with the spectrum of these additional substitutions conferring genotypic resistance and the increased level of circulating HBV DNA [25].

There is a high genetic barrier to resistance to ETV in nucleoside-naive patients and <1% experience virologic breakthrough with ETVr through 4 years of therapy [15]. However, in LVD-refractory patients, the barrier to resistance is lower because the suppression of HBV replication is not as great and these patients mostly harbor virus with two of the three substitutions required for high-level ETVr [26]. This results in virologic breakthrough with ETVr in LVD-refractory patients at 1% in the first year but increasing to 39.5% after 4 years of therapy [15].

In this article, we report two cases with confirmed genotypic resistance to ETV, virologic rebound, and biochemical breakthrough during long-term ETV treatment for nucleoside-naive CHB patients. In the first case, the patient received a lower dose of ETV (0.1 mg daily for 52 weeks) than is currently recommended in product labeling. It was shown that LVD–ADV combination therapy was apparently effective for the ETV-resistant strain, presumably because there is no cross-resistance between ETV and ADV [26, 27].

SNP-PCR analysis for resistance substitutions revealed that the LVDr M204V(GTG) and the ETVr S202G(GGT) substitutions were negative at baseline and emerged simultaneously at week 124 in both patients. The three resistance substitutions L180M, M204V, and S202G appeared to be genetically linked and did not arise in a stepwise manner in nucleoside-naive patients, as has been described previously.

ETV displays several properties for consideration as the first-line nucleoside analogue because of its potent antiviral activity and a lower frequency of drug resistance than LVD, ADV, or telbivudine [13]. Although ETV is effective in LVD-refractory patients, the potency is reduced somewhat and the barrier to resistance is diminished by the presence of rtM204I/V and rtL180M substitutions. The fact that ETVr may develop in nucleoside-naive patients, even if the chance is small, is noteworthy. In case 1, the patient received a lower dose of ETV (0.1 mg daily), which may be a possible contributing factor to resistance. The common features of our two cases were: HBeAg-positivity, male, high viral load, slow decrease of HBV DNA, and persistently detectable HBV DNA by PCR (>2.6 $\log_{10}$ copies/ml) during the treatment course; however, these characteristics were also present in some other patients who did not develop ETVr. Patient compliance with prescribed therapy also should be assessed in such situations. It is believed that some subpopulations of HBV that proliferate very actively and are not completely suppressed by ETV may have a chance of being selected for the resistance substitutions required for ETV virologic failure. Accordingly, such cases with persistent HBV DNA after extended ETV treatment should be evaluated for emergence of drug-resistance substitutions with close monitoring of HBV DNA level, even in nucleoside-naive patients.

The rate at which resistant mutants are selected is related to pretreatment serum HBV DNA level, rapidity of viral suppression, duration of treatment, and prior exposure to nucleoside analogue therapies [12]. For the management of the emergence of drug resistance in nucleoside analogue treatment of CHB and cirrhosis, prediction and early detection of drug-resistant HBV by close monitoring of serum viral load and genotypic resistance are necessary. Keeffe et al. [28] reported the "road-map concept," that is, on-treatment monitoring strategy, for selection of nucleoside analogues by early prediction of efficacy and resistance using assessment of viral responses at weeks 12 and 24. Although this "concept" seems imperfect because the probability of emergence of resistance to particular nucleoside analogue is not taken into account, a similar strategy for management of drug resistance by close monitoring of viral load and confirming genotypic resistance with consideration of the property of each nucleoside analogue should be established for antiviral treatment of CHB using nucleoside analogues.

Conclusions

We reported two cases of emergence of genotypic resistance to ETV accompanied by virologic breakthrough in nucleoside-naive CHB patients. One patient was treated with a lower than recommended dose of ETV. Although development of ETVr-related gene mutations is rare in nucleoside-naive patients, the patients with a slow decline of HBV DNA or persistent HBV DNA (>2.6 $\log_{10}$ copies/ml) after ETV administration should be evaluated carefully for the potential emergence of ETVr.

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Distribution of Hepatitis B Virus Genotypes among Patients with Chronic Infection in Japan Shifting toward an Increase of Genotype A▽

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Acute hepatitis B virus (HBV) infection has been increasing through promiscuous sexual contacts, and HBV genotype A (HBV/A) is frequent in patients with acute hepatitis B (AHB) in Japan. To compare the geographic distribution of HBV genotypes in patients with chronic hepatitis B (CHB) in Japan between 2005 and 2006 and between 2000 and 2001, with special attention to changes in the proportion of HBV/A, a cohort study was performed to survey changes in genotypes of CHB patients at 16 hospitals throughout Japan. Furthermore, we investigated the clinical characteristics of each genotype and examined the genomic characteristics of HBV/A isolates by molecular evolutionary analyses. Of the 1,271 patients, 3.5%, 14.1%, and 82.3% were infected with HBV/A, -B, and -C, respectively. In comparison with our previous survey during 2000 and 2001, HBV/A was twice as frequent (3.5% versus 1.7%; $P = 0.02$). The mean age was lower in the patients with HBV/A than in those with HBV/B or -C. Based on phylogenetic analyses of 11 full-length genomes and 29 pre-S2/S region sequences from patients, HBV/A isolates were imported from Europe and the United States, as well as the Philippines and India. They clustered with HBV/A from AHB patients and have spread throughout Japan. HBV/A has been increasing in CHB patients in Japan as a consequence of AHB spreading in the younger generation through promiscuous sexual contacts, aided by a tendency of HBV/A to induce chronic hepatitis. The spread of HBV/A infection in Japan should be prevented by universal vaccination programs.

Hepatitis B virus (HBV), a member of the *Hepadnaviridae*, is a circular, partially double-stranded DNA virus and is one of the major causes of chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC).

The HBV genome is composed of approximately 3,200 nucleotides. HBV is classified into eight genotypes, designated A to H, based on an intergroup divergence of 8% or more in the complete nucleotide sequence (3, 23, 26, 37). They have distinct geographical distributions and are associated with differences in clinical and virological characteristics, such as severity of liver disease and response to antiviral therapies (7, 8, 12, 13, 22, 28). Furthermore, subgenotypes have been reported for HBV/A, -B, and -C and named A1 to -3 (17, 38), B1 to -6 (31, 32, 40), and C1 to -6 (20, 31, 45). Equally, other genotypes are classified into subgenotypes. There have been increasing lines of evidence to indicate influences of HBV subgenotypes on the outcome of liver disease and the response to antiviral therapies (1, 39, 44).

In 2001, we reported the geographic distribution of HBV genotypes in Japan (27). Of the 720 Japanese patients with chronic HBV infection (CHB), 12 (1.7%) harbored HBV/A, 88 (12.2%) HBV/B, 610 (84.7%) HBV/C, 3 (0.4%) HBV/D, and 7 (1.0%) mixed genotypes. HBV/C was detected in over 94%

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of patients on the Japanese mainland, while HBV/B was found in 64% of those in Okinawa, the southernmost islands, and 44% of those in the Tohoku area in the northern part of the mainland.

Recently, acute HBV infection (AHB) has been increasing in Japan, predominantly through promiscuous sexual contacts. In addition, it was reported that HBV/A was more frequent in patients with acute hepatitis than in those with chronic hepatitis (29, 41, 49). Recent studies suggest that the chances for progression to chronic disease may differ among patients acutely infected with HBV of distinct genotypes (21, 25); patients infected with HBV/A run an increased risk of becoming HBV carriers. Hence, it is of utmost concern whether chronic HBV/A infection is increasing in Japan.

In the present study, we compared the geographic distribution of HBV genotypes in Japan during 2005 and 2006 with 2000 and 2001, with special attention to changes in the proportion of HBV/A. Furthermore, we investigated the clinical characteristics of each genotype and examined the genomic characteristics of HBV/A isolates by molecular evolutionary analyses.

TABLE 1. Characteristics of 1,271 CHB patients

Parameter	Value
Characteristic	
Male gender [no. (%)]	766 (60.3)
Age (yr; mean ± SD)	51.4 ± 14.0
Diagnosis	
Inactive carrier state [no. (%)]	206 (16.2)
Chronic hepatitis [no. (%)]	786 (61.8)
Cirrhosis [no. (%)]	175 (13.8)
HCC [no. (%)]	104 (8.2)
Antiviral treatment [no. (%)]	577 (45.4)
Blood tests	
Platelets (10^4/mm^3)	21.4 ± 30.2
ALT (IU/liter)	59.8 ± 103.0
ALP (IU/liter)	270.4 ± 136.0
γ−GTP (IU/liter)	47.4 ± 66.1
HBV markers	
HBeAg [no. (%)]	399 (31.4)
HBV DNA (median [range] [log copies/ml])	4.2 (<2.6 to >7.6)

MATERIALS AND METHODS

Patients. From September 2005 to October 2006, sera were collected from 1,370 consecutive patients with CHB at 16 representative hospitals that were liver centers in their respective regions throughout Japan for the purpose of investigating the geographic distribution of HBV genotypes in Japan. All of the patients were diagnosed after they had been followed for at least 12 months. Patients diagnosed with AHB were excluded from the study; they had a sudden onset of clinical symptoms of hepatitis, along with high-titer antibody to HBV core antigen of the immunoglobulin M class in serum. Their sera were tested for alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), and hepatitis B e antigen (HBeAg), as well as antibody to HBeAg (anti-HBe) (Dinabot, Tokyo, Japan). Four clinical diagnoses were established for them. The inactive carrier state was defined by the presence of HBV surface antigen (HBsAg) with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of portal hypertension. Chronic hepatitis was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/liter]) persisting over 6 months (with at least three bimonthly tests). Cirrhosis was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges, and hypersplenism), platelet counts of <100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy, or a combination thereof.

The study protocol conformed to the 1975 declaration of Helsinki and was approved by the ethics committees of the respective institutions. Every patient or his/her next of kin gave informed consent to the purpose of the study.

Genotypes and subgenotypes of HBV. The six HBV genotypes (A to F) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV Genotype EIA; Institutes of Immunology Co., Ltd., Tokyo, Japan). The method depends on the combination of epitopes on pre-S2 region products detected by monoclonal antibodies that were specific for each of them (46, 47). Subgenotypes of HBV/A, designated A1 and A2, were determined by direct sequencing of the pre-S2/S gene, followed by a phylogenetic analysis.

Quantification of HBV DNA and sequencing. HBV DNA levels in sera were quantitated with a commercial kit (Amplicor HBV Monitor; Roche Diagnostics, Basel, Switzerland) with a detection range from 2.6 to 7.6 log copies/ml. Nucleic acids were extracted from 100 μl of serum using the Qiaamp DNA Blood Minikit (Qiagen GmbH, Hilden, Germany). Eleven complete HBV/A genomes and 29 pre-S2/S region sequences were amplified by PCR with appropriate primer sets, as described previously (40). The amplified HBV DNA fragments were directly sequenced using the ABI Prism Big Dye kit version 3.0 (Applied Biosystems, Foster City, CA) in an ABI 3100 automated DNA sequencer (Applied Biosystems). All sequences were analyzed in both forward and reverse directions. Complete and partial HBV genome sequences were aligned using GENETYX version 11.0 (Software Development Co., Ltd., Tokyo, Japan).

Molecular evolutionary analysis of HBV. Reference sequences were retrieved from the DDBJ/EMBL/GenBank databases with their accession numbers for identification. To investigate the relationship between HBV isolates from patients with chronic and acute hepatitis B in Japan, HBV/A isolates (AH1 to -10) were randomly retrieved from them and sequenced in our previous study (29). Nucleotide sequences of HBV DNA were aligned by the program CLUSTAL X, and genetic distance was estimated by the six-parameter method (10) in the Hepatitis Virus Database (36). Based on these values, phylogenetic trees were constructed by the neighbor-joining method (30) with the midpoint rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1,000 times.

Statistical analysis. Categorical variables were compared between groups by the χ^2 test or Fisher's exact test and noncategorical variables by the Mann-Whitney U test. A P value of less than 0.05 was considered significant.

Nucleotide sequence accession numbers. The DDBJ/EMBL/GenBank accession numbers of the complete genome sequences of HBV isolates JPN_CH1 to -11 are AB453979 to AB453989.

RESULTS

Distribution of HBV genotypes among patients with CHB. Of the 1,370 serum samples, the genotype could not be determined for 99 (7.2%) by EIA due to low HBsAg levels, leaving 1,271 for analysis in this study (Table 1). Of these, 206 (16.2%) were inactive carriers, 786 (61.8%) had chronic hepatitis, 175 (13.8%) cirrhosis, and 104 (8.2%) HCC. They had a mean age of 51.4 ± 14.0 years and included 766 (60.3%) men. They had a median HBV DNA level of 4.2 log copies/ml, and 399 (31.4%) of them were positive for HBeAg. Antiviral treatment had been given to 577 (45.4%) of them with interferon, lamivudine, adefovir pivoxil, or entecavir.

The genotypes were HBV/A in 44 (3.5%), HBV/B in 179 (14.1%), HBV/C in 1,046 (82.2%), and HBV/D in 2 (0.2%) (Table 2). In comparison with our previous report on the distribution of genotypes in Japan in 2001 (27), HBV/A was more frequent in this study (3.5% versus 1.7%; $P = 0.02$). Of the 16 hospitals in this study, 10 overlapped with those in our previous report from 2001. In these 10 hospitals, HBV/A was more frequent in the present than in the previous survey (3.6% versus 1.7%; $P = 0.04$).

The distribution of HBV genotypes in Japan differed by

TABLE 2. Distribution of HBV Genotypes

Genotype	No. (%)	
	2005–2006 (n = 1,271)	2000–2001[a] (n = 720)
A	44 (3.5[b])	12 (1.7)
B	179 (14.1)	88 (12.2)
C	1,046 (82.3)	610 (84.7)
D	2 (0.2)	3 (0.4)
Mixed	0 (0.0)	7 (1.0)

[a] From Orito et al. (27).
[b] P = 0.02.

geographic location (Fig. 1). HBV/C was the most prevalent in the majority of areas. In the Tohoku area, the northern part of the Japanese mainland (Honshu), HBV/B was more prevalent than in the other areas of the Japanese mainland. In Okinawa, the southernmost islands of Japan, HBV/B was predominant. Of note, HBV/A was more frequent in the Kanto area (9.5%), the metropolitan area, and Okinawa (9.1%) than in the other areas.

Clinical differences among HBV/A, -B, and -C. Clinical backgrounds were compared among the patients infected with HBV/A, -B, and -C (Table 3). HBeAg was significantly less prevalent in the patients infected with HBV/B than in those infected with HBV/A or -C ($P < 0.01$ for each). When the positivity of HBeAg was stratified by age, HBeAg was markedly less common in patients infected with HBV/B than in those infected with HBV/A or -C who were older than 40 years of age (7/157 [4.5%] versus 4/19 [21.1%] [$P < 0.05$] or 215/755 [28.5%] [$P < 0.01$]) (Fig. 2). There were no significant differences in HBV DNA levels among patients infected with the three genotypes. As antiviral treatments might have influenced the severity of liver disease, clinical states were compared among patients infected with HBV/A, -B, and -C who did and did not receive it; antiviral treatments did not affect the above-mentioned trends represented in Table 3 in age, diagnosis, and HBeAg, as well as ALT and HBV DNA levels (data not shown).

Additionally, we compared the distributions of age and liver diseases in patients infected with HBV/A, -B, and -C. In patients infected with HBV/C, the prevalence of cirrhosis and HCC increased in those older than 50 years of age compared to younger patients (Fig. 3), whereas in the patients infected with HBV/B, cirrhosis and HCC were rare in elderly patients. The proportion of patients younger than 40 years of age was higher in those infected with HBV/A than in those infected with HBV/B or -C (25/44 [56.8%] versus 22/179 [12.3%] or 288/1,046 [27.5%]; $P < 0.01$ for each), while cirrhosis and HCC were also found in those older than 50 years of age infected with HBV/A.

Coinfection with human immunodeficiency virus type 1 (HIV-1) was found in 6 of the 44 (13.6%) patients infected with HBV/A compared to only 3 of the 1,046 (0.3%) patients infected with HBV/C ($P < 0.0001$); it occurred in none of the 179 patients infected with HBV/B.

Phylogenetic analyses. Among the 44 HBV/A isolates, the complete genome was sequenced successfully in 11 (JPN_CH1 to -11). Seven of them were classified as HBV/A2 and four as HBV/A1. A phylogenetic tree was constructed based on the complete genome sequences of these 11 isolates, along with those from two patients with AHB and those from 40 HBV/A isolates retrieved from the database (Fig. 4). Of the seven HBV/A2 isolates, the four from patients with CHB in this study formed a cluster with the Japanese isolates retrieved from the database and two from patients with AHB. Of the other three isolates, JPN_CH5 clustered with French and U.S. isolates, JPN_CH6 with German isolates, and JPN_CH7 with

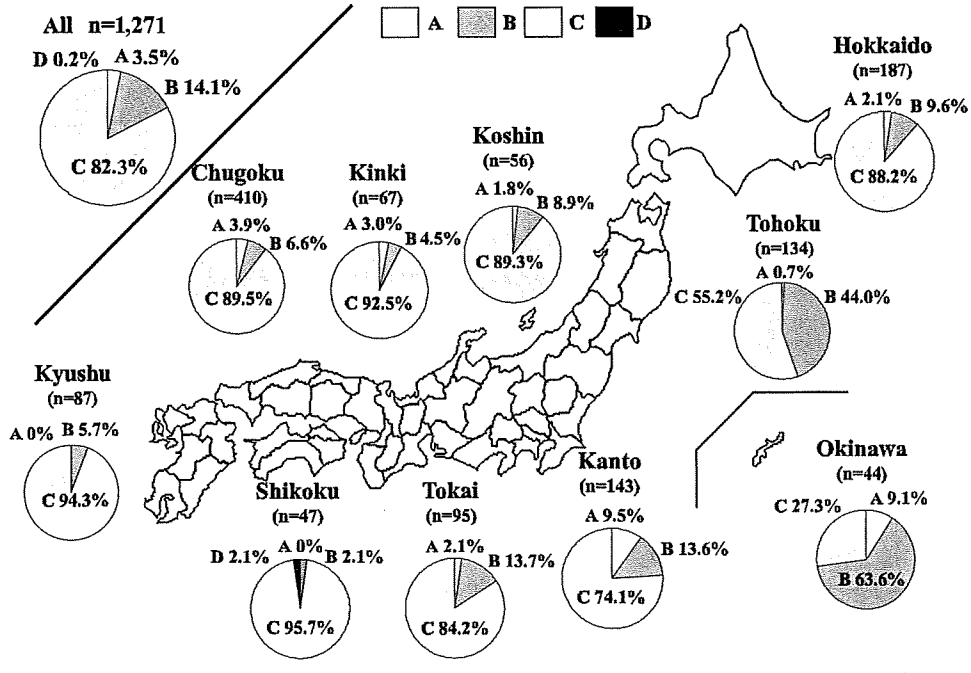

FIG. 1. Geographic distribution of HBV genotypes in patients with chronic HBV infection in Japan during 2005 and 2006.

TABLE 3. Clinical characteristics of individuals chronically infected with HBV of different genotypes

Parameter	Value for genotype:		
	A (n = 44)	B (n = 179)	C (n = 1,046)
Male gender [no. (%)]	32 (72.7)	112 (62.6)	621 (59.4)
Age (yr [mean ± SD])	41.3 ± 14.9[a]	55.8 ± 13.7[b]	48.8 ± 13.3
Diagnosis			
Inactive carrier state [no. (%)]	13 (29.5)[c]	63 (35.2)[b]	129 (12.3)
Chronic hepatitis [no. (%)]	26 (59)	103 (57.5)	656 (62.7)
Cirrhosis [no. (%)]	3 (6.8)	10 (5.6)[b]	162 (15.5)
HCC [no. (%)]	2 (4.5)	3 (1.7)[b]	99 (9.5)
Anti viral treatment [no. (%)]	13 (29.5)[d]	48 (26.8)[b]	516 (49.3)
Blood tests			
Platelet (10^4/mm^3)	23.3 ± 21.9	25.9 ± 35.9[e]	20.6 ± 29.5
ALT (IU/liter)	56.2 ± 83.8	42.2 ± 104.2[e]	63.0 ± 103.3
ALP (U/liter)	247.1 ± 123.0	255.5 ± 97.9	273.9 ± 141.9
γ-GTP (U/liter)	39.6 ± 34.6	49.3 ± 63.4	47.5 ± 67.6
HBV markers			
HBeAg [positive rate(%)]	15 (34.0)[f]	17 (9.5)[b]	367 (35.1)
HBV DNA (median [range]) (log copies/ml)	4.2 (<2.6–>7.6)	4.1 (<2.6–>7.6)	4.2 (<2.6–>7.6)

[a] $P < 0.01$, A versus B or C.
[b] $P < 0.01$, B versus C.
[c] $P < 0.01$, A versus C.
[d] $P < 0.05$, A versus C.
[e] $P < 0.05$, B versusC.
[f] $P < 0.01$, A versus B.

Spanish and Italian isolates. All four HBV/A1 isolates in this study formed a cluster with Philippine and Indian isolates.

In addition, the pre-S2/S region sequences of a total of 29 isolates were determined, including the 11 isolates whose complete genomes were sequenced. Of these, 21 (72%) were classified as HBV/A2 and the remaining 8 as HBV/A1. A phylogenetic tree was constructed based on the pre-S2/S region sequences from the 29 isolates, along with those from 10 patients with AHB infected with HBV/A and 47 HBV/A isolates retrieved from the database (Fig. 5). The 21 HBV/A2 isolates in the present study formed a cluster with Japanese, American, and European isolates retrieved from the database and those from patients with acute hepatitis. In addition, some of them were highly homologous with each other. Likewise, HBV/A1 isolates from eight patients with chronic hepatitis in this study were highly homologous with those from two patients with acute hepatitis and isolates from the Philippines and India. Based on the phylogenetic analyses, HBV/A isolates were imported from Europe and the United States, as well as the Philippines and India, and had infiltrated throughout Japan.

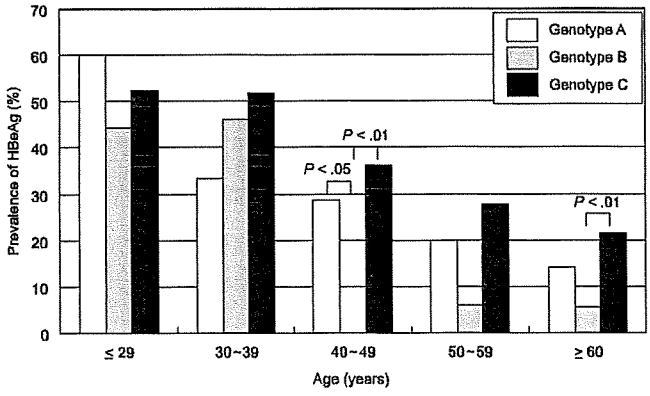

FIG. 2. Prevalence of HBeAg among patients infected with HBV of different genotypes stratified by the age.

DISCUSSION

Perinatal transmission from carrier mothers to their babies has been the principal route for establishing persistent HBV infection in Asian countries (19). In Japan, passive and active immunoprophylaxis with HBV immune globulin and vaccine has been mandated for babies born to HBeAg-positive carrier mothers since 1986; this was extended to HBeAg-negative carrier mothers in 1995. As a result, HBsAg has become rare in Japanese born after 1986; it was detected in only 0.2% of first-time blood donors younger than 19 years of age in 2000 (24). However, AHB has been increasing in Japan, predominantly through promiscuous sexual contacts.

In Japan, HBV/A is detected rarely among patients with CHB but is frequent in those with acute hepatitis (14, 25, 29, 41, 43). Yotsuyanagi et al. reported the distribution of genotypes in 145 Japanese patients with AHB and found HBV/A in 27 (19%), HBV/B in 8 (5%), and HBV/C in 109 (75%) (49). HBV/A is more frequent in metropolitan areas than other areas. The majority of patients with HBV/A infection in metropolitan areas have had extramarital sexual contacts with multiple irregular partners, through which they could have contracted infection. In support of this view, among men who have sex with men (MSM) who are coinfected with HBV and HIV-1 in Tokyo, most were infected with HBV/A (15, 35).

In Japan, AHB in adulthood becomes chronic in only ~1%

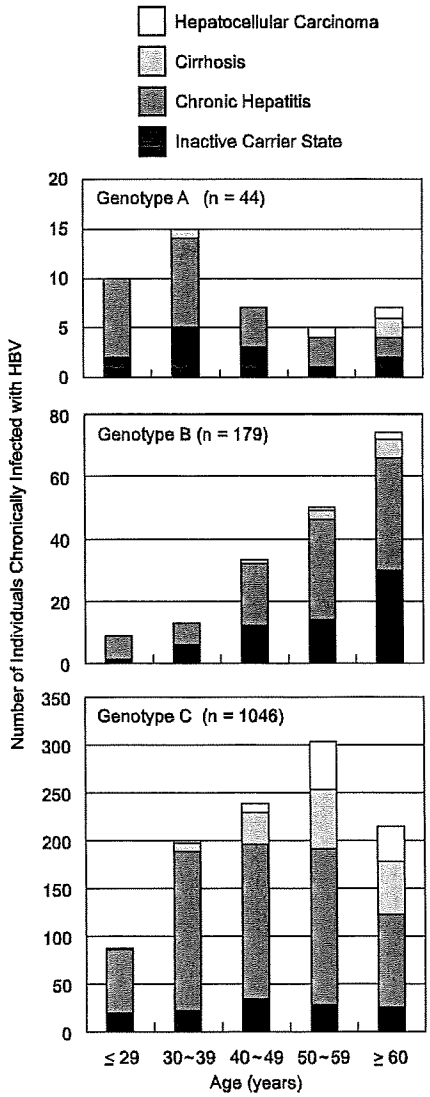

FIG. 3. Distribution of HCC, cirrhosis, chronic hepatitis, and inactive carrier state among the 1,271 patients infected with HBV of different genotypes stratified by the age.

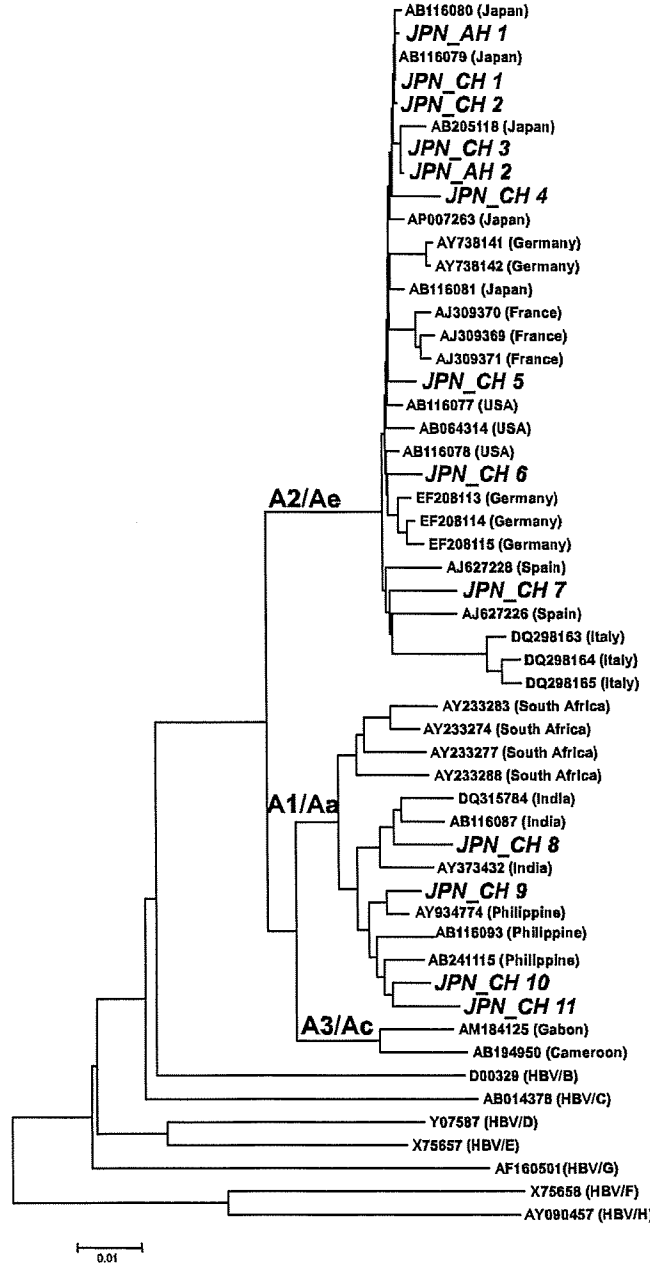

FIG. 4. Phylogenetic tree constructed based on the complete genome sequences of HBV/A isolates. Those from 11 patients with chronic infection in this study are shown in boldface italic (JPN_CH1 to -11), along with two isolates (JPN_AH1 and -2) from patients with acute hepatitis in Japan reported in our previous study (17). Representative isolates were retrieved from the DDBJ/EMBL/GenBank databases, including 21 HBV/Ae, 10 HBV/Aa, and 2 HBV/Ac isolates, along with 7 HBV isolates representative of the other seven genotypes. Isolates from the databases are identified by accession numbers, followed by the country of origin. The bar at the bottom spans 0.01 nucleotide substitutions per site.

of cases. This is much less than the progression to chronic disease (close to 10%) in Europe and the United States, where HBV/A prevails (34). Recent studies have suggested that the chances for persistence may differ among patients acutely infected with HBV of distinct genotypes (21, 25). In particular, acute infection with HBV/A may bring about an increased risk of progression to chronic disease. Therefore, an increase of acute infection with HBV/A would result in a surge of HBV/A among patients with CHB in Japan. In actuality, in comparison with our previous results during 2000 and 2001 (27), HBV/A was twice as frequent in this study (3.5% versus 1.7%; $P = 0.02$). HBV/A has been increasing in patients with CHB in the Kanto area, where HBV/A in patients with acute hepatitis is more frequent than in the other areas. In the islands of Okinawa, also, HBV/A was found to be prevalent in this study. Of the four patients infected with HBV/A there, two were coinfected with HIV-1. They were both MSM, and they were suspected to have been infected with HIV through sexual contacts on the Japanese mainland. It has been reported that HIV infection increases the probability that AHBs will become chronic (2, 11, 33, 48). Because they share routes of transmission and the risk for HIV-1 and HBV infections, approximately

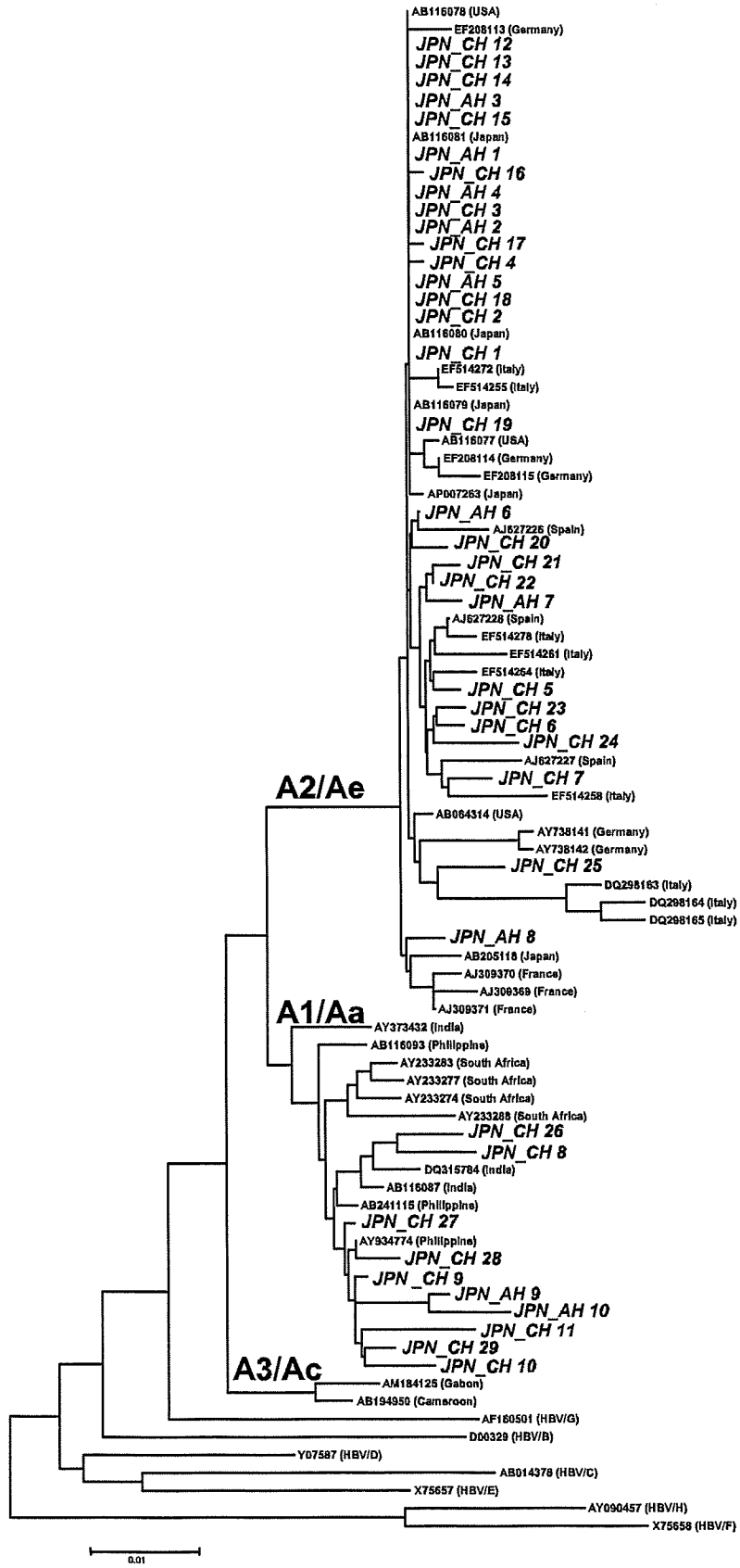

FIG. 5. Phylogenetic tree constructed based on pre-S2/S region sequences of HBV/A isolates. Those from 29 patients with chronic infection in this study are shown in boldface italic (JPN_CH1 to -29), along with 10 isolates (JPN_AH1 to -10) from patients with acute hepatitis in Japan reported in our previous study (17). Representative isolates were retrieved from the DDBJ/EMBL/GenBank databases, including 28 HBV/Ae, 10 HBV/Aa, and 2 HBV/Ac isolates and 7 HBV isolates representative of the other seven genotypes. Isolates from the databases are identified by accession numbers, followed by the country of origin. The bar at the bottom spans 0.01 nucleotide substitutions per site.

90% of patients with AIDS have markers of past or ongoing HBV infection (18). Thus, HBV carriers are more frequent in the HIV-1-positive than in the HIV-1-negative population (4, 9). Among patients with HIV infection in Japan, 6.3% are HBsAg positive, in particular, 8.3% of HIV-infected MSM (16). In this study, coinfection with HIV was found in 6 of the 44 (13.6%) patients infected with HBV/A. All of them were men. Their median age was 27.7 ± 4.1 years, and five patients were positive for HBeAg. Thus, there is a possibility that HIV-1 and HBV/A coinfections are increasing among young people in Japan, and the high rate of HBeAg positivity may be influenced by immune suppression due to HIV infection.

In the phylogenetic analysis, the HBV/A2 isolates recovered in this study were homologous to those from Europe and the United States, and some of them clustered with the Japanese isolates. On the other hand, there were HBV/A1 isolates that formed a cluster with those from the Philippines and India. Furthermore, some isolates from patients with acute hepatitis who were infected with HBV/A in Japan were highly homologous to HBV/A isolates from patients with chronic hepatitis. This invites speculation that some HBV/A isolates were introduced into Japan from foreign countries, while others have already settled down there and spread from patients with chronic infection to their contacts. HBV/A would have been infiltrating throughout Japan by these two different routes.

Clinical differences among patients infected with HBV/A, -B, and -C were observed. The mean age was lower in the patients infected with HBV/A than in those infected with HBV/B or -C. As mentioned above, AHB patients infected with HBV/A have been increasing in the younger generation in Japan, and around 10% of them would have progressed to chronic infection. This is one of the reasons why the patients infected with HBV/A are younger than those infected with HBV/B or -C. Most patients infected with HBV/B were negative for HBeAg, while a high proportion of the patients infected with HBV/A and -C had it. In particular, this difference was remarkable in the patients who were older than 40 years of age. Thus, the seroconversion rate for the loss of HBeAg among younger people may be higher in infection with HBV/B than in that with HBV/A or -C. Inactive carriers were commoner in HBV/A than in HBV/C infection, as well.

These lines of evidence indicate that the activity of hepatitis is lower in HBV/B than HBV/C infection, and patients with HBV/B seroconvert from HBeAg to anti-HBe at young ages. In addition, cirrhosis and HCC were less frequent in the patients infected with HBV/B than in those infected with HBV/C. Therefore, the prognosis would be better in the patients infected with HBV/B than in those infected with HBV/C. These results are in accord with previous reports (5, 13, 28, 42). There have been few reports on the clinical features of patients with chronic hepatitis infected with HBV/A in Japan. Chu et al. have reported the distribution of HBV genotypes with reference to clinical characteristics in the United States (6). They have shown that HBV/A and HBV/C infections are accompanied by a higher frequency of HBeAg than HBV/B infection, while HBV/B is associated with a lower rate of hepatic decompensation than HBV/A and -C. In our study, inactive carriers were commoner, while cirrhosis and HCC were found less often in HBV/A than in HBV/C infection. HBeAg was more prevalent in the patients infected with HBV/A than in those infected with HBV/B who were older than 40 years of age. Therefore, it can be said that the prognosis is better for patients infected with HBV/A than for those infected with HBV/C; it may be poorer than for those infected with HBV/B.

In conclusion, HBV/A has been increasing among CHB patients in Japan. On the basis of phylogenetic analyses, some HBV/A isolates appear to have been imported from foreign countries. They clustered with HBV/A from AHB patients and have infiltrated throughout Japan. It is very likely that acute and chronic infections with HBV/A have been increasing in Japan. Obviously, immunoprophylaxis of perinatal HBV infection, implemented since 1986 on a national basis, has been insufficient to prevent horizontal HBV/A infection diffusing among high-risk groups by transmission routes shared by HIV infection. The foreseeable spread of HBV/A infection in Japan should be prevented by universal vaccination programs extended to high-risk groups or the general population.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study

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Abstract
Background Chronic hepatitis C (CHC) genotype 1b patients with high viral load are resistant to peginterferon (PEG-IFN) and ribavirin (RBV) combination therapy, especially older and female patients.
Methods To elucidate the factors affecting early and sustained viral responses (EVR and SVR), 409 genotype 1b patients CHC with high viral loads who had received 48 weeks of PEG-IFN/RBV therapy were enrolled. The amino acid (aa) sequences of the HCV core at positions 70 and 91 and of the interferon sensitivity determining region (ISDR) were analyzed. Host factors, viral factors, and treatment-related factors were subjected to multivariate analysis.
Results Male gender, low HCV RNA load, high platelet count, two or more aa mutations of ISDR, and wild type of core aa 70 were independent predictive factors for SVR. In patients with over 80% adherences to both PEG-IFN and RBV, male gender, mild fibrosis stage, and wild type of core aa 70 were independent predictors for SVR.
Conclusions Independent predictive factors for SVR were: no aa substitution at core aa 70, two or more aa mutations in the ISDR, low viral load, high values of platelet count, mild liver fibrosis and male gender.

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Keywords Chronic hepatitis C · Peginterferon and ribavirin · Core amino acid · Interferon sensitivity determining region

Abbreviations

CHC	Chronic hepatitis C
PEG-IFN	Peginterferon
RBV	Ribavirin
RVR	Rapid viral response
cEVR	Complete early viral response
LVR	Late viral response
ETR	End of treatment response
NR	Non response
SVR	Sustained viral response
ISDR	Interferon sensitivity determining region
Aa	Amino acid
ALT	Alanine aminotransferase
PLT	Platelet
HCC	Hepatocellular carcinoma

Introduction

A combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy for 48 weeks achieves a sustained viral response (SVR) rate of 40–50% in chronic hepatitis C (CHC) patients with a high viral load of genotype 1 [1–4]. The dose-reduction rate and the frequency of discontinuation of this treatment are high in aged patients [5]. The SVR rate of the therapy is lower in females than males, especially in older patients in Japan [6].

Around 30% of HCV carriers have serum alanine aminotransferase (ALT) levels within the upper limit of normal ranges [7, 8] and HCV carriers with persistently normal serum ALT (PNALT) and serum platelet (PLT) counts of over $15 \times 10^4/mm^3$ show low grade hepatic fibrosis and good prognosis [9]. Before treating HCV carriers, it is very important to predict non-response to PEG-IFN plus RBV therapy because of its medical cost, adverse effects, and its impact on the long term quality of life.

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There are many factors affecting response to IFN monotherapy and PEG-IFN/RBV therapy, including body mass index (BMI) [10, 11], steatosis [12, 13], insulin resistance [14], stage of liver fibrosis [15, 16], total cholesterol (T. Chol), triglyceride (TG), adherence to both PEG-IFN and RBV [17], race [18, 19], age [1, 2, 20], and viral factors including serum quantity of HCV RNA, HCV genotype and substitution of amino acids (aa) in the interferon sensitivity determining region (ISDR, 2209–2248) of the nonstructural protein 5A (NS5A) [21] and in the core protein [22, 23]. Early viral response is an important predictive factor in PEG-IFN/RBV therapy for CHC patients with genotype 1 and high viral loads [24–27].

The aim of this study was to elucidate the valuable predictive factors of SVR in Japanese patients with HCV genotype 1b high viral loads following 48 weeks of PEG-IFN/RBV therapy, focusing on the relationship between aa substitutions in the ISDR and at core aa 70 and 91 and early viral kinetics.

Patients and methods

Selection of patients

This retrospective study was conducted at 15 clinical sites in Japan which are part of the Study Group of Optimal Treatment of Viral Hepatitis supported by the Ministry of Health, Labor and Welfare, Japan. Eligible subjects were CHC patients, who (1) had received liver biopsy; (2) were genotype 1b with high viral load ($\geq$100 KIU/ml by Cobas Amplicor Hepatitis C Virus Test, version 2.0) at the start of PEG-IFN/RBV therapy; (3) received weekly injections of PEG-IFN-α-2b (PEG-INTRON; Shering-Plough, Kenilworth, NJ) of 1.5 μg/kg bw and oral administration of RBV (Rebetol; Shering-Plough) for 48 weeks. The amount of RBV was adjusted based on the subject's body weight; (600 mg for $\leq$60 kg bw, 800 mg for 60–80 kg bw, 1,000 mg for $>$ 80 kg bw); (4) were examined serially for quantitative and qualitative HCV RNA; and (5) the aa sequences at positions 70 and 91 in the core region and of the ISDR in the NS5A had been determined in pretreatment sera.

Hepatitis B virus (HBV) infection, human immunodeficiency virus (HIV) infection, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease were excluded. Histopathological diagnosis was based on the scoring system of Desmet et al. [28]. The definition of alcohol abuse included patients having a history of more than 100 kg of total ethanol intake. Complete blood counts, liver function tests, serum lipids, serum ferritin, serum fibrosis markers, fasting plasma glucose (FPG), and immune reactive insulin (IRI) were examined in most cases. Written informed consent was obtained from all

patients before treatment, and the protocol was approved by the ethics committees in each site.

Study design

Four hundred and nine patients who completed 48 weeks of treatment and were followed for more than 24 weeks after treatment were enrolled in the first study (*Study design 1*).

To elucidate the effect of aa substitution of HCV core and in the ISDR on HCV dynamics, including a rapid viral response (RVR), complete early viral response (cEVR), a late viral response (LVR) and SVR, according to gender and age (<60 years ≥ 60 years), 201 of the 409 patients maintaining over 80% adherences to both PEG-IFN and RBV were enrolled in the second study (*Study design 2*).

Nucleotide sequencing of the core and NA5A gene

The nucleotide sequences encoding aa 1–191 (HCV core) and aa 2209–2248 (ISDR) were analyzed by direct sequencing as described by Akuta et al. [22, 27] and Enomoto et al. [21]. In brief, RNA was extracted from the sera and converted to cDNA and two nested rounds of polymerase chain reaction (PCR) were performed. Primers used in the PCR were as follows; (a) Nucleotide sequences of the core region: the first-round PCR was performed with CC11 (sense) and e14 (antisense) primers [22, 27], and the second-round PCR with CC9 (sense) and e14 (antisense) primers [22, 27]. (b) Nucleotide sequences of the ISDR in NS5A: the first-round PCR was performed with ISDR1 (sense) and ISDR2 (antisense) primers [21], and the second-round PCR with ISDR3 (sense) and ISDR4 (antisense) primers [21]. These sequences were compared with the consensus sequence of genotype 1b (HCV-J) [29]. Wild types virus encoded arginine and leucine at aa 70 and 91, respectively, and the aa substitutions were glutamine or histidine at aa 70 and methionine at aa 91.

Viral kinetic study

Serum HCV RNA levels were measured by PCR (Amplicor HCV RNA kit, version 2.0, Roche Diagnostics) using samples taken before treatment and at 4, 12, 24, and 48 weeks after the therapy. SVR was defined as HCV RNA negativity by qualitative analysis by PCR at 24 weeks after the treatment. RVR was defined as HCV RNA negativity at 4 weeks, cEVR as HCV RNA negativity at 12 weeks, LVR as HCV RNA negativity during 13–24 weeks and an end of treatment response (ETR) as HCV RNA negativity at the end of treatment. Patients who remained positive for HCV RNA at the end of the treatment and at 24 weeks after the therapy were defined as non-responders (NR).

Adherences to PEG-IFN and RBV

Adherences to PEG-IFN and RBV were assessed by separately calculating the actual doses of PEG-IFN and RBV received as percentages of the intended dosages. Adherences to PEG-IFN and RBV were divided into two groups; 80%≤ and <80%.

Statistical analysis

All data analyses were conducted using the SAS version 9.1.3 statistical analysis packages (SAS Institute, Cary, NC, USA). Individual characteristics between groups were evaluated by Mann–Whitney U test for numerical variables or Fisher's exact test for categorical variables. Variables exhibiting values of $p < 0.1$ in the univariate analysis were subjected to stepwise multivariate logistic regression analysis. The grade of steatosis and iron deposition in liver tissue, BMI, albumin (Alb), low density lipoprotein-cholesterol (LDL-C), homeostasis model assesment-insulin resistance (HOMA-IR), ferritin, and hyaluronic acid were excluded from multivariate logistic regression analysis because of the absence of those data in more than 10% of the patients. All p values of $p < 0.05$ by the two-tailed test were considered statistically significant.

Results

Study design 1

Baseline backgrounds, characteristics and adherences of peginterferon and ribavirin in males and females

The treatment outcome of PEG-IFN and RBV combination therapy depends on gender in Japanese patients, so in addition to aa substitutions in the ISDR in NS5A [21] or at HCV core 70 and 91 [22, 27], we compared the baseline characteristics according to gender (Table 1). Males were younger and the grade of hepatic inflammation was milder in males. The serum levels of LDL-C, PLT count, and aa substitutions of ISDR and at core 70 and 91 did not differ significantly different between males and females. The frequency of no alcohol abuse was significantly ($p < 0.0001$) higher in females than males (Some of them are not described in Table 1).

The rates of over 80% adherences to PEG-IFN and RBV were significantly lower ($p = 0.0066$, $p < 0.00001$, respectively) in females than males. Only in those above 60 years did the rate of over 80% adherence to PEG-IFN not differ significantly between males and females, but the rate of over 80% adherence to RBV was significantly lower ($p = 0.035$) in females than males (Table 1).

Table 1 Backgrounds and characteristics of male and female patients

Factors	Gender		*p* value
	Male	Female	
No. of patients	256 (62.6%)	153 (37.4%)	
Age			
Median (range)	53 (18–73)	59 (23–75)	0.00001
F stage			
F0–2	206 (80.5%)	119 (77.8%)	0.592
F3–4	50 (19.5%)	34 (22.2%)	
Grade (A factor)			
A0–1	163 (63. 7%)	79 (51.6%)	0.026
A2–3	93 (36.3%)	74 (48.4%)	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1500 (100–5000 <)	1280 (100–5000<)	0.384
ALT 0 week (IU/L)			
Median (range)	74.5 (16–504)	59 (19–391)	0.001
BMI			
Median (range)	23.6 (17.5–31.2)	22.1 (16.1–33.9)	0.00033
Alb (g/dL)			
Median (range)	4.0 (3.0–5.2)	3.8 (3.0–4.8)	0.011
LDL-C (mg/dL)			
Median (range)	97 (30–185)	90 (34–174)	0.612
T-Chol (mg/dL)			
Median (range)	167 (85–273)	176 (114–261)	0.0016
PLT count ($\times 10^4$/mm^3)			
Median (range)	17.0 (8.0–31.9)	16.4 (8.1–39.9)	0.350
Amino acid mutation of ISDR			
0–1	200 (78.1%)	121 (79.1%)	0.608
2≤	56 (21.9%)	32 (20.9%)	
Amino acid substitution of core 70			
Wild	177 (69.1%)	114 (74.5%)	0.261
Mutant	79 (30.9%)	39 (25.5%)	
Amino acid substitution of core 91			
Wild	153 (59.8%)	98 (64.1%)	0.403
Mutant	103 (40.2%)	55 (35.9%)	
PEG-IFN adherence			
<80%	41 (17.7%)	42 (30.4%)	0.0066
80%≤	190 (82.3%)	96 (69.6%)	
Ribavirin adherence			
<80%	54 (23.6%)	73 (52.1%)	<0.00001
80%≤	175 (76.4%)	67 (47.9%)	
Age: <60 years			
PEG adherence			
<80%	30 (17.8%)	23 (31.5%)	0.027
80%≤	139 (82.2%)	50 (68.5%)	
Ribavirin adherence			
<80%	27 (16.2%)	31 (42.5%)	0.000029
80%≤	140 (83.8%)	42 (57.5%)	
Age: 60 years≤			
PEG adherence			
<80%	11 (17.7%)	19 (29.2%)	0.147
80%≤	51 (82.3%)	46 (70.8%)	
Ribavirin adherence			
<80%	27 (43.5%)	42 (62.7%)	0.035
80%≤	35 (56.5%)	25 (37.3%)	

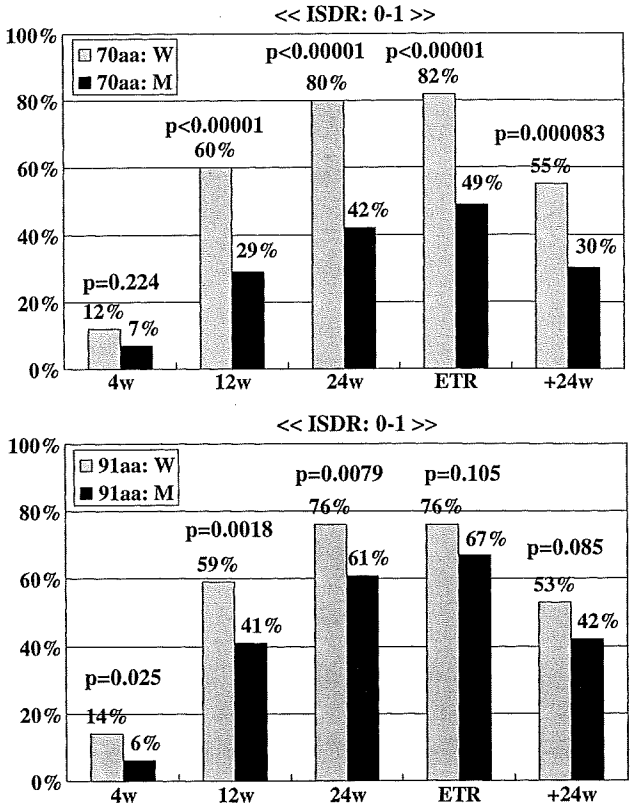

Fig. 1 Relationship between time course of serum HCV RNA negativity and amino acid substitutions in the ISDR and core amino acids 70 and 91. For cases with no or only one amino acid (aa) change in the ISDR, the rates of cEVR, LVR, ETR and SVR were significantly higher in patients with wild type core aa 70 but only the rates of RVR, cEVR, and LVR were significantly higher in patients with wild type core aa 91

Amino acid substitutions

There were no significant differences in the frequency of aa substitutions in the ISDR between males and females. Core aa substitutions at positions 70 and 91 were as follows; 291 (71.1%) were wild type and 118 (28.9%) were mutant at core aa 70, and 251 (61.4%) were wild type and 158 (38.6%) were mutant at core aa 91. There were no significant differences between males and females and between patients below and above 60 years of age.

Virological responses and aa substitutions

The rate of RVR did not differ significantly between males and females. However, more male patients showed HCV RNA negativity at 12 weeks (males vs. females; 60.7 vs. 48.4%, $p = 0.018$), 24 weeks (76.8 vs. 64.2%, $p = 0.0078$) and 48 weeks (78.2 vs. 68.6%, $p = 0.049$), and the proportion of male patients in SVR was significantly higher than that of females (61.3 vs. 37.3%, $p < 0.00001$).

RVR, cEVR and SVR rates were significantly higher in patients with two or more aa mutations in the ISDR compared to patients having no or one aa substitution in that region (20 vs. 11%, $p = 0.044$; 71 vs. 52%, $p = 0.0021$; 66 vs. 49%, $p = 0.0054$, respectively). AA substitution at core position 70 resulted in significantly lower rate of cEVR, LVR, ETR, and SVR (40 vs. 63%, $p = 0.000037$; 51 vs. 81%, $p < 0.00001$; 56 vs. 83%, 41 vs. 57%; $p < 0.00001$, $p = 0.0031$, respectively). Although the patients with the wild type aa at core 91 showed significantly higher rates of RVR and cEVR, the rate of SVR was not significantly higher in those patients ($p = 0.054$).

SVR rates were 30% for patients with no or one aa substitution in the ISDR and the core aa 70 substitution, and were significantly lower compared to those with the wild type aa core 70 (Fig. 1). These findings were not confirmed in patients with no or one aa substitution in the ISDR and the core aa 91 substitution (Fig. 1).

Factors affecting SVR by univariate analysis

Univariate analysis identified nine parameters that influenced non-SVR significantly: female gender, older age, advanced staged liver fibrosis, high viral load, low serum Alb level, low PLT count, no or one aa substitution in the ISDR, aa substitution at core aa 70, and low adherence to RBV (Table 2). The frequency of steatosis and HOMA-IR were significantly ($p = 0.0057$, $p < 0.00001$, respectively) lower in patients with SVR compared with non-SVR (data not shown). However, these factors were not entered in the multivariate analysis because of the absence of the data in many cases.

Factors affecting RVR, cEVR, and SVR by multivariate logistic regression analysis

Multivariate analysis identified four parameters that influenced RVR independently: low HCV RNA load, low serum ALT level, two or more aa mutations in the ISDR and the wild type aa at core position 91 (Table 3).

Concerning cEVR, male gender, mild fibrosis stage, low HCV RNA load, two or more aa mutations in the ISDR, and the wild type aa at core positions 70 and 91 were independent predictors (Table 3).

Concerning SVR, male gender ($p < 0.0001$), low HCV RNA load ($p = 0.013$), high PLT count ($p = 0.0019$), two or more aa mutations in the ISDR ($p = 0.024$), and wild type core aa 70 ($p = 0.0045$) were found to be independent predictors (Table 3).

The predictive values of the combination of gender, PLT count, ISDR and core aa 70 are shown in Fig. 2a. In male patients having PLT of $<15 \times 10^4/mm^3$, and, no or one aa substitution in the ISDR, the SVR rate was 68% when core 70

Table 2 Univariate analysis to identify the factors of SVR

Factors	Negative of HCV RNA after 24 weeks		*p* value
	(−)	(+)	
No. of patients	214 (52.3%)	195	
Gender			
Male	157 (61.3%)	99	<0.00001
Female	57 (37.3%)	96	
Age			
Median (range)	52.5 (18–75)	58 (20–74)	<0.00001
<60 years	155 (58.1%)	112	0.0018
60 years≤	59 (41.5%)	83	
Age: <60 years			
Male	118 (63.4%)	68	0.010
Female	37 (45.7%)	44	
Age: 60 years≤			
Male	39 (55.7%)	31	0.0011
Female	20 (27.8%)	52	
F stage			
F0–2	190 (58.5%)	135	0.000013
F3–4	25 (29.8%)	59	
Grade (A factor)			
A0–1	138 (56.8%)	104	0.130
A2–3	81 (48.5%)	86	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (100–5000<)	1700 (130–5000<)	0.016
ALT 0 week (IU/L)			
Median (range)	66 (16–391)	67 (19–504)	0.892
BMI			
Median (range)	23.0 (17.3–32.4)	23.25 (16.1–33.9)	0.714
Alb (g/dL)			
Median (range)	4.0 (3.2–5.2)	3.8 (3.0–4.8)	0.0088
LDL-C (mg/dL)			
Median (range)	94.5 (31–185)	97.5 (30–182)	0.611
T-Chol (mg/dL)			
Median (range)	169.5 (85–257)	170 (103–273)	0.511
PLT count ($\times 10^4$/mm^3)			
Median (range)	18.2 (8.7–39.9)	15.1 (8.0–31.9)	<0.00001
<15	54 (36.5%)	94	<0.00001
15≤	160 (61.3%)	101	
Amino acid mutation of ISDR			
0–1	156 (48.6%)	165	0.0054
2≤	58 (65.9%)	30	
Amino acid substitution of core 70			
Wild	166 (57.0%)	125	0.0031
Mutant	48 (40.7%)	70	
Amino acid substitution of core 91			
Wild	141 (56.2%)	110	0.054
Mutant	73 (46.2%)	85	
PEG-IFN adherence			
<80%	35 (42.2%)	48	0.063
80%≤	154 (53.8%)	132	
Ribavirin adherence			
<80%	55 (43.3%)	72	0.048
80%≤	132 (54.5%)	110	

Table 3 Multivariate logistic regression analysis to identify independent predictive factors of RVR, cEVR, and SVR

	Odds ratio	95% CI	*p* value
RVR factors selected by stepwise method			
F stage			
F0–2/F3–4	2.924	0.988–8.696	0.053
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	2.151	1.130–4.082	0.020
ALT 0 week (IU/L)			
<60/60≤	2.165	1.127–4.149	0.020
Amino acid mutation of ISDR			
2≤/0–1	2.371	1.187–4.735	0.014
Amino acid substitution of core 91			
W/M	2.137	1.021–4.464	0.044
cEVR factors selected by stepwise method			
Gender			
Male/female	1.912	1.209–3.021	0.0055
F stage			
F0–2/F3–4	2.079	1.133–3.817	0.018
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	1.608	1.002–2.577	0.049
PLT count ($\times 10^4$/mm^3)			
15≤/<15	1.427	0.882–2.309	0.148
Amino acid mutation of ISDR			
2≤/0–1	2.512	1.407–4.485	0.0018
Amino acid substitution of core 70			
W/M	2.513	1.508–4.184	0.0004
Amino acid substitution of core 91			
W/M	1.965	1.241–3.115	0.004
SVR factors selected by stepwise method			
Gender			
Male/female	3.704	2.132–6.410	<0.0001
F stage			
F0–2/F3–4	1.812	0.888–3.690	0.103
HCV RNA load0 week (KIU/mL)			
<1000/1000≤	2.024	1.163–3.534	0.013
PLT count ($\times 10^4$/mm^3)			
15≤/<15	2.469	1.394–4.372	0.0019
Amino acid mutation of ISDR			
2≤/0–1	2.148	1.107–4.170	0.024
Amino acid substitution of core 70			
W/M	2.415	1.316–4.444	0.0045
Amino acid substitution of core 91			
W/M	1.433	0.828–2.481	0.199
PEG adherence (%)			
80≤/<80	1.562	0.834–2.926	0.164

W Wild, *M* Mutant

was a wild type but only 16% in patients with mutant at core 70. In female patients, no or one aa substitution in ISDR and $<15 \times 10^4$/mm^3 of PLT count, the SVR rates were as low as 10 or 8%, irrespective of aa substitution at core 70. SVR was only 24% in patients with substitution of core aa 70 even when the PLT count was $\geq 15 \times 10^4$/mm^3. In this study, the combination analysis of PLT count, ISDR, and core aa substitution was useful for predicting non-SVR.

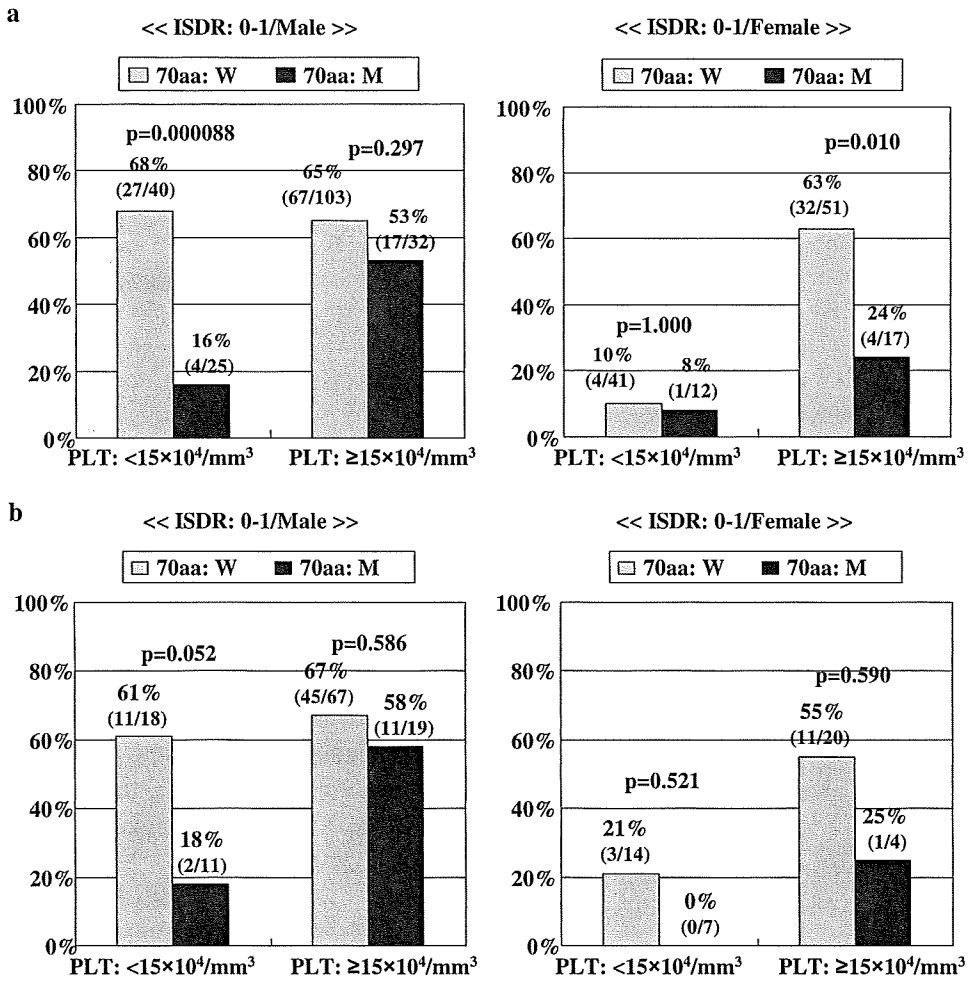

Fig. 2 Relationship between SVR rate and amino acid substitutions in the ISDR and core amino acids 70 and 91, PLT counts and gender difference. The two figures of **a** show the results of *Study 1* and the two figures of **b** show the results of *Study 2*. In male patients with no or only one amino acid (aa) substitution in the ISDR and PLT count of less than $15 \times 10^4/mm^3$, the SVR rate was 68% in those with wild type core aa 70, but only 16% in patients with mutant type of core aa 70, which is significantly different ($p = 0.000088$). There were no significant differences between wild type and mutant type of core aa 70 in the patients with no or one aa substitution in the ISDR and PLT count of over $15 \times 10^4/mm^3$. By contrast, in female patients with no or one aa substitution in the ISDR, there were no significant differences between wild type and mutant type of core aa 70 with PLT count of less than $15 \times 10^4/mm^3$, but there were significant differences between wild type and mutant type of core aa 70 with PLT counts of less than $15 \times 10^4/mm^3$ (**a**). For the patients maintaining over 80% adherences to both PEG-IFN and RBV, in males having no or one aa substitution in the ISDR and PLT counts of less than $15 \times 10^4/mm^3$, a wild type of core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). However, in male patients with PLT counts of over $15 \times 10^4/mm^3$, core aa 70 was not a useful marker for predicting SVR and non-SVR. The number of female patients with no or one aa substitution in ISDR was too small to reach a definite conclusion (**b**)

Study design 2

The basic features of 201 patients achieving 80% adherences to both PEG-IFN and RBV are as follows: the females were significantly ($p = 0.00006$) older than the males. Iron deposition in liver tissue, alcohol abuse, BMI, serum albumin level, serum ferritin level, and PLT count were significantly higher in males than females. Inflammatory activity was significantly ($p = 0.046$) higher in females than males (data not shown).

AA substitutions in the ISDR were as follows; in males 33 (22.3%) had two or more aa substitutions, in females 8 (15.1%) had two or more aa substitutions. The analysis of core aa position 70 and 91 sequences showed no significant differences in aa substitutions of either core aa 70 or 91 between males and females (data not shown).

In patients less than 60 years of age, SVR rate was significantly higher ($p = 0.0042$) in males than females, but no significant difference was noted between males and females over 60 years old. However, the number of patients over 60 years was small (Table 4).

Table 4 Univariate analysis to identify the significantly different factors between SVR and non-SVR (201 patients received over 80% adherences of both PEG-IFN and RBV)

Factors	Negative of HCV RNA after 24 weeks		*p* value
	(−)	(+)	
No. of patients	111 (55.2%)	90	
Gender			
Male	93 (62.8%)	55	0.00037
Female	18 (34.0%)	35	
Age			
Median (range)	51 (18–70)	56 (23–74)	0.00025
<60 years	91 (60.3%)	60	0.014
60 years≤	20 (40.0%)	30	
Age: <60 years			
Male	79 (66.4%)	40	0.0042
Female	12 (37.5%)	20	
Age: 60 years≤			
Male	14 (48.3%)	15	0.243
Female	6 (28.6%)	15	
F stage			
F0–2	103 (60.9%)	67	0.0012
F3–4	8 (25.8%)	23	
Grade (A factor)			
A0–1	80 (59.3%)	55	0.189
A2–3	31 (47.0%)	35	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (110–5000<)	1280 (130–5000<)	0.351
ALT 0 week (IU/L)			
Median (range)	74 (16–268)	67.5 (19–504)	0.752
BMI			
Median (range)	23.1 (17.3–31.0)	23.6 (16.1–33.9)	0.626
Alb (g/dL)			
Median (range)	3.95 (3.3–5.2)	3.9 (3.0–4.8)	0.079
LDL-C (mg/dL)			
Median (range)	96 (31–185)	97.5 (30–182)	0.865
T-Chol (mg/dL)			
Median (range)	170 (85–248)	170 (105–273)	0.624
PLT count ($\times 10^4$/mm^3)			
Median (range)	18.9 (8.7–30.9)	15.55 (7.2–28.4)	0.00003
<15	23 (35.9%)	41	0.00024
15≤	88 (64.2%)	49	
Amino acid mutation of ISDR			
0–1	84 (52.5%)	76	0.159
2≤	27 (65.9%)	14	
Amino acid substitution of core 70			
Wild	91 (61.5%)	57	0.0037
Mutant	20 (37.7%)	33	
Amino acid substitution of core 91			
Wild	73 (60.3%)	48	0.083
Mutant	38 (47.5%)	42	

Virological responses and aa substitution

The rates of RVR, cEVR, LVR, ETR and SVR in males and females were 12.5 versus 11.3% ($p = 1.000$), 59.6 versus 43.4% ($p = 0.053$), 74.3 versus 50.0% ($p = 0.0018$), 76.2 versus 66.7% ($p = 0.198$), and 62.8 versus 34.0% ($p = 0.00037$), respectively (data not shown). The backgrounds and characteristics of SVR and non SVR patients are shown in Table 4. There were significant differences in gender (male vs. female; $p = 0.00037$), age (<60 years vs. ≥60 years; $p = 0.014$), F stage (F0-2 vs. F3,4; $p = 0.0012$), PLT count ($<15 \times 10^4/mm^3$ vs. $15 \times 10^4/mm^3 \leq$; $p = 0.00024$), and substitution of core aa 70 (wild type vs. mutant, p = 0.0037) between SVR and non-SVR patients. The distribution of fatty change in liver tissue (≤10% vs. 11–33% vs. 34%≤; $p = 0.046$) and the grade of HOMA-IR (1.7 vs. 3.9, $p = 0.0018$) were significantly different between SVR and non-SVR (data not described in Table 4).

Factors affecting SVR by multivariate logistic regression analysis

Male gender ($p = 0.0006$), mild fibrosis stage ($p = 0.027$), and wild type of core aa 70 ($p = 0.043$) were independent predictors of SVR.

Valuable markers for predictions of sustained virological response to peginterferon and ribavirin therapy

Two or more aa mutations in the ISDR, wild type core aa 70, $\geq 15 \times 10^4/mm^3$ of PLT count, and male gender were selected statistically as independent predictors of SVR. We show here SVR rates of the patients having over 80% adherences to both PEG-IFN and RBV (Fig. 2b). In males having no or one aa substitution in the ISRD and PLT count of $<15 \times 10^4/mm^3$, wild type core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). In females, the SVR rate was very low in those who had substitution of core aa 70, but there was no significant difference between patients with wild type and substitution of core aa 70. The number of female patients was too small to provide a definite conclusion.

Discussion

The present multivariate logistic regression analysis revealed that male gender, low HCV RNA load, high PLT count, and two or more aa mutations in the ISDR and wild type core aa 70 were independent predictors for SVR. PLT count significantly decreased corresponding to the progression to the stage of liver fibrosis in CHC [9, 30, 31].

It has been considered that the low adherence level to PEG-IFN/RBV is a major cause of a significantly lower SVR rate in females and older patients [32]. The percentage of patients having over 80% adherences to both PEG-IFN and RBV was significantly lower in females than males, however, differences in the adherence to PEG-IFN/RBV between males and females were not independent predictive factors of non-SVR.

A recent report from Japan showed six or more mutations in the variable region 3 (V3) of nonstructural protein 5A (NS5A) plus upstream flanking region NS5A (aa 2334–2379), referred to as the IFN/RBV resistance determining region (IRRDR), was a useful marker for predicting SVR, but the ISDR sequence was not valuable for predicting SVR [33]. However, the number of subjects in that study was too small ($n = 45$) to reach an acceptable conclusion.

To elucidate the factors affecting low SVR rate in older female patients, we performed a multivariate logistic regression analysis using patients who achieved ≥80% adherence to both PEG-IFN and RBV. Male gender, stage of mild liver fibrosis, and wild type core aa 70 were independent predictors of SVR. In this study, blood concentration of RBV was determined in fewer than 50% of cases during treatment. Thus we cannot exclude the possibility of the effect of the blood concentration of RBV during treatment on the low SVR rate in females and older patients.

From the present analysis, it was clear that ALT, BMI, Alb, T. Chol, and adherence to RBV differed significantly between males and females, however, these factors were not independent predictors of SVR. There is a report that steatosis is an important cofactor that reduces the SVR rate in genotype 1 infected patients [34], however, such an effect was not seen in this study. Thus we could not identify the factors associated with a significantly lower SVR rate in females than males.

In the present multivariate logistic regression analyses, patients having wild type core aa 91 had significantly higher rates of RVR and cEVR, but not SVR, and patients with wild type core aa 70 had significantly higher rates of cEVR and SVR, but not RVR. Patients having two or more aa substitutions in the ISDR had significantly higher rates of RVR, cEVR, and SVR. Although several possibilities have been considered concerning the effects of aa substitutions of core protein on SVR in PEG-IFN/RBV therapy for CHC patients, the exact mechanisms have not yet been elucidated.

Recent reports have indicated that low serum IP-10 (interferon-γ inducible protein 10 kDa) [35], a higher HCV-specific CD8 cell proliferation potential [36], and a high ratio of Th1/Th2 [37] are good predictors of SVR to