg levels above the upper range were diluted in a stepwise fashion to 1:20 and 1:500 with Architect diluents using the information supplied by the manufacturer. HBeAg was determined by enzyme-linked immunosorbent assay kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). Serum HBcrAg level was measured using a Cleia HBcrAg assay kit (Fujirebio, Tokyo, Japan) using a fully automated analyzer system (Lumipulse System; Fujirebio). The cut-off value of HBcrAg was 3.0 log U/ml. HBV DNA was quantified using the Cobas TaqMan HBV v.2.0 (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.1–9.0 log copies/ml.

A commercial kit (HBV Genotype EIA; Institute of Immunology) was used to determine serologically the HBV genotypes using the combination of epitopes expressed on the pre-S2 region product, which is specific to each of the major genotypes.

Follow-Up and Diagnosis of Future Hepatocellular Carcinoma

After the initial visit, patients were followed-up once or three times a month. Imaging studies (ultrasonography, computed tomography, or magnetic resonance imaging) were conducted once or more per year.

Statistical Analysis

Non-parametric tests (Mann–Whitney *U*-test, chi-squared test and Fisher's exact probability test) were used to compare differences between two groups. Correlation analysis was evaluated by the Spearman rank correlation test. The cumulative rate of hepatocarcinogenesis was calculated using the Kaplan–Meier technique; differences between cumulative carcinogenesis curves between groups were tested using the log-rank test. Statistical analyses of the rate of hepatocarcinogenesis according to groups were calculated using the period from the initial visit. Univariate and multivariate logistic regression analyses were used to determine the independent surrogate markers of elevated AFP at the initial visit. The odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated. A two-tailed *P*-value less than 0.05 was considered significant. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors for elevated AFP. Potential surrogate markers of elevated AFP at the initial visit included the following pretreatment variables: age, sex, family history of liver disease, lifetime cumulative alcohol intake, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, gamma-glutamyl transpeptidase (GGT), hemoglobin, platelet count, HBV genotype, HBeAg, HBsAg levels, HBcrAg levels, and HBV DNA levels. Statistical analyses were performed using the Statistical Package for Social Sciences software (SPSS, Inc., Chicago, IL).

RESULTS

Cumulative Rate of Hepatocarcinogenesis According to the AFP Level at the Initial Visit

A total of 1,061 patients naïve to antiviral therapy from the initial visit until the last visit were evaluated for the rate of development of HCC based on the AFP levels at the initial visit. During the follow-up period, HCC was diagnosed in 31 of 905 patients (3.4%) with a low AFP level (≤10 μg/L; below the upper limit of normal) and 37 of 156 patients (23.7%) with a high AFP level (≥11 μg/L) at the initial visit. The cumulative hepatocarcinogenesis rates for patients with low and high AFP levels at the initial visit were 4.7% and 30.2% at the end of 10-year follow-up; 9.1% and 36.5% at the end of 20-year follow-up; and 13.2% and 42.9% at the end of 30-year follow-up, respectively. These results indicate that the rate of hepatocarcinogenesis is significantly higher in patients with HBV infection and high AFP levels than their counterparts with low AFP levels ($P < 0.001$; Log-rank test) (Fig. 1).

HBsAg and AFP Levels at the Initial Visit

Blood samples from all patients were analyzed to determine the relationship between the HBsAg and the AFP levels at the initial visit. The proportions of patients with high AFP levels among those with the HBsAg levels below 500 IU/ml, from 500 to 1,999 IU/ml, from 2,000 to 6,999 IU/ml, from 7,000 to 24,999 IU/ml, and above 25,000 IU/ml were 12.6% (42 of 333 patients), 26.7% (89 of 333), 22.6% (94 of 416), 10.4% (29 of 278), and 6.4% (16 of 250), respectively (Fig. 2A). The relationship between the HBsAg and

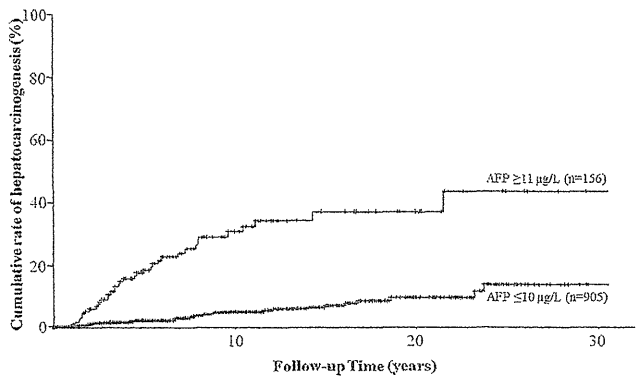

Fig. 1. Cumulative rate of hepatocarcinogenesis according to the AFP level at the initial visit in patients naïve to antiviral therapy from the initial visit until the last visit. The rate of hepatocarcinogenesis was significantly higher in patients with high AFP levels (≥11 μg/L) than in those with low levels (≤10 μg/L) at the initial visit ($P < 0.001$; Log-rank test).

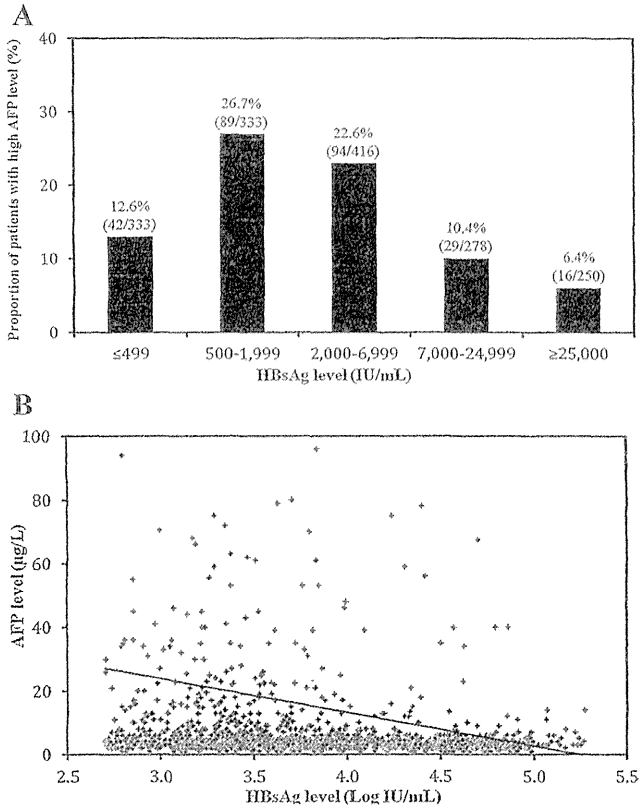

Fig. 2. **A**: Proportions of patients with the high AFP levels (≥11 μg/L) at the initial visit, stratified according to the HBsAg levels. Patients with the HBsAg levels above 500 IU/ml included a significantly lower proportions of patients with the high AFP levels and the HBsAg levels above 7,000 IU/ml (8.5%) than those with the HBsAg levels below 7,000 IU/ml (24.4%) ($P < 0.001$). **B**: Analysis of data of patients with the HBsAg levels above 500 IU/ml at the initial visit, showed a significant negative correlation between logarithmically transformed HBsAg and AFP levels ($r = -0.225$, $P < 0.001$).

the AFP levels at the initial visit suggested the presence of two distinct populations within the study group. Especially, in 1,277 patients with the HBsAg levels above 500 IU/ml, a significantly smaller proportion of patients with high AFP levels were noted among those with HBsAg of more than 7,000 IU/ml (8.5%) than those with the HBsAg levels less than 7,000 IU/ml (24.4%) ($P < 0.001$). Furthermore, the HBsAg levels correlated negatively but significantly with the AFP levels ($r = -0.225$, $P < 0.001$) (Fig. 2B).

The HBsAg Levels and the Platelet Count at the Initial Visit

Blood samples from all patients were analyzed to determine the relationship between the HBsAg levels and the platelet count at the initial visit. The median platelet counts among patients with the HBsAg levels below 500 IU/ml, from 500 to 1,999 IU/ml, from 2,000 to 6,999 IU/ml, from 7,000 to 24,999 IU/ml, and above 25,000 IU/ml were $19.1 \times 10^4/mm^3$, $17.2 \times 10^4/mm^3$, $18.0 \times 10^4/mm^3$, $20.9 \times 10^4/mm^3$, and $21.2 \times 10^4/mm^3$, respectively (Fig. 3A). The relationship between the HBsAg levels and the platelet count at the initial visit also suggested the presence of two distinct populations within the study group. Especially, in 1,277 patients with the HBsAg levels of more than 500 IU/ml, significantly higher platelet counts were noted among those with the HBsAg levels of more than 7,000 IU/ml (the median platelet count; $21.0 \times 10^4/mm^3$) than those with the HBsAg levels less than 7,000 IU/ml (the median platelet count; $17.6 \times 10^4/mm^3$) ($P < 0.001$). Furthermore, the HBsAg

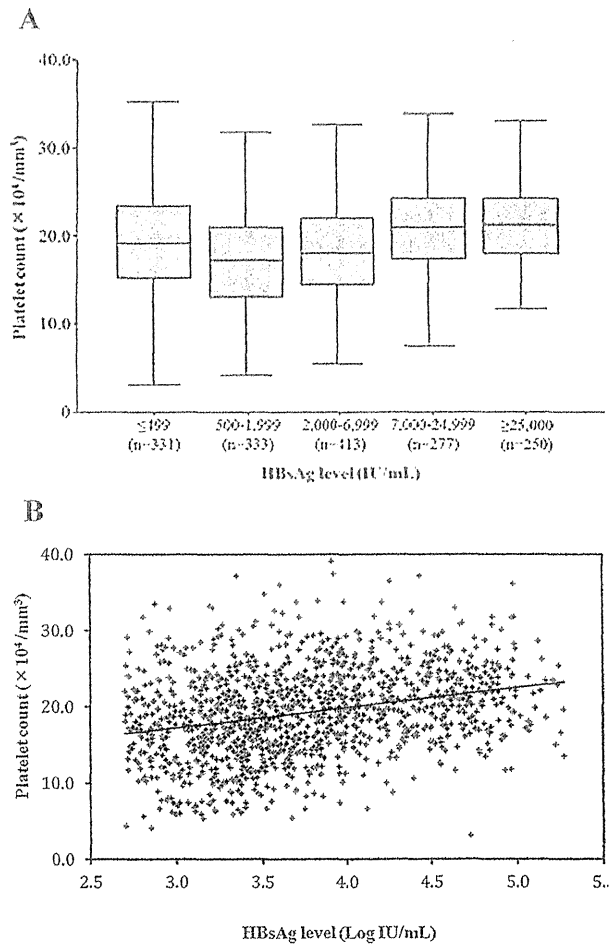

Fig. 3. **A**: The platelet count at the initial visit, stratified according to the HBsAg levels. Bars within the boxes indicate the median platelet count. The boxes denote the 25th to 75th percentiles, the lower and upper bars the 10th and 90th percentiles, respectively. Among patients with the HBsAg levels above 500 IU/ml at the initial visit, those with the HBsAg levels above 7,000 IU/ml had significantly higher platelet count (the median platelet count; $21.0 \times 10^4/mm^3$) compared to those with the HBsAg levels below 7,000 IU/ml (the median platelet count; $17.6 \times 10^4/mm^3$) ($P < 0.001$). **B**: Among patients with the HBsAg levels above 500 IU/ml at the initial visit, logarithmically transformed the HBsAg levels correlated significantly with the platelet count ($r = 0.293$, $P < 0.001$).

levels correlated significantly and positively with the platelet count ($r = 0.293$, $P < 0.001$) (Fig. 3B).

Clinical Profiles and Laboratory Data According to the HBsAg Level at the Initial Visit

Table II summarizes the clinical profiles and laboratory data according to the HBsAg level at the initial visit of 1,610 patients infected with HBV. Patients with the HBsAg levels below 500 IU/ml were significantly older and exhibited lower inflammatory activity (lower levels of AST and ALT), and had lower viral levels (they were HBeAg negative and had lower levels of HBcrAg/HBV DNA), compared to those with the HBsAg levels above 500 IU/ml ($P < 0.001$).

Factors Associated With High AFP Levels at the Initial Visit, Stratified According to the HBsAg Levels

Blood samples from all 1,610 patients were analyzed to determine the factors that affect the AFP level at the initial visit. Among 1,277 patients with the HBsAg levels more than 500 IU/ml at the initial visit, high AFP levels were detected in 228 (17.9%) patients. Univariate analysis identified 12 parameters that correlated significantly with a high AFP level at the initial visit. These included age (≥30 years; $P < 0.001$), AST (≥34 IU/L; $P < 0.001$), ALT (≥43 IU/L; $P < 0.001$), albumin (<3.9 g/dl; $P < 0.001$), GGT (≥50 IU/L; $P < 0.001$), total bilirubin (≥1.0 mg/dl; $P < 0.001$), platelet count ($<20.0 \times 10^4/mm^3$; $P < 0.001$), HBV genotype (C; $P < 0.001$), HBsAg levels (<7,000 IU/ml; $P < 0.001$), HBeAg (positive; $P < 0.001$), HBV DNA (≥5.0 log copies/ml; $P < 0.001$), and HBcrAg (≥3.0 log U/ml; $P < 0.001$). Multivariate analysis that included the above variables identified seven factors that influenced independently the elevated AFP level at the initial visit. These included HBsAg level (<7,000 IU/ml; OR 3.69, $P < 0.001$), albumin (<3.9 g/dl; OR 3.09, $P < 0.001$), platelet count ($<20.0 \times 10^4/mm^3$; OR 2.50, $P = 0.001$), GGT (≥50 IU/L; OR 2.28, $P = 0.001$), AST (≥34 IU/L; OR 2.77, $P = 0.003$), HBeAg (positive; OR 2.07, $P = 0.005$), and HBcrAg (≥3.0 log U/ml; OR 5.10, $P = 0.031$) (Table III).

Among 333 patients with the HBsAg levels less than 500 IU/ml, a high AFP at the initial visit was detected in 42 (12.6%) patients. Univariate analysis identified nine parameters that correlated significantly with a high AFP level at the initial visit. These included AST (≥34 IU/L; $P < 0.001$), ALT (≥43 IU/L; $P = 0.001$), albumin (<3.9 g/dl; $P < 0.001$), GGT (≥50 IU/L; $P < 0.001$), platelet count ($<20.0 \times 10^4/mm^3$; $P = 0.001$), HBV genotype (C; $P < 0.001$), HBeAg (positive; $P < 0.001$), HBV DNA (≥5.0 log copies/ml; $P = 0.001$), and HBcrAg (≥3.0 log U/ml; $P < 0.001$). Multivariate analysis that included the above variables identified three factors that influenced independently the elevated AFP level at the initial visit. These included albumin (<3.9 g/dl; OR 12.8, $P < 0.001$), GGT (≥50 IU/L; OR 6.95, $P = 0.002$), and HBcrAg (≥3.0 log U/ml; OR 5.62, $P = 0.010$) (Table III).

Factors Associated With High AFP Levels at the Initial Visit According to the HBsAg Levels in Patients With Low Transaminase Levels

To minimize the effect of inflammatory activity, we examined the data of 618 (among 1,610 patients) who

TABLE II. Profiles and Laboratory Data of Patients Infected With HBV According to the HBsAg Level at the Initial Visit

	HBsAg <500 IU/L	HBsAg ≥500 IU/L	*P*
Demographic data			
Number of patients	333	1,277	
Sex (male/female)	227/106	820/457	NS
Age (years)*	49 (18–75)	38 (18–83)	<0.001
Family history of liver disease[a]	130 (39.0%)	706 (55.3%)	<0.001
Lifetime cumulative alcohol intake (≥500 kg)	32 (9.6%)	80 (6.3%)	0.037
Laboratory data*			
Total bilirubin (mg/dl)	0.7 (0.2–2.9)	0.6 (0.1–2.9)	0.033
Aspartate aminotransferase (IU/L)	29 (12–175)	40 (5–220)	<0.001
Alanine aminotransferase (IU/L)	32 (7–289)	56 (5–297)	<0.001
Albumin (g/dl)	4.2 (1.1–5.6)	4.2 (1.0–5.5)	NS
Gamma-glutamyl transpeptidase (IU/L)	36 (2–2,370)	38 (4–1,638)	NS
Hemoglobin (g/dl)	14.4 (8.4–17.4)	14.6 (6.9–18.2)	NS
Platelet count ($\times 10^4/mm^3$)	19.1 (2.7–39.6)	19.2 (3.1–44.7)	NS
Alpha-fetoprotein (μg/L)	4 (1–968)	4 (1–1,770)	0.005
Virological data			
HBeAg (No. of positive)	37 (11.1%)	653 (51.1%)	<0.001
HBsAg (IU/ml)*	123 (0.09–498)	4,680 (503 to >125,000)	<0.001
HBcrAg (log U/ml)*	<3.0 (<3.0 to >6.8)	5.9 (<3.0 to >6.8)	<0.001
HBV DNA (log copies/ml)*	3.7 (<2.1 to >9.1)	6.6 (<2.1 to >9.1)	<0.001
HBV genotype (A/B/C/others/ND)	7/104/141/0/81	58/114/978/6/121	<0.001

NS; not significant.
Data are number/percentages of patients, except those denoted by *, which represent the median (range) values.
[a]Family history of positivity for hepatitis B surface antigen including third-degree relatives.

TABLE III. Results of Multivariate Logistic Analysis for Factors Associated With the High AFP Levels at the Initial Visit

Factor	Category	Risk ratio (95%CI)	P
Patients with the HBsAg levels above 500 IU/ml (n = 1,277)			
HBsAg (IU/ml)	1: ≥7,000	1	
	2: <7,000	3.69 (2.12–6.41)	<0.001
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	3.09 (1.88–5.05)	<0.001
Platelet count ($\times 10^4$/mm^3)	1: ≥20.0	1	
	2: <20.0	2.50 (1.47–4.24)	0.001
Gamma-glutamyl transpeptidase (IU/L)	1: <50	1	
	2: ≥50	2.28 (1.40–3.72)	0.001
Aspartate aminotransferase (IU/L)	1: <34	1	
	2: ≥34	2.77 (1.42–5.39)	0.003
HBeAg	1: Negative	1	
	2: Positive	2.07 (1.24–3.45)	0.005
HBcrAg (log U/ml)	1: <3.0	1	
	2: ≥3.0	5.10 (1.16–22.4)	0.031
Patients with the HBsAg levels below 500 IU/ml (n = 333)			
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	12.8 (4.02–41.7)	<0.001
Gamma-glutamyl transpeptidase (IU/L)	1: <50	1	
	2: ≥50	6.95 (2.06–23.5)	0.002
HBcrAg (log U/ml)	1: <3.0	1	
	2: ≥3.0	5.62 (1.51–21.0)	0.010

Low transaminase levels were defined as transaminase levels below the upper limit of normal.

had low transaminase levels (AST ≤33 IU/L and ALT ≤42 IU/L, i.e., below the upper limits of normal) to further determine those factors that determine the high level of AFP at the initial visit. High AFP was detected in 26 (6.1%) patients among 426 with the HBsAg levels above 500 IU/ml and low transaminase levels. Using the data of these patients, univariate analysis identified three parameters that correlated significantly with a high AFP level at the initial visit. These included albumin (<3.9 g/dl; $P = 0.004$), platelet count (<20.0 × 10^4/mm^3; $P = 0.012$), and HBsAg levels (<7,000 IU/ml; $P = 0.004$). Multivariate analysis that included the above variables identified albumin (<3.9 g/dl; OR 3.92, $P = 0.001$) and HBsAg levels (<7,000 IU/ml; OR 4.33, $P = 0.004$) as independent determinants of a high AFP level at the initial visit (Table IV).

Among 192 patients with the HBsAg levels below 500 IU/ml and low transaminase levels, high AFP levels were detected at the initial visit in 12 (6.3%). Univariate analysis identified three parameters that influenced significantly the elevated AFP level at the initial visit. These included albumin (<3.9 g/dl; $P = 0.010$), GGT (≥50 IU/L; $P = 0.011$), and platelet count (<20.0 × 10^4/mm^3; $P = 0.020$). Multivariate analysis that included these variables identified albumin (<3.9 g/dl; OR 7.19, $P = 0.004$) as the only independent determinant of a high AFP level at the initial visit (Table IV).

TABLE IV. Results of Multivariate Analysis for Factors Associated With the High AFP Levels at the Initial Visit

Factor	Category	Risk ratio (95%CI)	P
Patients with HBsAg >500 IU/ml and low transaminase levels (n = 426)			
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	3.92 (1.71–9.01)	0.001
HBsAg (IU/ml)	1: ≥7,000	1	
	2: <7,000	4.33 (1.58–11.9)	0.004
Patients with HBsAg <500 IU/ml and low transaminase levels (n = 192)			
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	7.19 (1.87–27.8)	0.004

Low transaminase levels were defined as transaminase levels below the upper limit of normal.

DISCUSSION

There is little information on the cutoff value of AFP that can be used to predict the future probability of HCC in patients with HBV infection. The present study followed-up patients naïve to antiviral therapy from the initial visit and showed that the rate of hepatocarcinogenesis was significantly higher in those with high AFP levels at the baseline than those with low levels. To our knowledge, the present study is the first to report the hepatocarcinogenesis rate stratified according to the AFP level in patients infected with HBV but free of HCC at the initial visit, based on a large-scale long-term follow-up cohort. The results indicated that patients with high AFP levels at the initial visit are at high risk of HCC, and emphasize the need to determine the factors that could affect the AFP level as surrogate markers of early hepatocarcinogenesis. Previous studies in patients with HCV infection indicated that suppression of the AFP level by treatment with interferon reduced the HCC risk even in those without complete eradication of HCV [Arase et al., 2007; Asahina et al., 2013]. However, there is little

evidence that suppression of the AFP level by antiviral therapy reduces the HCC risk in patients with HBV infection. Further prospective studies are needed to investigate this issue in detail.

In the present study, the relationship between the HBsAg levels and the AFP levels detected at the initial visit suggested the presence of two distinct groups within the study patients. Interestingly, in patients with the HBsAg levels above 500 IU/ml, a significant negative correlation was observed between the HBsAg and the AFP levels, and a significant positive correlation was observed between the HBsAg and the platelet count. Previous studies indicated that high serum AFP levels correlated with liver fibrosis Stage 3 and 4 [Bayati et al., 1998; Chu et al., 2001; Hu et al., 2002, 2004], and that lower thrombocytopenia was closely associated with advanced liver disease [Ikeda et al., 2009; Akuta et al., 2012]. Considered together, these results emphasize the importance of hyper-α-fetoproteinemia and thrombocytopenia in the prediction of severe liver fibrosis, respectively. Based on the present results and the recent reports suggesting the potential correlation between the HBsAg level and the stage of liver fibrosis [Seto et al., 2012; Martinot-Peignoux et al., 2013], it is possible that HBsAg levels could correlate with the stage of fibrosis in patients with the HBsAg levels above 500 IU/ml. Further studies are needed to determine the value of hyper-α-fetoproteinemia in patients with low and high HBsAgemia.

In addition to the HBsAg level, multivariate analysis also identified HBcrAg as another viral factor that influenced independently the AFP level at the baseline. HBcrAg comprises HBcAg, HBeAg and a 22-kDa precore protein coded with the precore/core gene [Kimura et al., 2002, 2005]. Previous studies reported a significant correlation between serum HBcrAg concentrations and intrahepatic levels of covalently closed circular DNA (cccDNA) [Wong et al., 2007; Suzuki et al., 2009]. Other studies indicated that HBcrAg is a useful predictor of HCC during antiviral therapy [Kumada et al., 2013], and post-treatment recurrence of HCC during antiviral therapy [Hosaka et al., 2010]. The present study, based on patients naïve to antiviral therapy showed that high serum HBcrAg concentrations also correlated with high AFP at the initial visit. This is the first report demonstrating the potential usefulness of HBcrAg as a surrogate marker for early hepatocarcinogenesis.

The impact of the HBsAg level on hepatocarcinogenesis is not clear at this stage. In this study, the effect of the HBsAg levels at the initial visit on HCC was assessed in 1,061 consecutive antiviral therapy-naive patients infected with HBV. Analysis of data of 794 patients with the HBsAg levels above 500 IU/ml at the initial visit (after exclusion of patients on antiviral therapy) showed a significantly lower cumulative HCC rate in patients with the HBsAg levels above 7,000 IU/ml than those with levels below 7,000 IU/ml ($P < 0.001$, Log-rank test, Fig. 4). This

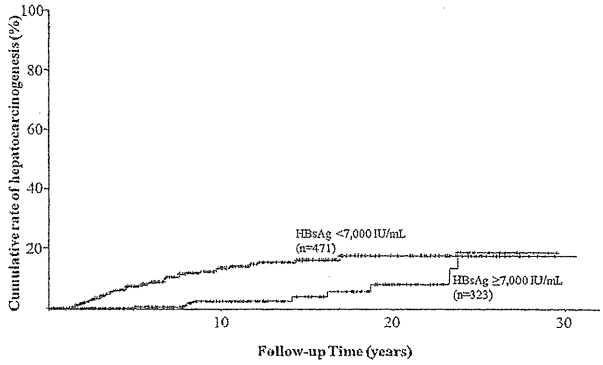

Fig. 4. Cumulative rate of hepatocarcinogenesis stratified according to the HBsAg levels at the initial visit in patients naïve to antiviral therapy from the initial visit until last visit. In a preliminary study based on 794 patients with the HBsAg levels above 500 IU/ml at the initial visit, the cumulative hepatocarcinogenesis rate for patients with the HBsAg levels more than 7,000 IU/ml was significantly lower than for those with levels below 7,000 IU/ml ($P < 0.001$; Log-rank test).

result suggests that HBsAg levels at the baseline do not only influence AFP, but also play a role in hepatocarcinogenesis. Further studies need to be performed to determine the pathomechanisms of HBsAg in hepatocarcinogenesis.

The present study has certain limitations. First, the study did not examine the effects of other genotypes, apart from HBV genotype B or C. Second, the study population was limited to Japanese and did not include other races, and thus generalization of the results to other races cannot be made based on the results. Third, the study did not investigate the effects of antiviral therapy (interferon and/or nucleot(s)ide analogs) on the outcome since such therapy suppressed the AFP levels and thus reduce the risk of HCC in patients with HBV infection.

In conclusion, the present studies demonstrated that the HBsAg level seem to influence the AFP levels and can be used as a surrogate marker for early hepatocarcinogenesis in patients with hepatitis B viral infection.

REFERENCES

Akuta N, Suzuki F, Seko Y, Kawamura Y, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Hara T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2012. Complicated relationships of amino acid substitution in hepatitis C virus core region and IL28B genotype influencing hepatocarcinogenesis. Hepatology 56:2134–2141.

Alpert E, Feller ER. 1978. α-fetoprotein (AF) in benign liver disease. Gastroenterology 74:856–858.

Arase Y, Ikeda K, Suzuki F, Suzuki Y, Kobayashi M, Akuta N, Hosaka T, Sezaki H, Yatsuji H, Kawamura Y, Kobayashi M, Kumada H. 2007. Prolonged-interferon therapy reduces hepatocarcinogenesis in aged-patients with chronic hepatitis C. J Med Virol 79:1095–1102.

Asahina Y, Tsuchiya K, Nishimura T, Muraoka M, Suzuki Y, Tamaki N, Yasui Y, Hosokawa T, Ueda K, Nakanishi H, Itakura J, Takahashi Y, Kurosaki M, Enomoto N, Nakagawa M, Kakinuma S, Watanabe M, Izumi N. 2013. α-Fetoprotein levels after interferon therapy and risk of hepatocarcinogenesis in chronic hepatitis C. Hepatology 58:1253–1262.

Bayati N, Silverman Al, Gordon SC. 1998. Serum alpha-fetoprotein levels and liver histology in patients with chronic hepatitis C. Am J Gastroenterol 93:2452–2456.

Bergstrand CG, Czar B. 1956. Demonstration of a new protein fraction in serum from the human fetus. Scand J Clin Lab Invest 8:174.

Bruix J, Sherman M. 2005. Practice guidelines committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. Hepatology 42:1208–1236.

Brunetto MR, Moriconi F, Bonino F, Lau GK, Farci P, Yurdaydin C, Piratvisuth T, Luo K, Wang Y, Hadziyannis S, Wolf E, McCloud P, Batrla R, Marcellin P. 2009. Hepatitis B virus surface antigen levels: A guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. Hepatology 49:1141–1150.

Brunetto MR, Oliveri F, Colombatto P, Moriconi F, Ciccorossi P, Coco B, Romagnoli V, Cherubini B, Moscato G, Maina AM, Cavallone D, Bonino F. 2010. Hepatitis B surface antigen serum levels help to distinguish active from inactive hepatitis B virus genotype D carriers. Gastroenterology 139:483–490.

Chan HL. 2012. Identifying hepatitis B carriers at low risk for hepatocellular carcinoma. Gastroenterology 142:1057–1060.

Chan HL, Wong VW, Wong GL, Tse CH, Chan HY, Sung JJ. 2010. A longitudinal study on the natural history of serum hepatitis B surface antigen changes in chronic hepatitis B. Hepatology 52:1232–1241.

Chan HL, Thompson A, Martinot-Peignoux M, Piratvisuth T, Cornberg M, Brunetto MR, Tillmann HL, Kao JH, Jia JD, Wedemeyer H, Locarnini S, Janssen HL, Marcellin P. 2011. Hepatitis B surface antigen quantification: Why and how to use it in 2011—A core group report. J Hepatol 55:1121–1131.

Cheema AW, Hirschtritt T, Van Thiel DH. 2004. Markedly elevated alpha-fetoprotein levels without hepatocellular carcinoma. Hepatogastroenterology 51:1676–1678.

Chen DS, Sung JL. 1979. Relationship of hepatitis B surface antigen to serum alpha-fetoprotein in nonmalignant diseases of the liver. Cancer 44:984–992.

Chu CW, Hwang SJ, Luo JC, Lai CR, Tsay SH, Li CP, Wu JC, Chang FY, Lee SD. 2001. Clinical, virological, and pathologic significance of elevated serum alpha-fetoprotein levels in patients with chronic hepatitis C. J Clin Gastroenterol 32:240–244.

Di Bisceglie AM, Hoofnagle JH. 1989. Elevations in serum alpha-fetoprotein levels in patients with chronic hepatitis B. Cancer 64:2117–2120.

Ebara M, Ohto M, Shinagawa T, Sugiura N, Kimura K, Matsutani S, Morita M, Saisho H, Tsuchiya Y, Okuda K. 1986. Natural history of minute hepatocellular carcinoma smaller than three centimeters complicating cirrhosis. A study in 22 patients. Gastroenterology 90:289–298.

Elftherious N, Heathcote J, Thomas HC, Sherlock S. 1977. Serum alpha-fetoprotein levels in patients with acute and chronic liver disease. J Clin Pathol 30:704–708.

Hosaka T, Suzuki F, Kobayashi M, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, Akuta N, Suzuki Y, Saitoh S, Arase Y, Ikeda K, Kobayashi M, Kumada H. 2010. HBcrAg is a predictor of post-treatment recurrence of hepatocellular carcinoma during antiviral therapy. Liver Int 30:1461–1470.

Hu KQ, Esrailian E, Thompson K, Chase R, Kyulo N, Hassen M, Abdelhalim F, Hillebrand DJ, Runyon BA. 2002. Hepatic steatosis is associated with disease progression of chronic hepatitis C: A large cohort study in the United States. Hepatology 36:349A.

Hu KQ, Kyulo N, Lim N, Elhazin B, Hillebrand DJ, Bock T. 2004. Clinical significance of elevated alpha-fetoprotein (AFP) in patients with chronic hepatitis C, but not hepatocellular carcinoma. Am J Gastroenterol 99:860–865.

Ikeda K, Arase Y, Kawamura Y, Yatsuji H, Sezaki H, Hosaka T, Akuta N, Kobayashi M, Saitoh S, Suzuki F, Suzuki Y, Kumada H. 2009. Necessities of interferon therapy in elderly patients with chronic hepatitis C. Am J Med 122:479–486.

Jaroszewicz J, Calle Serrano B, Wursthorn K, Deterding K, Schlue J, Raupach R, Flisiak R, Bock CT, Manns MP, Wedemeyer H, Cornberg M. 2010. Hepatitis B surface antigen (HBsAg) levels in the natural history of hepatitis B virus (HBV)-infection: A European perspective. J Hepatol 52:514–522.

Johnson PJ. 2001. The role of serum alpha-fetoprotein estimation in the diagnosis and management of hepatocellular carcinoma. Clin Liv Dis 5:145–159.

Kew MC, Purves LR, Bersohn I. 1973. Serum alpha-fetoprotein levels in acute viral hepatitis. Gut 14:939–942.

Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa K, Maki N. 2002. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J Clin Microbiol 40:439–445.

Kimura T, Ohno N, Terada N, Rokuhara A, Matsumoto A, Yagi S, Tanaka E, Kiyosawa K, Ohno S, Maki N. 2005. Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. J Biol Chem 280:21713–21719.

Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, Kobayashi M, Suzuki F, Akuta N, Someya T, Matsuda M, Sato J, Kumada H. 2002. Clinical characteristics of patients infected with hepatitis B virus genotypes A, B, and C. J Gastroenterol 37:35–39.

Kumada T, Toyoda H, Tada T, Kiriyama S, Tanikawa M, Hisanaga Y, Kanamori A, Niinomi T, Yasuda S, Andou Y, Yamamoto K, Tanaka J. 2013. Effect of nucleos(t)ide analogue therapy on hepatocarcinogenesis in chronic hepatitis B patients: A propensity score analysis. J Hepatol 58:427–433.

Liaw YF. 2011. Clinical utility of hepatitis B surface antigen quantitation in patients with chronic hepatitis B: A review. Hepatology 54:E1–E9.

Martinot-Peignoux M, Lada O, Cardoso AC, Lapalus M, Boyer N, Ripault MP, Asselah T, Marcellin P. 2010. Quantitative HBsAg: A new specific marker for the diagnosis of HBsAg inactive carriage. Hepatology 52:992A.

Martinot-Peignoux M, Carvalho-Filho R, Lapalus M, Netto-Cardoso AC, Lada O, Batrla R, Krause F, Asselah T, Marcellin P. 2013. Hepatitis B surface antigen serum level is associated with fibrosis severity in treatment-naïve, E antigen-positive patients. J Hepatol 58:1089–1095.

Moucari R, Mackiewicz V, Lada O, Ripault MP, Castelnau C, Martinot-Peignoux M, Dauvergne A, Asselah T, Boyer N, Bedossa P, Valla D, Vidaud M, Nicolas-Chanoine MH, Marcellin P. 2009. Early serum HBsAg drop: A strong predictor of sustained virological response to pegylated interferon alfa-2a in HBeAg-negative patients. Hepatology 49:1151–1157.

Nguyen T, Thompson AJ, Bowden S, Croagh C, Bell S, Desmond PV, Levy M, Locarnini SA. 2010. Hepatitis B surface antigen levels during the natural history of chronic hepatitis B: A perspective on Asia. J Hepatol 52:508–513.

Sato Y, Nakata K, Kato Y, Shima M, Ishii N, Koji T, Taketa K, Endo Y, Nagataki S. 1993. Early recognition of hepatocellular carcinoma based on altered profiles of alpha-fetoprotein. N Engl J Med 328:1802–1806.

Seto WK, Wong DK, Fung J, Ip PP, Yuen JC, Hung IF, Lai CL, Yuen MF. 2012. High hepatitis B surface antigen levels predict insignificant fibrosis in hepatitis B e antigen positive chronic hepatitis B. PLoS ONE 7:e43087.

Shinagawa T, Ohto M, Kimura K, Tsunetomi S, Morita M, Saisho H, Tsuchiya Y, Saotome N, Karasawa E, Miki M. 1984. Diagnosis and clinical features of small hepatocellular carcinoma with emphasis on the utility of real-time ultrasonography. A study in 51 patients. Gastroenterology 86:495–502.

Silver HK, Gold P, Shuster J, Javitt NB, Freedman SO, Finlayson ND. 1974. Alpha 1-fetoprotein in chronic liver disease. N Engl J Med 291:506–508.

Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. 2009. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. J Med Virol 81:27–33.

Tseng TC, Liu CJ, Yang HC, Su TH, Wang CC, Chen CL, Kuo SF, Liu CH, Chen PJ, Chen DS, Kao JH. 2012. High levels of hepatitis B surface antigen increase risk of hepatocellular carcinoma in patients with low HBV load. Gastroenterology 142:1140–1149.

Viola LA, Barrison IG, Coleman JC, Paradinas FJ, Fluker JL, Evans BA, Murray-Lyon IM. 1981. Natural history of liver disease in chronic hepatitis B surface antigen carriers. Survey of 100 patients from Great Britain. Lancet 2:1156–1159.

Wong DK, Tanaka Y, Lai CL, Mizokami M, Fung J, Yuen MF. 2007. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. J Clin Microbiol 45:3942–3947.

Yao FY. 2003. Dramatic reduction of the alpha-fetoprotein level after lamivudine treatment of patients with chronic hepatitis B virus infection and cirrhosis. J Clin Gastroenterol 36:440–442.

Journal of Medical Virology 86:169–175 (2014)

Impact of Virus Clearance for the Development of Hemorrhagic Stroke in Chronic Hepatitis C

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The aim of this retrospective cohort study was to assess the cumulative incidence and predictive factors for intracerebral hemorrhagic stroke after the termination of interferon (IFN) therapy in Japanese patients with hepatitis C virus (HCV). A total of 4,649 HCV-positive patients treated with IFN were enrolled. The primary goal is the first onset of intracerebral hemorrhagic stroke. The mean observation period was 8.0 years. Evaluation was performed using the Kaplan–Meier method and the Cox proportional hazard model. A *P*-value of less than 0.05 was considered statistically significant. A total of 28 developed intracerebral hemorrhagic stroke. The cumulative incidence of intracerebral hemorrhagic stroke was 0.3% at 5 years, 0.8% at 10 years, and 1.7% at 15 years. Intracerebral hemorrhagic stroke occurred when patients had age increments of 10 years (hazard ratio: 2.77; 95% confidence interval (CI) 1.48–5.18; $P=0.001$), hypertension (hazard ratio: 2.30; 95% CI 1.09–4.83; $P=0.021$), liver cirrhosis (hazard ratio: 4.50; 95% CI 2.07–9.78; $P<0.001$), and HCV non-clearance (hazard ratio: 3.22; 95% CI 1.22–8.53; $P=0.018$). On the intracerebral hemorrhagic stroke based on the difference of liver fibrosis and efficacy of IFN therapy, HCV clearance reduced to 24.3% (1/4.11) compared to HCV non-clearance in cirrhotic patients ($P=0.040$). In conclusion, HCV clearance reduced the development of intracerebral hemorrhagic stroke. In particular, HCV clearance reduced intracerebral hemorrhagic stroke to about one-fourth in cirrhotic patients. ***J. Med. Virol. 86:169–175, 2014.***

KEY WORDS: hepatitis C virus; interferon therapy; hemorrhagic stroke

INTRODUCTION

There are 170 million people affected with chronic hepatitis C virus (HCV) infection worldwide, which may cause an insidiously progressive form of liver disease that relentlessly but silently progresses to cirrhosis in 20–50% of cases over a period of 10–30 years [Kiyosawa and Furuta, 1991; Alter et al., 1992]. In addition, HCV is a major risk for hepatocellular carcinoma (HCC) [Hasan et al., 1990; Kew et al., 1990; Ikeda et al., 1993; Tsukuma et al., 1993; Arase et al., 2012]. In addition, several authors have reported that HCV clearance decreases the rate of fibrosis progression and the development of HCC in patients with chronic HCV infection [Kasahara et al., 1998; Yoshida et al., 2002; Arase et al., 2013].

On the other hand, hemorrhagic stroke is a medical emergency and can cause permanent neurological damage and death [Truelsen et al., 2003; Iso et al., 2007; Donnan et al., 2008]. It is becoming a great health burden in most countries. However, there is a little information on the incidence and risk factors on the incidence of hemorrhagic stroke in HCV patients treated with interferon (IFN). Furthermore, it is not clear whether the HCV clearance is useful for

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; CT, computed tomography; GGT, gamma-glutamyltransferase; HbA_{1C}, hemoglobin A_{1C}; HCV, hepatitis C virus; HDL, high density lipoprotein; IFN, interferon; LDL, low density lipoprotein

Grant sponsor: Japanese Ministry of Health, Labour and Welfare (partial support)

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Accepted 9 August 2013

DOI 10.1002/jmv.23777
Published online 24 October 2013 in Wiley Online Library (wileyonlinelibrary.com).

reducing the development of hemorrhagic stroke in HCV patients.

With this background in mind, the present retrospective cohort study was initiated to investigate the cumulative incidence and risk factors of cerebral stroke after prolonged follow-up in HCV patients treated with IFN. The strengths of the current study are the large numbers of patients included and the long-term follow-up of patients.

PATIENTS AND METHODS

Patients

The number of patients who were diagnosed with chronic HCV infection and treated for the first time with IFN monotherapy or combination therapy between September 1990 and May 2010 in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan was 7,635. Of these, 4,649 patients satisfied with the following enrolled criteria: (1) features of chronic hepatitis or cirrhosis diagnosed via laparoscopy and/or liver biopsy within 1 year before the initiation of IFN therapy; (2) positivity for serum HCV-RNA before the initiation of IFN therapy; (3) period of $\geq$1 month to $\leq$1 year of IFN therapy; (4) negativity for hepatitis B surface antigens (HBsAg), antibody to hepatitis B core, or antimitochondrial antibodies in serum, as determined by radioimmunoassay, enzyme-linked immunosorbent assay or indirect immunofluorescence assay; (5) age of $\geq$30 to $\leq$80 years; and (6) no autoimmune systemic disease, such as systemic lupus erythematosus or rheumatic arthritis. Patients with either of the following criteria were excluded from the study: (1) they had illnesses that could seriously reduce their life expectancy; (2) they had a history of coronary and/or cerebrovascular disease; (3) they had a history of carcinogenesis; and (4) they had been given anticoagulant and antiplatelet drugs.

The primary outcome is the first development of hemorrhagic stroke. Hemorrhagic stroke was regarded as intracerebral hemorrhagic stroke in the present study. Thus, patients with subarachnoid hemorrhagic stroke or subdural hematoma were excluded from analyses. The development of hemorrhagic stroke was diagnosed by clinical symptoms and imaging (computed tomography and/or magnetic resonance imaging) based on the World Health Organization definition [Truelsen et al., 2003; Iso et al., 2007; Donnan et al., 2008]. All of the studies were performed retrospectively by collecting and analyzing data from the patient records. The physicians in charge explained the purpose, method, and side effect of IFN therapy to each patient and/or patients' family. In addition, the physicians in charge got permission of serum stores and future uses of stored serum. Informed consent for IFN therapy and future uses of stored serum was obtained from all patients. This study had been approved by Institutional Review Board of our hospital.

Medical Evaluation

Body weight was measured in light clothing and without shoes to the nearest 0.1 kg. Height was measured to the nearest 0.1 cm. Height and weight were recorded at baseline, and the body mass index (BMI) was calculated as kg/m^2. All patients were interviewed by physicians or nurse staff in the Toranomon Hospital using a questionnaire that gathered information on demographic characteristics, medical history, and health-related habits including questions on alcohol intake and smoking history.

Hemoglobin A_{1C} (HbA_{1C}) was estimated as National Glycohemoglobin Standardization Program equivalent value (%) and fasting plasma glucose [American Diabetes Association, 2010]. Patients were defined as having type 2 diabetes mellitus when HbA_{1C} level was $\geq$6.5% and/or fasting plasma glucose level was $\geq$126 mg/dl. Patients were defined as hypertensive when blood pressure was $\geq$140/90 mmHg or pharmacological treatment for high blood pressure was given. Smoking index (package per day $\times$ year) and total alcohol intake were evaluated by the sum of before, during, and after the IFN therapy.

Laboratory Investigation

Diagnosis of HCV infection was based on detection of serum HCV antibody and positive RNA. Anti-HCV was detected using an enzyme-linked immunosorbent assay (ELISA II) (Abbott Laboratories, North Chicago, IL). HCV-genotype was examined via polymerase chain reaction assay, using a mixture of primers for the six subtypes known to exist in Japan, as reported [Dusheiko et al., 1994]. HCV-RNA was determined by the COBAS TaqMan HCV test (Roche Diagnostics, Basel, Switzerland). The serum samples stored at -80°C before IFN therapy were used. The linear dynamic range of the assay was 1.2–7.8 log IU/ml, and the undetectable samples were defined as negative. A HCV clearance was defined as clearance of HCV RNA using the COBAS TaqMan HCV test 6 months after the cessation of IFN therapy.

Evaluation of Liver Cirrhosis

Status of liver was mainly determined on the basis of peritoneoscopy and/or liver biopsy. Liver biopsy specimens were obtained using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The size of specimens for examination was more than six portal areas [Desmet et al., 1994].

Follow-Up

The observation starting point was 6 months after the termination of IFN therapy. After that, patients were followed up at least twice a year in our hospital.

Biochemical tests were conducted at each examination together with regular check-up. Four hundred fifty patients were lost to follow-up. The final date of follow-up in 452 patients with loss of follow-up was regarded as last consulting day.

Patients with either of the following criteria during follow-up were regarded as censored data in statistical analysis [Fleming et al., 1984]: (1) they were retreated with IFN (N = 949); (2) they had new onset of carcinogenesis (N = 645); and (3) they had been given anticoagulant and antiplatelet drugs (N = 28). The final date of follow-up in these patients with censored data was regarded as the time of the initiation of criteria described above. The mean follow-up period was 6.7 [standard deviation (SD) 4.3] years in 452 patients with loss of follow-up and 7.4 (SD 4.7) years in 1,722 patients who had censored data. Patients with loss of follow-up and censored data were counted in the analysis.

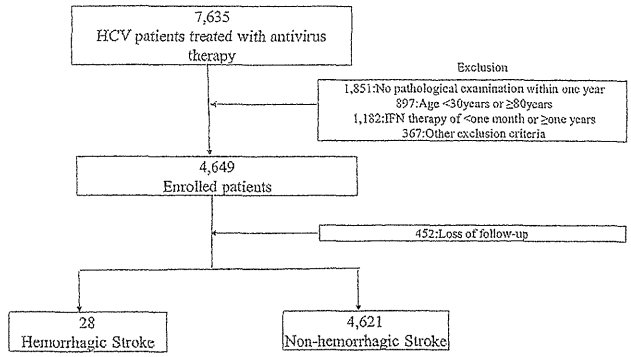


Fig. 1. An algorithm of the study population.

Statistical Analysis

Clinical differences between patients with hemorrhagic stroke and those without events were evaluated using Mann–Whitney test. The cumulative incidence of hemorrhagic stroke were calculated by using the Kaplan–Meier technique, and differences in the curves were tested using the log-rank test [Kaplan and Meier, 1958; Harrington and Fleming, 1983]. Independent risk factors associated with hemorrhagic stroke were studied using the stepwise Cox regression analysis [Cox, 1972]. The following

TABLE I. Clinical Backgrounds at the Initiation of Follow-Up in Enrolled Patients

	Total	Hemorrhagic stroke group	Without events group	*P*-value
N	4,649	28	4,621	
Age (years)	51.9 ± 11.8	60.4 ± 6.7	51.8 ± 11.9	<0.001
Gender (M/F)	2,966/1,883	16/12	2,950/1,871	0.781
Height (cm)	163.1 ± 9.2	159.5 ± 9.4	163.2 ± 9.2	0.171
Weight (kg)	61.4 ± 12.8	57.9 ± 8.0	61.4 ± 12.7	0.113
BMI	22.7 ± 3.1	23.4 ± 2.8	22.7 ± 3.1	0.582
BP (systolic, mmHg)	128 ± 18	140 ± 20	127 ± 18	0.007
BP (diastolic, mmHg)	77 ± 13	86 ± 15	77 ± 13	0.001
Total alcohol intake (kg)[a]	95 ± 92	148 ± 105	94 ± 92	0.002
Smoking index[a]	6.5 ± 9.5	11.8 ± 12.4	6.4 ± 9.4	<0.001
AST (IU/L)	41 ± 43	48 ± 28	41 ± 43	<0.001
ALT (IU/L)	44 ± 53	53 ± 38	43 ± 52	0.004
GGT (IU/L)	53 ± 60	59 ± 47	52 ± 61	0.078
Albumin (g/dl)	4.0 ± 0.3	3.5 ± 0.4	4.0 ± 0.3	0.110
Triglyceride (mg/dl)	101 ± 52	108 ± 46	100 ± 52	0.097
Cholesterol (mg/dl)	170 ± 31	171 ± 27	170 ± 31	0.893
HDL-C (mg/dl)	48 ± 14	45 ± 12	48 ± 14	0.002
LDL-C (mg/dl)	104 ± 29	108 ± 37	103 ± 29	0.049
Fasting plasma glucose (mg/dl)	99 ± 22	103 ± 23	100 ± 22	0.093
HbA_{1C} (%)	5.7 ± 1.1	5.9 ± 1.2	5.7 ± 1.1	0.024
Platelet ($\times 10^4/mm^3$)	17.2 ± 5.2	14.1 ± 6.2	17.3 ± 5.4	0.001
Staging (cirrhosis/non-cirrhosis)[b]	485/4,164	12/16	473/4,148	<0.001
HCV genotype (1b/2a/2b/other)[b]	2,859/1,109/497/184	22/5/1/0	2,837/1,104/496/184	0.104
HCV RNA (log IU/ml)[b]	6.07 ± 1.05	6.03 ± 1.03	6.08 ± 1.05	0.387
IFN monotherapy/combination therapy[c]	3,000/1,649	24/4	2,976/1,645	<0.001
Efficacy (HCV; clearance/non-clearance)	2,103/2,546	5/23	2,098/2,523	0.006

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BP, blood pressure; GGT, gamma-glutamyltransferase; HbA_{1C}; hemoglobin A_{1C}; HCV, hepatitis C virus; HDL, high density lipoprotein; IFN, interferon.
Data are number of patients or mean ± standard deviation.
[a]Smoking index is defined as package per day × year; total alcohol intake and smoking index indicate the sum before and after first consultation.
[b]Value before IFN treatment.
[c]Outbreak of IFN monotherapy: recombinant IFN alpha 2a, 238 cases; recombinant IFN alpha 2b, 183 cases; natural IFN alpha, 1,750 cases; natural IFN beta, 750 cases; total dose of IFN = 554 ± 164 MU. Outbreak of peg IFN monotherapy: peg IFN alpha 2a, 93 cases, total dose of peg IFN = 7.54 ± 2.20 mg.
Outbreak of combination therapy: recombinant IFN alpha 2b + ribavirin, 335 cases, total dose of IFN = 508 ± 184 MU, total dose of ribavirin = 160 ± 68 g; natural IFN beta + ribavirin, 127 cases, total dose of IFN = 502 ± 177 MU, total dose of ribavirin = 155 ± 67 g; peg IFN alpha 2b + ribavirin, 1,173 cases, total dose of peg IFN = 4.12 ± 1.10 mg, total dose of ribavirin = 205 ± 58 g.

variables were analyzed for potential covariates for incidence of primary outcome: (1) age, gender, type 2 diabetes mellitus, hypertension, BMI at the initiation time of follow-up, (2) HCV genotype, HCV load, and hepatic fibrosis before IFN therapy, (3) average value of aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride, total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, and platelet during follow-up, (4) sum value of smoking and alcohol before, during, and after the IFN therapy, (5) efficacy of IFN therapy, combination of ribavirin, type of IFN, and total dose of IFN. A P-value of less than 0.05 was considered statistically significant. Data analysis was performed using SPSS 11.5 for Windows (SPSS, Chicago, IL).

RESULTS

Patients Characteristics

Figure 1 shows the algorithm of the study population. For the mean observation period of 8.0 years, 28 of 4,649 patients developed hemorrhagic stroke. Table I shows the baseline characteristics of the enrolled 4,649 patients at the initiation of follow-up. The patients are divided into two groups of patients with hemorrhagic stroke and without event. There are significant differences in several baseline characteristics between the two groups. The HCV clearance rate was 34.7% (1,042/3,000) in IFN monotherapy and 64.3% (1,061/1,649) in combination therapy of IFN and ribavirin. Thus, the number of patients with HCV clearance was 2,103. The mean follow-up was 8.0 (SD 5.0) years. The 28-day vascular disease-related mortality rate was 33% (10/28) in hemorrhagic stroke.

Predictive Factors for the Development of Intracerebral Hemorrhagic Stroke

The cumulative incidence of intracerebral hemorrhagic stroke was 0.3% at 5 years, 0.8% at 10 years, and 1.7% at 15 years (Fig. 2A). The factors associated with the development of intracerebral hemorrhagic stroke are shown in Table II. Intracerebral hemorrhagic stroke occurred when patients had age increments of 10 years [hazard ratio: 2.77; 95% confidence interval (CI) 1.48–5.18; $P = 0.001$], hypertension

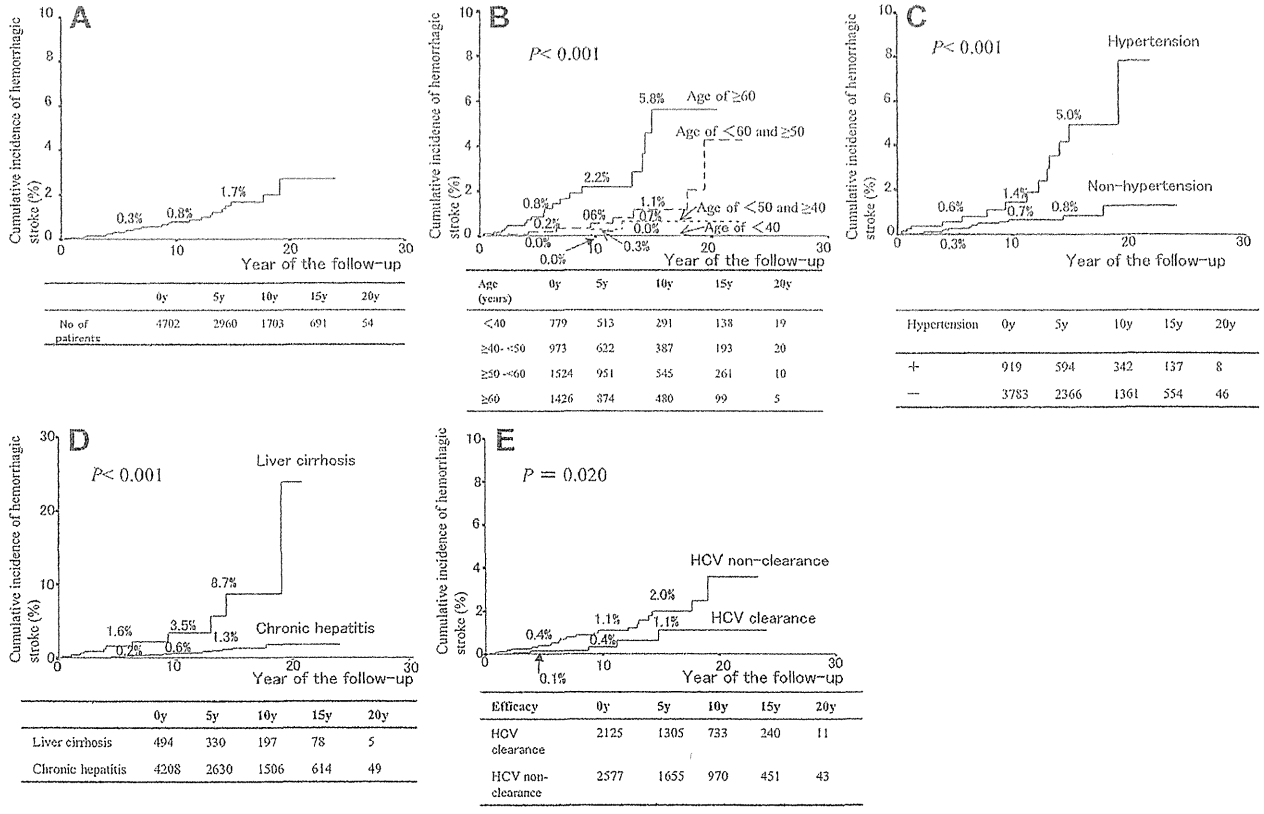


Fig. 2. **Panel A**: Cumulative development rate of intracerebral hemorrhagic stroke in total HCV patients treated with IFN therapy. **Panel B**: Cumulative development rate of intracerebral hemorrhagic stroke based on difference of age. **Panel C**: Cumulative development rate of ischemic stroke based on the difference of blood pressure. **Panel D**: Cumulative development rate of intracerebral hemorrhagic stroke based on difference of liver fibrosis. **Panel E**: Cumulative development rate of intracerebral hemorrhagic stroke based on difference of interferon efficacy.

TABLE II. Predictive Factors for the Development of Intracerebral Hemorrhagic Stroke

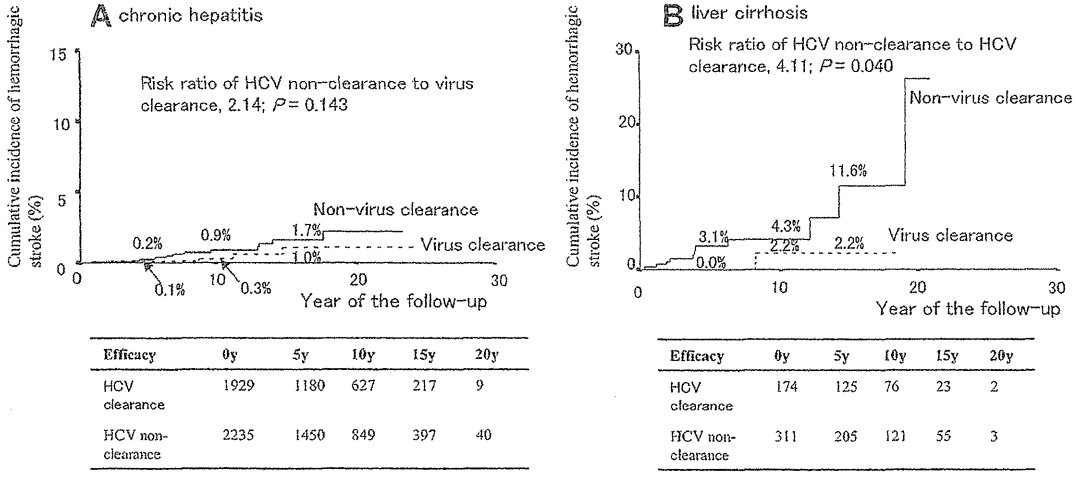
	Univariate analysis		Cox regression	
Variables	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years, per 10)	3.55 (1.96–6.43)	<0.001	2.77 (1.48–5.18)	0.001
Gender (M/F)	1.26 (0.65–2.44)	0.334		
BMI (≥22/<22)	0.97 (0.75–1.24)	0.767		
Diabetes (+/−)	3.40 (1.26–9.15)	0.015		
Hypertension (+/−)	4.07 (1.94–8.54)	<0.001	2.30 (1.09–4.83)	0.021
Smoking index (≥20/<20)[a]	2.12 (0.95–4.76)	0.068		
Total alcohol intake (kg, ≥200/<200)[a]	1.10 (0.53–4.37)	0.138		
AST (IU/L, ≥34/<34)	2.79 (1.17–6.66)	0.020		
ALT (IU/L, ≥36/<36)	2.68 (1.14–6.29)	0.023		
GGT (IU/L, ≥109/<109)	1.28 (0.610–1.89)	0.655		
Albumin (g/dl, <3.9/≥3.9)	2.96 (1.24–7.09)	0.015		
Triglyceride (mg/dl, ≥100/<100)	1.19 (0.83–1.49)	0.283		
Total cholesterol (mg/dl, <150/≥150)	1.06 (0.48–1.91)	0.936		
HDL-C (mg/dl, ≥40/<40)	0.96 (0.38–2.50)	0.960		
LDL-C (mg/dl, ≥120/<120)	0.81 (0.50–2.51)	0.572		
Platelet ($\times 10^4/mm^3$, <15/≥15)	3.22 (1.41–7.35)	0.005		
Histological diagnosis (cirrhosis/non-cirrhosis)	7.40 (3.30–16.77)	<0.001	4.50 (2.07–9.78)	<0.001
Combination of ribavirin (+/−)	0.80 (0.25–2.54)	0.701		
Type of IFN (α/β)	1.29 (0.65–2.33)	0.116		
Total dose of IFN (MU, ≥500/<500)	0.87 (0.39–1.99)	0.744		
HCV genotype (1/2)	1.53 (0.62–3.80)	0.360		
HCV RNA (log IU/ml, ≥5/<5)	1.35 (1.02–1.79)	0.035		
Efficacy (HCV: non-clearance/clearance)	2.98 (1.13–6.59)	0.020	3.22 (1.22–8.53)	0.018

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; GGT, gamma-glutamyltransferase; HCV, hepatitis C virus; IFN, interferon.
[a]Smoking index is defined as package per day × year; total alcohol intake and smoking index indicate the sum before and after first consultation.

(hazard ratio: 2.30; 95% CI 1.09–4.83; $P=0.021$), liver cirrhosis (hazard ratio: 4.50; 95% 2.07–9.78; $P<0.001$), and HCV non-clearance (hazard ratio: 3.22; 95% CI 1.22–8.53; $P=0.018$). Figure 2B–E shows the cumulative incidence of hemorrhagic stroke based on difference of age, blood pressure, liver fibrosis, and efficacy of IFN therapy.

Hemorrhagic Stroke Based on the Difference of Liver Fibrosis and Efficacy

Figure 3A,B shows the cumulative incidence of intracerebral hemorrhagic stroke based on the difference of liver fibrosis and efficacy of IFN therapy. As shown in Figure 3B, HCV clearance reduced



Efficacy	0y	5y	10y	15y	20y
HCV clearance	1929	1180	627	217	9
HCV non-clearance	2235	1450	849	397	40

Efficacy	0y	5y	10y	15y	20y
HCV clearance	174	125	76	23	2
HCV non-clearance	311	205	121	55	3

Fig. 3. **Panel A:** Cumulative development rate of intracerebral hemorrhagic stroke based on difference of efficacy after interferon treatment in HCV patients with chronic hepatitis. **Panel B:** Cumulative development rate of intracerebral hemorrhagic stroke based on the difference of efficacy after interferon treatment in HCV patients with liver cirrhosis.

TABLE III. Comparison in Clinical Backgrounds Between HCV Clearance and HCV Non-Clearance in Patients With Liver Cirrhosis

	HCV clearance group	HCV non-clearance group	*P*-value
N	174	311	
Age (years)	56.7 ± 9.6	57.0 ± 9.9	0.721
Gender (M/F)	108/66	184/127	0.562
BMI	23.8 ± 3.7	23.6 ± 3.5	0.479
BP (systolic, mmHg)	132 ± 18	131 ± 17	0.791
BP (diastolic, mmHg)	80 ± 11	79 ± 12	0.775
Total alcohol intake (kg)[a]	112 ± 97	128 ± 101	0.057
Smoking index[a]	6.2 ± 10.7	5.9 ± 10.2	0.129
AST (IU/L)	33 ± 20	73 ± 47	<0.001
ALT (IU/L)	34 ± 28	79 ± 61	<0.001
GGT (IU/L)	24 ± 26	61 ± 65	<0.001
Albumin (g/dl)	3.7 ± 0.4	3.5 ± 0.4	0.149
Triglyceride (mg/dl)	110 ± 47	104 ± 45	0.243
Cholesterol (mg/dl)	157 ± 29	161 ± 31	0.373
HDL-C (mg/dl)	42 ± 12	45 ± 12	0.257
LDL-C (mg/dl)	96 ± 26	95 ± 30	0.748
Fasting plasma glucose (mg/dl)	104 ± 22	109 ± 26	0.085
HbA_{1C} (%)	5.7 ± 1.2	6.0 ± 1.3	0.024
Platelet ($\times 10^4/mm^3$)	14.1 ± 6.2	17.3 ± 5.4	0.097
HCV genotype (1b/2a/2b/other)[b]	75/72/24/3	209/54/15/33	<0.001
HCV RNA (log IU/ml)[b]	5.32 ± 1.12	6.38 ± 1.00	<0.001
IFN monotherapy/combination therapy[c]	110/64	232/79	0.012

Data are number of patients or mean ± standard deviation, ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BP, blood pressure; GGT, gamma-glutamyltransferase; HbA_{1C}, hemoglobin A_{1C}; HCV, hepatitis C virus; HDL, high density lipoprotein; IFN, interferon.
[a]Smoking index is defined as package per day × year; total alcohol intake and smoking index indicate the sum before and after first consultation.
[b]Value before IFN treatment.
[c]Outbreak of IFN monotherapy: natural IFN alpha, 252 cases; natural IFN beta, 90 cases; total dose of IFN = 518 ± 156 MU.
Outbreak of combination therapy: natural IFN beta + ribavirin, 41 cases, total dose of IFN = 490 ± 171 MU, total dose of ribavirin = 151 ± 64 g; peg IFN alpha 2b + ribavirin, 102 cases, total dose of peg IFN = 3.96 ± 1.03 mg, total dose of ribavirin = 188 ± 51 g.

hemorrhagic stroke to one-fourth in cirrhotic patients. Table III shows the clinical backgrounds between HCV clearance and HCV non-clearance in patients with liver cirrhosis. There are significant differences in AST, ALT, GGT, HCV genotype, HCV RNA, and HbA_{1C} between HCV clearance group and HCV non-clearance group. However, there are no significant differences in age and hypertension between HCV clearance group and HCV non-clearance group.

DISCUSSION

The incidence of hemorrhagic stroke after the termination of IFN therapy in HCV patients has been described in the present study. The strengths of the present study are a prolonged follow-up in the large numbers of patients included.

The present study shows several findings with regard to the cumulative incidence and predictive factors for hemorrhagic stroke after IFN therapy for HCV patients. First, intracranial hemorrhagic stroke occurred significantly when patients had advanced age of ≥60 years, hypertension, liver cirrhosis, and HCV non-clearance. Several authors have reported that the most common risk factor for hemorrhagic stroke is aging, high levels of blood pressure [Turin et al., 2010; O'Donnell et al., 2010; Naidech, 2011; Cervera et al., 2012]. In addition, antiplatelet and anticoagulant medications also increase the risk of hemorrhagic stroke [Cervera et al., 2012]. Our results evaluated hemorrhagic stroke in HCV patients agreed with these reports concerning aging and hypertension.

Second, HCV clearance reduced hemorrhagic stroke to about one-fourth in cirrhotic patients. In general, patients with advanced liver fibrosis have often the hemorrhagic tendency due to prothrombin deficit and platelets diminution. Thus, our result suggests that the HCV clearance prevent the aggravation of prothrombin deficit and platelets diminution. Our previous reports have indicated that HCV clearance reduces type 2 diabetes mellitus [Arase et al., 2009], bone fracture [Arase et al., 2010], and chronic kidney disease [Arase et al., 2011]. In the present study, HCV clearance reduced the incidence of intracerebral hemorrhagic stroke. In particular, HCV clearance reduced intracerebral hemorrhagic stroke to about one-fourth in cirrhotic patients.

A hemorrhagic stroke is the rapid loss of brain function due to hemorrhage. As a result, a hemorrhagic stroke is a medical emergency and can cause permanent neurological damage and death. Recently, the life span has been long in Japan. Thus, in near the future, a large number of patients with HCV will be >60 years of age. A hemorrhagic stroke might be increasing in HCV positive patients in aging society. Our results show that physicians in charge of HCV