the decision tree topology and a significant interaction between sex and treatment duration, it appears that 72 weeks of treatment may be most beneficial in women between the ages of 46 and 58 years who have low cholesterol. In general, patients who are younger, male, have cholesterol over 130 mg/dl, or who have wild-type core aa70 or mutant ISDR are the most likely to achieve an SVR.

Because each of the above values can be determined prior to treatment and are interpretable by clinicians, they may be useful as a guide when establishing a treatment regimen in the case of potentially difficult-to-treat patients. Once IFN treatment has been started, early and/or rapid viral response is likely to be the strongest predictor of SVR [33], and slow responders have been shown to be the most likely to benefit from extended treatment [34, 35]. However, because of the expense, low success rate, and potential side effects of IFN-based therapy, predictors available prior to treatment are also needed. Factors predictive of NR may help guide the decision to avoid or discontinue IFN therapy in patients with a low probability of SVR, and factors predictive of SVR may help identify subsets of patients who are likely to achieve an SVR if treated longer than the standard 48-week regimen.

Several other recent studies have examined predictors for SVR for 72 weeks of treatment, although nearly all focus on on-treatment predictors and conclude that 72-week therapy significantly improves SVR rates in slow responders [9, 10, 35]. Ferenci et al. [11] also showed that extension to 72 weeks decreased the relapse rate among early viral responders. In a large retrospective cohort study, Watanabe et al. [36] dissected a complex relationship between SVR and age, sex, and viral load similar to that reported here, although results are difficult to compare because they did not measure cholesterol or viral substitutions. While they recommend 72-week therapy for all slow-responding patients regardless of sex or age, they note that the SVR rate was surprisingly high among elderly female patients following 72-week treatment, noting that the SVR for 48-week treatment was typically low among older female patients in Japan, which they suggest could be related to the development of insulin resistance associated with menopause [36]. Other studies discourage the use of 72-week therapy for all patients except in the specific case of slow responders [8]. Moreover, in a large prospective study, Buti et al. [34] conclude that 48-week combination therapy should remain the standard of care even for slow responders, due to the increased cost and incidence of adverse events relative to a modest increase in the SVR rate. They clarify, however, that patients with a less than 2 log decline at week 8 and undetectable HCV RNA at week 24 are the most likely to benefit from 72-week treatment. Unfortunately they did not examine other predictors in a multivariate analysis. Because each of these studies hinges on rapid versus slow viral response and an on-treatment predictor requiring up to 24 weeks of treatment to establish, pretreatment predictors of early viral kinetics, including those presented here (e.g., viral substitutions and baseline cholesterol levels [12]), may be useful for predicting the outcome of extended therapy prior to treatment [17].

The combination of multiple approaches to identify predictive factors should help improve confidence in the results and partially protect against the bias inherent in any single approach. Comparing the results of a standard analysis with an alternative technique may reveal which variables are robust and which are sensitive to methodological differences. There are many different classification tools, including neural networks, Bayesian networks, and support vector machines, but models based on these may be more difficult to interpret or apply in clinical practice. On the other hand, decision tree approaches such as C4.5 and CART are widely used in biomedical studies [37–39] and provide a simple and intuitive hierarchical format that in many cases can be used without a computer.

The lack of randomized assignment of patients to duration of treatment limits the conclusions that can be drawn from the present study, and additional predictive factors, particularly interleukin (IL) 28B single-nucleotide polymorphism (SNP) genotype and viral kinetics, should be included in future prospective studies. Comparison of ROC curves suggests that the performance of the two models in the present study is similar, although neither is sufficiently sensitive or specific for accurate clinical prediction based on the number of patients analyzed. Nonetheless the strong overlap between the variables selected by each method suggests that several patient factors, including age, sex, and cholesterol level, as well as several viral factors, including core aa70 and ISDR substitutions, are robust predictors for SVR. Differences in the variables selected between the two approaches suggest that several models with similar predictive ability are also possible. In the regression model, LDL cholesterol but not total cholesterol was an independent factor associated with SVR, whereas in the CART analysis total cholesterol was selected instead. This may be due to the hierarchical nature of decision tree models, which may yield better results in the face of missing data, higher-order interactions, or nonlinear relationships. Comparison of separate models for 48 and 72 weeks also suggests that age and ISDR substitutions are important predictors of SVR for patients undergoing 72 weeks of treatment, whereas the decision tree suggests that the 72-week treatment length is important mainly for a subgroup of female patients. Without greater understanding of the role of HCV core and ISDR substitutions, it is difficult to interpret the role of these predictors, as well as

potential interactions with cholesterol level and other clinical factors. Further studies should be performed to investigate these interactions and to better characterize the subgroup of patients who are most likely to respond to long-term IFN therapy.

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Conflict of interest None of the authors have conflicts of interest to declare.

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Original Article

Recommendation of lamivudine-to-entecavir switching treatment in chronic hepatitis B responders: Randomized controlled trial

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Aim: In the 2007–2008 guidelines of the study group (Ministry of Health, Labor and Welfare of Japan), lamivudine (LAM)-continuous treatment was recommended in patients treated with LAM for more than 3 years who maintained hepatitis B virus (HBV) DNA less than 2.6 log copies/mL, because in these patients LAM resistance might exist and switching treatment to entecavir (ETV) might cause ETV resistance. However, there was no evidence on whether switching treatment to ETV- or LAM-continuous treatment was better in those patients. In the present study, we performed a randomized controlled trial of LAM-to-ETV switching treatment.

Methods: Twenty-seven patients treated with LAM for more than 3 years whose HBV DNA levels were less than 2.6 log copies/mL were enrolled and randomly divided into two groups, LAM-continued group or switching to ETV group. Then, we examined incidence of virological breakthrough (VBT) and breakthrough hepatitis (BTH) in each group.

Results: There was no BTH in any of the patients. VBT was observed in six patients of the LAM group (6/15, 40%), and no patient of the ETV group (0/11, 0%) ($P = 0.02$). The differences of the proportion of cumulated VBT using a log–rank test with Kaplan–Meier analysis were significant between the LAM and ETV groups ($P = 0.025$).

Conclusion: In patients treated with LAM for more than 3 years maintaining HBV DNA less than 2.6 log copies/mL, switching treatment to ETV is recommended at least during the 2 years' follow-up period.

Key words: chronic hepatitis B, entecavir, lamivudine, lamivudine resistance, randomized controlled trial, switching treatment

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INTRODUCTION

OVER THE PAST two decades, treatment of chronic hepatitis B (CHB) has greatly improved with the availability of nucleos(t)ide analogs (NA), including lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine, clevudine and tenofovir. NA target

the reverse transcriptase of hepatitis B virus (HBV), and are highly effective in suppressing HBV replication and clinical progression to liver cirrhosis and hepatocellular carcinoma in CHB patients.[1–4]

Lamivudine, ADV and ETV are commonly available in Japan. LAM, the first approved NA, has been shown to provide benefit for CHB patients with respect to the reduction of HBV DNA, normalization of alanine aminotransferase (ALT) and improvement of liver histology.[5,6] However, a serious problem of LAM is the high incidence of drug resistance during long-term treatment. The detection rate of LAM resistance has been reported to be 24% at 1 year and 70% after 5 years of treatment.[7–10] Even when the HBV DNA level was maintained at less than 2.6 log copies/mL, the accumulated incidence of LAM resistance reached 65% in patients treated with LAM for a long period (3 to ~10 years).[11] LAM resistance is caused by amino acid substitution(s) at rtM204V/I within the reverse transcriptase domain of the HBV polymerase gene.[12–14] The emergence of a LAM-resistant strain leads to virological breakthrough (VBT) and breakthrough hepatitis (BTH).

Recently, ETV has been demonstrated to exert antiviral efficacy in both NA-naïve and LAM-resistant CHB patients.[15–17] The frequency of ETV resistance has been reported to be 1.2% after 5 years of treatment in NA-naïve CHB patients.[18,19] On the other hand, in switching treatment to ETV for LAM-resistant CHB patients, the cumulative probability of ETV resistance increases.[17,20] After 5 years of treatment, 51% of LAM-refractory patients treated with ETV showed genotypic ETV resistance.[21]

The 2007–2008 guidelines of the study group (Ministry of Health, Labor and Welfare of Japan) for patients on LAM therapy are summarized in Table 1.[22] Regardless of duration of LAM administration, in cases where HBV DNA is more than 2.6 log copies/mL with BTH, ADV add-on treatment was recommended. In patients treated with LAM for less than 3 years who maintained HBV DNA of less than 2.6 log copies/mL or HBV DNA of 2.6 log copies/mL or more without BTH, switching to ETV was recommended. On the other hand, in patients treated with LAM for more than 3 years who maintained HBV DNA of less than 2.6 log copies/mL or HBV DNA of 2.6 log copies/mL or more without BTH, LAM-continuous treatment was recommended because in these patients LAM resistance might exist, and switching treatment to ETV might cause ETV resistance. However, there is insufficient evidence on whether switching treatment to ETV- or LAM-continuous treatment is better for CHB patients treated with LAM for more than 3 years with HBV DNA of less than 2.6 log copies/mL.

In the present study, we performed a randomized controlled trial of LAM-to-ETV switching treatment in CHB patients treated with LAM for more than 3 years who maintained HBV DNA of less than 2.6 log copies/mL.

METHODS

Patients

A TOTAL OF 27 CHB patients (mean age 55 ± 9 years, 17 men) from 11 institutions all over Japan (Hokkaido University Hospital, Tohoku University Hospital, Akita City Hospital, Kuramitsu Clinic, Juntendo University Hospital, Chukyo Hospital, Nagoya City University Hospital, Okayama University Hospital, Kawasaki Medical University Hospital, Ehime University Hospital, Shin-Kokura Hospital) were enrolled from April 2008. All the patients were followed at least 6 months after they were diagnosed with CHB. Their characteristics are shown in Table 2. They were treated with LAM (100 mg/day) for more than 3 years (median 50 months, range 36–106 months). Before starting LAM administration, all patients were positive for hepatitis B surface antigen (HBsAg) in serum, abnormal for ALT, detectable for HBV DNA, and were not

Table 1 2007–2008 guidelines of the study group (Ministry of Health, Labor and Welfare of Japan) for patients on lamivudine treatment

Duration of lamivudine treatment HBV DNA		<3 years	≥3 years
<2.6 log copies/mL, persistently		May be switched to ETV 0.5 mg/day	LAM 100 mg/day
≥2.6 log copies/mL	No BTH†	May be switched to ETV 0.5 mg/day	LAM 100 mg/day
	With BTH	Add on ADV 10 mg/day	Add on ADV 10 mg/day

†After checking for absence of LAM resistance.
ADV, adefovir; BTH, breakthrough hepatitis; ETV, entecavir; HBV, hepatitis B virus; LAM, lamivudine.

Table 2 Characteristics of LAM continuous group and ETV switch group at baseline

	LAM ($n = 15$)	ETV ($n = 11$)	*P*-value
Male	10	6	NS
Age	53 ± 7	57 ± 7	NS
Duration of LAM administration (month)	59 ± 23	55 ± 18	NS
ALT (IU/L)	33 ± 29	28 ± 22	NS
HbeAg positive	1	1	NS

ALT, alanine aminotransferase; ETV, entecavir; HBeAg, hepatitis B e-antigen; LAM, lamivudine; NS, not significant.

infected with hepatitis C virus and HIV. Patients diagnosed with alcoholism, primary biliary cirrhosis or autoimmune hepatitis were excluded.

Study design

The overview of this study design is shown in Figure 1. Twenty-seven patients treated with LAM for more than 3 years were enrolled, who showed HBV DNA of less than 2.6 log copies/mL at entry. They were randomly divided into two groups by each institution, the LAM-continued group (LAM group) or switching to the ETV group (ETV group). The primary end-points were the incidences of VBT and BTH in each group. VBT was defined as having more than 1 log copies/mL increase of HBV DNA level from the nadir on at least two occasions after initial virological response. BTH was defined as showing abnormal ALT level due to LAM or ETV resistance. All subjects were monitored at least every 3-month intervals. At every visit, routine examination with biochemical (ALT, bilirubin, albumin) and virological (HBV DNA level, hepatitis B e-antigen [HBeAg], anti-HBe) assessments took place. The mean follow-up period was 24 ± 3 months.

This study was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) on 4 April 2008 as "A randomized trial of lamivudine continuous therapy and entecavir switching therapy for chronic hepatitis B patients treated with lamivudine monotherapy" (no. UMIN000001120).

The study protocol conformed to the Declaration of Helsinki, and was approved by the Committee for Ethics of Medical Experiments on Human Subjects of all the institutions, and written informed consent was obtained from every participant.

Serological and virological markers of HBV

Hepatitis B surface antigen, antibody against HBsAg (anti-HBs), HBeAg and antibody against HBeAg (anti-HBe) were determined using commercially available enzyme immunoassays. HBV DNA was determined by an Amplicor HBV Monitor (Roche Molecular Systems, Branchburg, NJ, USA; detection limit 2.6 log copies/mL)

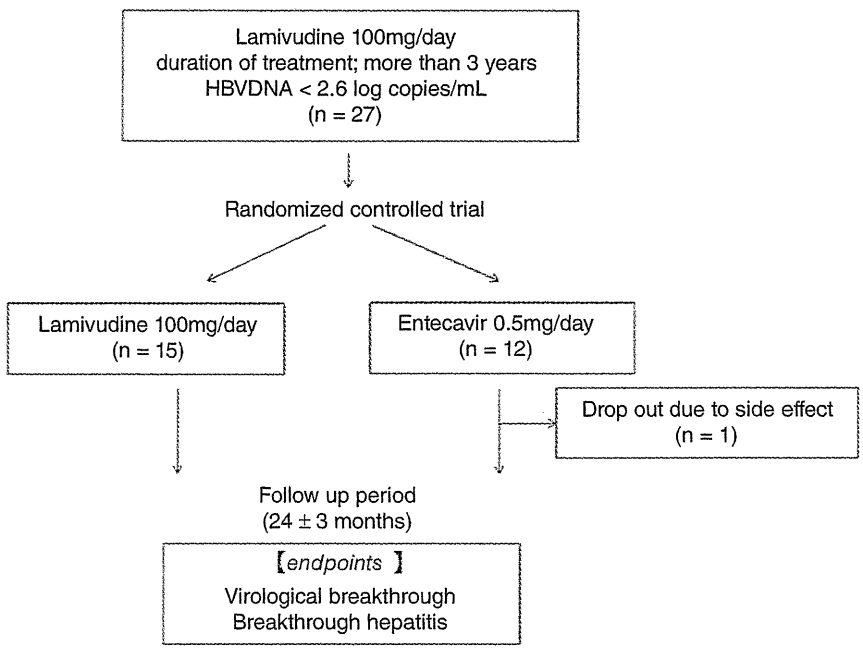

Figure 1 Overview of this study design. Twenty-seven patients treated with lamivudine for more than 3 years whose hepatitis B virus (HBV) DNA was maintained at <2.6 log copies/mL were enrolled. They were randomly divided into two groups by each institution, lamivudine-continued group or switching to entecavir group. We examined the incidence of virological breakthrough and breakthrough hepatitis in each group.

or COBAS AmpliPrep-COBAS TaqMan HBV test (Roche Molecular Systems; detection limit 2.1 log copies/mL). Positive results (signals) below the quantitative HBV DNA concentrations are referred to as "detected" and negative signals are "not detected" when registered by COBAS AmpliPrep-COBAS TaqMan HBV test. The presence of LAM-resistant rtM204V/I and rtL180M substitutions was analyzed by direct sequencing of the HBV DNA polymerase reverse transcriptase site.

Retrospective analysis

Using a conserved serum sample, we examined the existence of LAM-resistant rtM204V/I or rtL180M at baseline in patients with VBT. We also measured HBV DNA by COBAS AmpliPrep-COBAS TaqMan HBV test, and we evaluated the subsequent occurrence of VBT according to the DNA level (not detected/detected/2.1 to <2.6 log copies/mL).

Statistical analysis

Categorial variables were compared between groups by the χ^2-test or Fisher's exact test, and non-categorical variables by Mann–Whitney's *U*-test. The cumulated VBT rate was compared between each group using a log–rank test with Kaplan–Meier analysis. All data were analyzed using SPSS ver. 15.0J software. $P < 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics of the patients

BASED ON THIS randomized controlled trial, 12 patients were placed in an ETV group and 15 in a LAM group. One patient in the ETV group dropped out because of skin rash by ETV. The baseline characteristics of the patients are described in Table 2. At the entry, one patient was positive for HBeAg in each group. There was no difference in sex, age, duration of LAM administration and ALT level between the two groups.

Incidence of VBT and BTH

There was no BTH in any of the patients. The incidence of VBT was six patients out of 15 (40%) in the LAM group, and no patient in the ETV group ($P = 0.02$). The Kaplan–Meier curve for the proportion of cumulated VBT is shown in Figure 2. The differences in the rates of VBT were significant between the LAM and ETV groups (log–rank test $P = 0.025$).

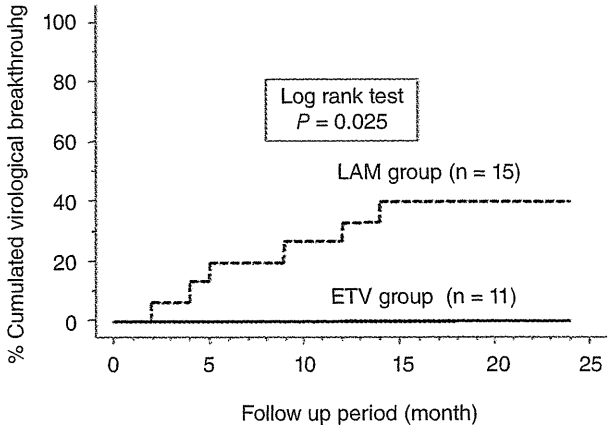

Figure 2 Proportion of cumulated virological breakthrough in lamivudine (LAM) and entecavir (ETV) group. The cumulated rate of virological breakthrough was higher in patients treated with LAM than those with ETV (40% vs 0%, $P = 0.025$ by log–rank test).

Characteristics of patients with VBT in LAM group

Details of the six VBT cases in the LAM group are described in Table 3. Assessment of LAM-resistant mutations at the time of VBT showed that both rtM204V and rtL180M were observed in all cases. For five of the six cases, HBV DNA was detected by COBAS AmpliPrep-COBAS TaqMan HBV test at baseline, although the HBV DNA level was very low. With respect to LAM-resistant mutation at baseline, rtM204V and rtL180M were observed in one of six cases. In contrast, no LAM-resistant mutations were observed in 20 non-VBT cases at baseline.

Incidence of VBT based on the HBV DNA level by COBAS AmpliPrep-COBAS TaqMan HBV test

Incidence of VBT based on the HBV DNA level according to COBAS AmpliPrep-COBAS TaqMan HBV test at baseline is shown in Figure 3. HBV DNA levels were less than 2.6 log copies/mL by Amplicor HBV Monitor in all cases. However, HBV DNA levels in the LAM group were "not detected" in five cases, "detected" in eight cases and 2.1 log copies/mL or more in two cases by COBAS AmpliPrep-COBAS TaqMan HBV test. VBT was observed in five of the 10 cases whose results were either "detected" or 2.1 log copies/mL or more and in one of the five "not detected" cases. On the other hand, although HBV DNA levels in the ETV group were

Table 3 Characteristics of patients with virological breakthrough in LAM group

At baseline						At virological breakthrough		
Age	Sex	Duration of LAM administration (month)	HBeAg	HBV DNA by TaqMan HBV (log copies/mL)	Mutant of LAM resistance	Period of VBT (months)	HBV DNA (log copies/mL)	Mutant of LAM resistance
49	M	37	Negative	Detected	None	14	4.9	L180M/M204V
54	F	106	Negative	Detected	None	5	2.8	L180M/M204V
63	F	81	Negative	Not detected	None	9	4.5	L180M/M204V
57	F	43	Negative	Detected	None	10	3	L180M/M204V
55	M	84	Negative	Detected	None	12	2.8	L180M/M204V
57	M	36	Negative	2.3	L180M/M204V	2	4	L180M/M204V

ALT, alanine aminotransferase; ETV, entecavir; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; LAM, lamivudine; VBT, virological breakthrough.

"detected" in six cases by COBAS AmpliPrep-COBAS TaqMan HBV test, there was no incidence of VBT: HBV DNA levels of five patients were undetectable and that of one patient was "detected" at the last follow-up point after switching to ETV.

DISCUSSION

AT PRESENT, LAM, ADV and ETV are only approved for treatment of CHB patients in Japan. ETV has become the first-line treatment for NA-naïve patients, because the ETV resistance is much less frequent than LAM-resistance.[8,23,24] On the other hand, in switching treatment to ETV for LAM-resistant CHB patients, the frequency of ETV resistance was increased.[17,20,25–27] It has also been reported that ADV add-on treatment suppressed HBV replication more effectively than ETV or ADV monotherapy in patients with LAM-resistant CHB.[25,28] Therefore, it is desirable to examine LAM-resistant mutants before switching to ETV in patients treated with LAM. However, as the assay for the LAM-resistant mutants is not covered by the Japanese health insurance system at present, the Japanese guidelines for CHB management after LAM therapy were based on HBV DNA, duration of LAM administration and incidence of BTH (Table 1).[22] In patients treated with LAM for more than 3 years, maintaining HBV DNA of less than 2.6 log copies/mL or HBV DNA of 2.6 log copies/mL or more without BTH, LAM-continuous treatment was recommended because in these patients, LAM-resistance might exist, and switching treatment to ETV might cause ETV-resistance. It was reported that although LAM-resistant strains were detected in 34% cases treated with LAM for more than 3 years and whose HBV DNA level was suppressed to less than 2.6 log copies/mL, switching to ETV maintained undetectable HBV DNA level over 2 years.[29] In addition, Kurashige *et al.* reported that LAM-to-ETV switching treatment maintained an undetectable HBV DNA level in patients with baseline HBV DNA of less than 2.6 and 2.6 to less than 4.0 log copies/mL for a period of ETV treatment ranging 10–23 (median 20) months.[30] In the present study, randomized controlled trial evidenced that switching treatment to ETV or LAM-continuous treatment would be recommended in CHB patients treated with LAM for more than 3 years and maintained HBV DNA of less than 2.6 log copies/mL. Interestingly, even though HBV DNA had been suppressed to less than 2.6 log copies/mL, a high rate of VBT was observed in the LAM group, whereas no VBT over 24 months was observed in the ETV group. Of the six patients with VBT,

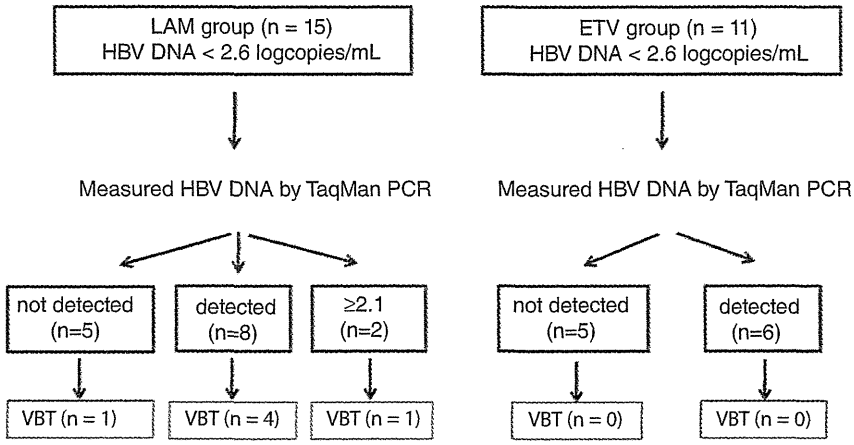

Figure 3 Incidence of virological breakthrough (VBT) based on the hepatitis B virus (HBV) DNA level at baseline by COBAS AmpliPrep-COBAS TaqMan HBV test (TaqMan PCR). The subsequent occurrence of VBT according to the DNA level by TaqMan PCR (not detected/detected/2.1 to <2.6 log copies/mL) was evaluated. In the lamivudine (LAM) group, VBT was observed in five of the 10 cases in which the results were either "detected" or ≥2.1 log copies/mL and in one of the five "not detected" cases. On the other hand, HBV DNA levels in the entecavir (ETV) group were "detected" in six, but there was no incidence of VBT.

five had no LAM resistance at baseline. However, the LAM resistance of rtM204V and rtL180M were found in all the patients with VBT in the LAM group. Moreover, a retrospective assessment by COBAS AmpliPrep-COBAS TaqMan HBV test showed that HBV DNA was detectable in 10 patients in the LAM group and six patients in the ETV group. Only five of the 10 patients in the LAM group had VBT, but none in the ETV group. In addition, one patient had VBT in the LAM group even though DNA was not detected by the TaqMan test, suggesting that switching to ETV was preferable. Hence, our data supported the 2010 Japanese guidelines which recommend switching to ETV in patients whose HBV DNA levels are less than 2.1 log copies/mL by TaqMan PCR.

A potential limitation of the present study is that the number of the cases was small. Nevertheless, our randomized controlled trial indicated significant difference in the incidence of VBT between the LAM and ETV groups. Therefore, this study is valuable for the purpose of verifying the 2007–2008 guidelines in Japan. In the present study, although no LAM-resistant mutant was observed in the ETV group at baseline, a very low level of LAM-resistant mutants may derive ETV resistance for long-term therapy. The results of switching to ETV in the present study were favorable during the 24-month observation period, but we have to be careful of possible emergence of ETV-resistant mutants in long-term follow up.

In conclusion, in patients treated with LAM for more than 3 years maintaining HBV DNA of less than 2.6 log copies/mL, switching treatment to ETV is recommended in at least a 2-year follow-up period.

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Prevalence of Hepatitis C Virus Genotype 1a in Japan and Correlation of Mutations in the NS5A Region and Single-Nucleotide Polymorphism of Interleukin-28B With the Response to Combination Therapy With Pegylated-Interferon-Alpha 2b and Ribavirin

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Hepatitis C virus (HCV) genotype 1a is rare in Japanese patients and the clinical characteristics of this genotype remain unclear. The interferon (IFN) sensitivity-determining region (ISDR) and single-nucleotide polymorphisms (SNPs) of interleukin-28B (IL28B) among patients with HCV genotype 1b are associated with IFN response, but associations among patients with genotype 1a are largely unknown. This study investigated the clinical characteristics of genotype 1a and examined whether genomic heterogeneity of the ISDR and SNPs of IL28B among patients with HCV genotype 1a affects response to combination therapy with pegylated-IFN-α2b and ribavirin. Subjects comprised 977 patients infected with HCV genotype 1, including 574 men and 412 women (mean age, 55.2 ± 10.6 years). HCV was genotyped by direct sequencing of the 5′-untranslated region and/or core regions and confirmed by direct sequencing of the NS5A region. HCV genotypes 1a (n = 32) and 1b (n = 945) were detected. Twenty-three (71.9%) of the 32 patients with genotype 1a were patients with hemophilia who had received imported clotting factors. Prevalence of genotype 1a after excluding patients with hemophilia was thus 0.9%. Of the 23 patients with genotype 1a who completed IFN therapy, 11 (47.8%) were defined as achieving sustained virological response. Factors related to sustained virological response by univariate analysis were IL28B and ISDR. In conclusion, HCV genotype 1a is rare in Japan. The presence of IL28B genotype TT, and more than two mutations, in the ISDR are associated with a good response to IFN therapy in patients with HCV genotype 1a. ***J. Med. Virol. 84:438–444, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: hepatitis C virus; genotype 1a; NS5A; IL 28B; interferon

INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family and causes chronic hepatitis that can develop into cirrhosis and hepatocellular carcinoma [Seeff, 2002]. HCV infection is a significant global health problem, affecting 170 million individuals worldwide. HCV can be divided into six genotypes and several subtypes according to genomic heterogeneity [Simmonds et al., 2005]. Each genotype shows a unique distribution and clinical characteristics such

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as interferon (IFN) responsiveness [Ghany et al., 2009]. HCV genotypes 1b, 2a, and 2b are the major types encountered in Japan [Enomoto et al., 1990; Hayashi et al., 2003]. Genotype 1a is common worldwide, but is rare in Japan except among individuals with hemophilia who have received imported clotting factors [Fujimura et al., 1996; Otagiri et al., 2002; Hayashi et al., 2003]. The prevalence and clinical characteristics, including IFN responsiveness, of Japanese patients with HCV genotype 1a are unclear. HCV NS5A protein reportedly includes a domain associated with IFN response. This domain, located in the NS5A region of HCV genotype 1b, is closely associated with response to IFN therapy and is known as the IFN sensitivity-determining region (ISDR) [Enomoto et al., 1996]. IFN acts to inhibit viral replication by inducing double-stranded RNA-dependent protein kinase (PKR). The ISDR is located at the 5′ end of the PKR-binding domain and is inhibited by PKR in vitro [Gale et al., 1998]. ISDR heterogeneity of genotype 1b is thus an important factor that may affect response to IFN [Enomoto et al., 1996; Nakano et al., 1999; Pascu et al., 2004; Hayashi et al., 2011a]. Several studies have reported a relationship between ISDR and IFN responsiveness among patients with HCV genotype 1a [Hofgärtner et al., 1997; Zeuzem et al., 1997; Kumthip et al., 2011; Yahoo et al., 2011]. However, this remains controversial for genotype 1a, and the utility of ISDR sequences for predicting IFN responsiveness has not been investigated for HCV genotype 1a in Japan due to the rarity of this genotype. Both genetic heterogeneity of the HCV genome and host genetics contribute to IFN responsiveness. Several genome-wide association studies have thus been performed to clarify host factors associated with IFN responsiveness, revealing that interleukin-28B (IL28B) polymorphisms are strongly associated with response to IFN therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Thomas et al., 2009]. Combined use of the single-nucleotide polymorphisms (SNPs) of IL28B and amino acid substitutions in the core region and ISDR could thus improve the prediction of response to IFN in patients with HCV genotype 1b [Akuta et al., 2011; Hayashi et al., 2011b; Kurosaki et al., 2011]. However, the effects of a combined evaluation of the SNPs of IL28B and amino acid substitutions in the ISDR in patients with HCV genotype 1a on IFN response are unclear. The aim of the present study was to determine whether genomic heterogeneity of the ISDR and SNPs of IL28B among patients with HCV genotype 1a affect response to combination therapy with pegylated-IFN-α2b and ribavirin.

PATIENTS AND METHODS

A total of 977 patients (569 men, 408 women) with chronic hepatitis C genotype 1 and high viral load (<100 KIU/ml) who were treated at Nagoya University Hospital and affiliated hospitals were enrolled in this study. Mean age of patients was 55.1 ± 12.2 years (range: 18–75 years). None of the patients had a history of chronic alcohol abuse, autoimmune disease, or metabolic disease. Patients with active intravenous drug use and immigrants were excluded from this study. The core region (aa 30–110) and ISDR (aa 2,209–2,248) of HCV were examined by direct sequencing. SNPs of IL28B (rs8099917) were identified using a real-time polymerase chain reaction (PCR) system. Patients received subcutaneous injections of pegylated-IFN-α2b (1.5 μg/kg) once each week along with oral ribavirin (600 mg/day for patients <60 kg, 800 mg/day for 60–80 kg, 1,000 mg/day for >80 kg) for 48 weeks. Patients who became negative for HCV-RNA between 16 and 36 weeks after initiating IFN treatment had the IFN treatment extended to 72 weeks, in accordance with Japanese guidelines [Kumada et al., 2010]. HCV-RNA levels in serum samples were examined at 12 weeks, at the end of IFN therapy, and at 6 months after the end of treatment. Serum was stored at −80°C for virological examination at pretreatment. Early virological response was defined as HCV-negative status at 12 weeks. Patients who were persistently negative for serum HCV-RNA at 24 weeks after withdrawal of IFN treatment were considered to show sustained virological response. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Virological Analysis

HCV-RNA quantitative viremia load was determined by PCR. HCV was genotyped by direct sequencing of the 5′-untranslated region and/or core regions as described previously and confirmed by direct sequencing of the NS5A region [Otagiri et al., 2002; Dal Pero et al., 2007; Hayashi et al., 2011a]. Genotypes were classified according to the nomenclature proposed by Simmonds et al. [2005]. Direct sequencing of the core and NS5A-ISDR regions was performed as reported previously [Dal Pero et al., 2007; Hayashi et al., 2011a]. In brief, RNA was extracted from 140 μl of serum using a commercial kit (QIAamp Viral RNA Kit; Qiagen, Valencia, CA) and dissolved in 50 μl of diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligos and random hexamer primers with a commercial kit (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA). The HCV core region and NS5A-ISDR were amplified by nested PCR. In brief, each 50-μl PCR reaction mixture contained 100 nM of each primer, 1 ng of template cDNA, 5 μl of GeneAmp 10× PCR buffer, 2 μl of dNTPs, and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). Primers for the core region were: sense, 5′-GGGAGGTCTCGTAGACCGTGCACCATG-3′ and antisense, 5′-GAGMGGKATRTACCCCATGAGRTCGGC-3′. Primers for the NS5A-ISDR were: sense, 5′-GCCTGGAGCCCTTGTAGTC-3′ and

TABLE I. Clinical Characteristic of Patients With HCV Genotype 1a

	N = 32
Age (y.o.)	36.4 ± 2.2
Sex: male/female	28/4
AST (IU/L)	48.8 ± 33.6
ALT (IU/L)	64.6 ± 57.8
Platelet (10^4/μl)	18.8 ± 6.0
HCV RNA level (KIU/ml)	2607.4 ± 3072.2
Source (clotting factor/BTF/unknown)	23/2/7

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

antisense, 5′-CTGCGTGAAGTGGTGGAATAC-3′. Amplification conditions consisted of 10 min at 94°C, followed by 40 cycles of 94°C for 10 sec, 55°C for 30 sec, and 72°C for 30 sec in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was performed using the same reaction buffer with the first-round PCR product as template, and the following sets of primers: for the core region, sense primer 5′-AGACCGTGCACCATGAGCAC-3′ and antisense 5′-TACGCCGGGGGTCAKTRGGGCCCCA-3′; and for the NS5A-ISDR, sense 5′-TGTTTCCCCCACG-CACTAC-3′ and antisense 5′-TGATGGGCAGTTTT-TGTTCTTC-3′. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers using a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems).

Genotyping Analysis

Detection of SNPs for IL28B (rs8099917) was conducted using a real-time PCR system. In brief, genomic DNA was extracted from 150 μl of whole blood with a commercial kit (QIAamp DNA Blood mini Kit; Qiagen) and dissolved in 50 μl of diethylpyrocarbonate-treated water. DNA (10 ng) was used for PCR and genotyping of IL28B SNP (rs8099917) was performed by TaqMan allelic discrimination (ABI-Prism 7300 SDS software; Applied Biosystems) with TaqMan SNP Genotyping Assays provided by Applied Biosystems (C__11710096_10).

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). The paired *t*-test was used to analyze differences in variables. A value of $P < 0.05$ was considered statistically significant. Statview 5.0 software (SAS Institute, Cary, NC) was used for all analyses.

RESULTS

Thirty-two of the 977 patients (3.3%) were infected by genotype 1a. Clinical characteristics of patients with genotype 1a are summarized in Table I. Twenty-three cases involved patients with hemophilia who had received imported clotting factors. The prevalence of genotype 1a after excluding patients with hemophilia was 0.9%. A comparison of clinical characteristics according to hemophilia status is shown in Table II. No significant differences were apparent among the two groups. Differences in clinical characteristics between genotypes 1a and 1b are shown in Table III. Males were more frequent among patients with genotype 1a (87.5%) than among those with genotype 1b (57.2%), as the majority of patients with genotype 1a were young male patients with hemophilia. Sequence alignments of the core region at codons 71 and 90 showed arginine and cysteine, respectively, in all patients. The HCV core region of genotype 1a was thus well-conserved, with no significant mutations at codons 71 or 90. This is not similar to previous findings for genotype 1b [Akuta et al., 2005, 2011; Hayashi et al., 2011a,b; Kurosaki et al., 2011]. Alignment of the amino acid sequence for NS5A-ISDR is shown in Figure 1. The sequence of the HCV-1 strain was defined as the consensus sequence of genotype 1a, and the number of mutations to the chosen consensus sequence in ISDR was used to analyze the ISDR system. Sequences of the HCV-1 strain and HCV-1 strain with only one amino acid substitution were defined as wild-type, while ISDR sequences with more than two amino acid substitutions were defined as mutant-type. Twenty-seven strains were defined as wild-type and 5 strains were defined as mutant-type. IL28B genotypes could be obtained for 25 patients, and IL28B alleles were TT (n = 14) and TG (n = 11). Twenty-three patients received pegylated-IFN-α2b plus ribavirin therapy. Twenty patients were treated for 48 weeks, and 1 patient was treated for 72 weeks. Two patients were withdrawn at 24 weeks due to a

TABLE II. Clinical Characteristic According to Hemophilia

	Patients with hemophilia (N = 23)	Patients without hemophilia (N = 9)	*P*-value
Age (y.o.)	37.1 ± 9.2	37.1 ± 16.3	0.9966
Sex: male/female	22/1	6/3	0.0572
AST (IU/L)	51.2 ± 34.8	41.9 ± 30.9	0.5072
ALT (IU/L)	68.2 ± 55.8	54.0 ± 66.1	0.5566
Platelet (10^4/μl)	18.4 ± 6.8	19.8 ± 3.0	0.5602
HCV levels (KIU/ml)	2599.6 ± 3108.0	2630.0 ± 3176.5	0.9812

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

TABLE III. Clinical Characteristic According to Genotypes

	Genotype 1a (N = 32)	Genotype 1b (N = 945)	*P*-value
Age (y.o.)	36.4 ± 2.2	55.9 ± 11.6	0.0001
Sex: male/female	28/4	546/408	0.0004
Patients with hemophilia	23	4	0.0001
AST (IU/L)	48.8 ± 33.6	59.9 ± 45.0	0.1745
ALT (IU/L)	64.6 ± 57.8	64.6 ± 57.8	0.9894
Platelet ($10^4/\mu l$)	18.8 ± 6.0	17.2 ± 6.0	0.0918
HCV levels (KIU/ml)	2607.4 ± 3072.2	2011.5 ± 1453.8	0.0642

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus.

lack of response to IFN therapy. Frequency of early virological response, characterized by undetectable HCV at 12 weeks, was 30.4% (7/23). Virological response rate at the end of treatment was 47.8% (11/23). Finally, 11 of 23 patients (47.8%) achieved sustained virological response. Clinical characteristics were compared between patients who achieved sustained virological response and patients who did not (Table IV), revealing significant differences in two factors on univariate analysis: IL28B and ISDR.

DISCUSSION

The present study investigated 977 patients with genotype 1 using direct sequencing of core and NS5A regions, revealing that genotype 1a is rare (3.3%) in Japan. Of the 33 patients with genotype 1a, 23 (71.9%) were patients with hemophilia, confirming that the majority of cases with genotype 1a involve patients with hemophilia who have received imported clotting factors, as previously reported [Fujimura et al., 1996; Otagiri et al., 2002; Hayashi et al., 2003]. Analysis after excluding patients with hemophilia revealed the prevalence of genotype 1a in Japan was 0.9% (9/954). Recently, the distributions of HBV genotypes have been changing in Japan due to international exchange [Hayashi et al., 2007; Matsuura et al., 2009]. However, prevalences of HCV genotypes have remained stable because of the different modes of infection involved. The present study revealed that 11 (47.8%) of 23 patients achieved sustained virological response. The IFN responsiveness of HCV genotype 1a in Japanese patients was reported in 1999 from Okinawa, a far southern island in Japan [Sakugawa et al., 1997]. That study reported that the rate of sustained virological response tended to be higher in patients with genotype 1a than in those with genotype 1b, but no significant differences were identified because of the small number of patients with genotype 1a. Low virological response rates in both genotypes 1a and 1b were confirmed in the present Japanese patients, as in Caucasian patients [Manns et al., 2001; McHutchison et al., 2009]. No significant differences in sustained virological response rate were seen between genotypes 1a and 1b. Discriminating between genotypes 1a and 1b thus seems to have little clinical relevance in terms of IFN responsiveness. Viral factors associated with sustained virological response, including HCV genotype, have been studied most frequently studied and mutations in the core and NS5A regions of HCV genotype 1b have been associated with response to IFN therapy [Akuta et al., 2005, 2010, 2011; Okanoue et al., 2009; Nakagawa et al., 2010; Toyoda et al., 2010; Hayashi et al., 2011a; Hayes et al., 2011; Kumthip et al., 2011; Kurosaki et al., 2011]. These viral factors could improve prediction of sustained virological response for genotype 1a, as in 1b. Amino acid substitutions at positions 70 and 91 of the HCV core region in genotype 1b have been related to IFN responsiveness, liver steatosis, hepatic oxidative stress, insulin resistance, and carcinogenesis [Akuta et al., 2005, 2007, 2009; Tachi et al., 2010]. These substitutions may have substantial impacts on

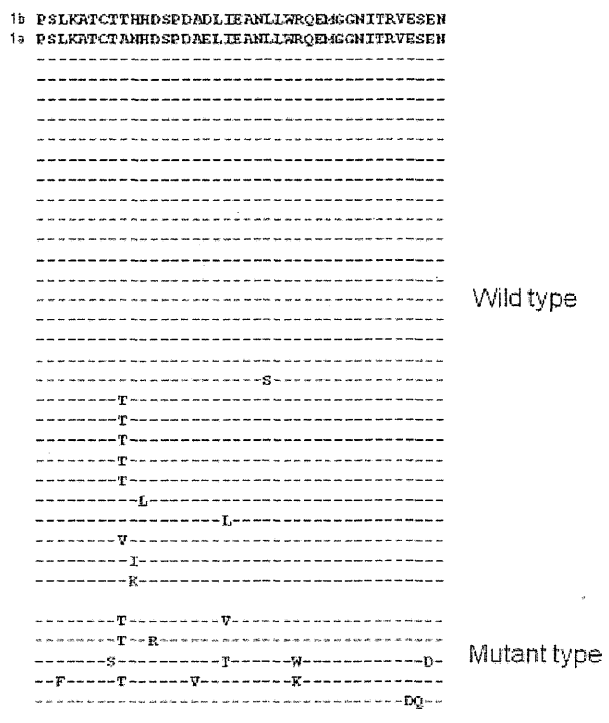

Fig. 1. Alignment of the amino acid sequence for the NS5A-ISDR. In the sequence alignment, dashes indicate amino acids identical to consensus sequence HCV1. Sequences of the HCV1 strain and HCV1 strains with one-nucleotide substitutions were defined as wild-type ISDR, and all other strains were defined as mutant-type ISDR. ISDR, interferon sensitivity-determining region.

TABLE IV. Univariate Analysis: Factors Predictive of Sustained Virologic Response

Factors	Sustained virologic response (n = 11)	Non-sustained virologic response (n = 12)	*P*-value
Age (y.o.)	37.9 ± 10.9	39.8 ± 11.3	0.6958
Gender: male/female	10/1	10/2	0.9999
ALT (IU/L)	78.2 ± 50.8	62.6 ± 68.1	0.5435
AST (IU/L)	51.4.4 ± 29.2	48.8 ± 40.4	0.8616
PLT ($\times 10^4/mm^3$)	19.0 ± 5.4	19.3 ± 5.7	0.8870
HCV RNA level (KIU/ml)	1323.1 ± 1077.3	2567.0 ± 2940.8	0.2481
ISDR: wild/mutant	7/4	12/0	0.0373
IL28B:TT/TG	9/1	4/8	0.0115

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; IL28B, interleukin 28B.

the pathogenesis of HCV genotype 1a infection. However, the HCV core region of genotype 1a is well-conserved and no significant mutations were seen in the core region, which is associated with IFN responsiveness. Several reports have also found that the HCV core region, including positions 70 and 91, of HCV genotype 1a is highly conserved [Alestig et al., 2011; Kumthip et al., 2011]. Mutations in the core region of genotype 1a would be rare, so this region might be unsuitable for routine clinical use, unlike in genotype 1b. However, the number of patients in this study was small, and large studies including from other countries are needed to clarify these issues. The ISDR in the NS5A region of HCV genotype 1b is closely associated with response to IFN therapy. ISDR mutations of genotype 1b are well known to be more important in predicting sustained virological response in Japanese patients than European patients [Hofgärtner et al., 1997; Zeuzem et al., 1997; Nakano et al., 1999; Pascu et al., 2004; Hayashi et al., 2011a]. European studies have failed to detect the specific amino acid substitutions in ISDR of genotype 1a associated with IFN responsiveness [Hofgärtner et al., 1997; Zeuzem et al., 1997]. In this study, sustained virological response was achieved in 36.8% of patients with wild-type ISDR and 100% of patients with mutant-type ($P = 0.0373$). The present analysis showed a close relationship between ISDR of genotype 1a and sustained virological response, as in genotype 1b. Recent investigations in Thailand and Iran have failed to identify the usefulness of ISDR for HCV genotype 1a in predicting sustained virological response [Kumthip et al., 2011; Yahoo et al., 2011]. The high virological response rate and low prevalence of patients with mutations in the ISDR do not favor the use of ISDR analysis in predicting IFN responsiveness [Herion and Hoofnagle, 1997; Yokozaki et al., 2011]. Rates of sustained virological response among these studies were much higher than those in the present study (68.4% and 75% vs. 47.8%). The mean number of mutations in patients who achieved sustained virological response in the studies by Kumthip et al. [2011] and Yahoo et al. [2011], and the present group were 1.4, 1.4, and 1.6, respectively. Differences in sustained virological response and the number of mutations to the ISDR might underpin this discrepancy in the evaluation of ISDR. Although the sample size in the present study was small, the results indicate that ISDR represents a strong indicator of progression to sustained virological response for patients with HCV genotype 1a. Amino acid substitutions in the ISDR of genotype 1a thus also play an important role in predicting sustained virological response in Japanese patients compared to patients from other countries. IL28B polymorphisms such as host genetics, as well as mutations in the HCV genome, contribute to IFN treatment outcomes. Rates of sustained virological response in patients in this study with TT and TG were 69.2% and 11.1%, respectively. The TG allele of the IL28B genotype was significantly associated with poor response to IFN therapy ($P = 0.0115$). SNPs of IL28B would regulate the expression of IFN-stimulated genes and affect IFN responsiveness. IL28B and ISDR thus exert independent effects on IFN responsiveness and both host and viral factors impacting IFN responsiveness would improve the prediction of sustained virological response. Several studies have thus reported that both the SNP of IL28B and mutations in the ISDR were associated with sustained virological response in patients with HCV genotype 1b [Akuta et al., 2011; Hayashi et al., 2011b; Kurosaki et al., 2011]. In the present study of HCV genotype 1a, among the 9 patients who had simultaneously the TG allele for IL28B and wild-type ISDR, only 1 achieved sustained virological response (11.1%). The best-sustained virological response was achieved in patients with mutant-type ISDR and the T allele (100%). The combination of SNPs for IL28B and mutations in ISDR may thus predict response to IFN therapy in patients with HCV genotype 1a as well as genotype 1b. Given the small sample size in this investigation, larger cohorts are needed to confirm the present results. Furthermore, infection with genotype 1a in Japanese patients is rare, making large-scale studies difficult to perform.

In conclusion, the prevalence of HCV genotype 1a is rare in Japan and the majority of cases involve patients with hemophilia. The TG genotype of IL28B is associated with poor response, while mutant-type ISDR is associated with good response to combination therapy with pegylated-IFN-α2b and ribavirin in patients with HCV genotype 1a. Combined use of both IL28B and ISDR could improve the prediction of IFN response.

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Mutations in the core and NS5A region of hepatitis C virus genotype 1b and correlation with response to pegylated-interferon-alpha 2b and ribavirin combination therapy

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SUMMARY. Mutations in two regions of hepatitis C virus (HCV) have been implicated in influencing response to interferon (IFN) therapy. Substitutions in the NS5A region of HCV have been associated with response to IFN therapy, and this region has been known as the IFN sensitivity-determining region (ISDR). The mutations in the core region of HCV have also been reported to predict IFN response. The aim of this study was to investigate whether amino acid substitutions in the core region and ISDR among patients with HCV genotype 1b affect the response to IFN therapy. A total of 213 patients who completed IFN treatment were randomly selected. All patients received pegylated-IFN-alpha 2b once each week, plus oral ribavirin daily for 48 weeks. Of the 213 patients, 117 (54.9%) showed early virologic response (EVR), with HCV-negativity, at 12 weeks. Factors related to EVR on multivariate analysis were non-Gln70 and Leu91 in the core region, and ISDR mutant-type. One hundred and two (47.9%) showed a sustained virologic response (SVR). SVR occurred more frequently in patients without Gln70 (55.4%) than in those with Gln70 (21.3%) ($P < 0.0001$). SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% of patients with mutant-type ($P = 0.0227$). Of the 34 patients who simultaneously had non-Gln70 and mutant-type ISDR, 26 (76.5%) achieved SVR. Factors related to SVR on multivariate analysis were non-Gln70 and ISDR mutant-type. In conclusion, amino acid substitutions in the core region and ISDR were useful for predicting the response to IFN in patients with HCV genotype 1b.

Keywords: core region, genotype 1b, hepatitis C virus, interferon sensitivity-determining region, interferon therapy, NS5A.

INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family and causes chronic hepatitis that can develop into potentially fatal cirrhosis and hepatocellular carcinoma [1]. It has been estimated that 170 million people are infected with HCV worldwide. Therefore, HCV infection is a major global health problem. HCV consists of four structural proteins (core, envelope 1, envelope 2 and p7) and six nonstructural proteins (NS2–NS5) [2]. HCV core protein was thought to inhibit the antiviral action of interferon (IFN) through down-regulation of transcription of IFN-induced antiviral genes [3,4]. The NS5A region includes the PKR-binding domain, which is associated with viral replication that is affected by IFN [5]. Thus, the core and NS5A regions of HCV appear to be important factors that may affect the response to IFN therapy, and mutations in the core and NS5A regions of HCV have been reported to affect response to IFN therapy [6–10]. The core region of HCV is well conserved, but substitutions of amino acid (aa) 70 and aa 91 are frequently found. Several studies reported a relation between these substitutions in the core region and IFN responsiveness [8,10]. The substitutions in the NS5A region of HCV have been closely associated with response to IFN therapy, and this region is known as the IFN sensitivity-determining region (ISDR) [6]. However, these

Abbreviations: Aa, amino acid; ALT, alanine aminotransferase; EVR, early virologic response; HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity-determining region; SVR, sustained virologic response.

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relationships are little known and still controversial [10]. The aim of this study was to investigate whether amino acid substitutions in the core region and ISDR among patients with HCV genotype 1b affect the response to pegylated-IFN-alpha 2b and ribavirin combination therapy.

MATERIAL AND METHODS

A total of 891 patients with chronic hepatitis C genotype 1b and high viral load who were treated at Nagoya University Hospital and Affiliated Hospitals were enrolled; 213 patients who completed IFN treatment were randomly selected for this study. The patients' clinical characteristics are summarized in Table 1. Patients whose HCV-RNA levels were <100 KIU/mL were excluded. The core region (aa 30–110) and ISDR (aa 2209–2248) were examined by direct sequencing. All patients received subcutaneous injections of pegylated-IFN-alpha 2b (1.5 μg/kg) once each week plus oral ribavirin daily for 48 weeks. HCV-RNA in serum samples was examined at 12 weeks, at the end of IFN therapy and at 6 months after the end of treatment. Serum was stored at −80 °C for virologic examination. Early virologic response (EVR) was defined as HCV-negative at 12 weeks. Patients who were persistently negative for serum HCV-RNA and who had a normal serum alanine aminotransferase (ALT) level at 24 weeks after withdrawal of IFN treatment were considered to have sustained virologic response (SVR). Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Virologic analysis

HCV-RNA quantitative viremia load was determined by polymerase chain reaction (PCR). HCV was genotyped by direct sequencing of the 5′-untranslated region and/or E1 regions as described previously [11,12]. Genotypes were classified according to the nomenclature proposed by Simmonds *et al.* [13]. Direct sequencing of the core and NS5A-ISDR region was carried out as reported previously, but with modifications [7,14]. In brief, RNA was extracted from 140 μL serum with a commercial kit (QIAamp Viral RNA Kit; Qiagen, Valencia, CA, USA) and dissolved in 50 μL diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligo and random hexamer primers with a commercial kit (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA, USA). HCV core region and NS5A-ISDR were amplified by nested PCR. In brief, each 50-μL PCR reaction contained 100 nM of each primer, 1 ng template cDNA, 5 μL GeneAmp 10 × PCR buffer, 2 μL dNTPs and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Primers for core region were sense 5′-GGGAGGTCTCGTAGACCGTGCACCATG-3′ and antisense 5′-GAGMGGKATRTACCCCATGAGRTCGGC-3′ and primers for the NS5A-ISDR were sense 5′-TGGATGGAGTGCGGTTGCACAGGTA-3′ and antisense 5′-TCTTTCTCCGTGGAGGTGGTATTG-3′. Amplification conditions consisted of 10 min at 94 °C, followed by 40 cycles of 94 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was performed in the same reaction buffer with the first-round PCR product as template, and the following sets of primers: for the core region, sense primer 5′-AGACCGTGCACCATGAGCAC-3′ and antisense 5′-TACGCCGGGGGTCAKTRGGGCCCCA-3′; and for the NS5A-ISDR, sense 5′-CAGGTACGCTCCGGCGTGCA-3′ and antisense 5′-GGGGCCTTGGTAGGTGGCAA-3′. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems). A mutation mixture was defined as viral mutants that constituted 50% or more of the total viral population.

Table 1 Clinical characteristics

Clinical characteristics	*N* =2 13
Age (years)	55.2 ± 10.6
Sex: male/female	120/93
AST(IU/L)	58.5 ± 37.7
ALT(IU/L)	66.0 ± 53.9
Platelet count (10^4/uL)	17.1 ± 5.1
HCV RNA level (KIU/mL)	1720 (100–7200)
Treatment: naive/retreatment	117/96
Body weight (kg)	55.3 ± 19.9

Data are expressed as mean ± standard deviation HCV RNA level was shown by median (range). AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

Statistical analysis

Data are expressed as means ± standard deviation (SD). The paired *t*-test, the chi-square and the Fisher's exact tests were used to analyze differences in variables. A *P*-value of <0.05 was considered statistically significant. Multiple logistic regression models were used to identify factors predictive of EVR and SVR. Statview 5.0 software (SAS Institute, Inc., Cary, NC, USA) was used for all analyses.

RESULTS

Genetic heterogeneity in NS5A-ISDR and core regions of the HCV genome

The mutations in the HCV core region were measured by direct sequencing. The core region of HCV is well conserved,

Table 2 Prevalence of amino acid substitutions at 70, 75, and 91

Core 70	
Histidine	$n = 6$
Glutamine	$n = 46$
Glutamine/Histidine	$n = 1$
Arginine	$n = 160$
Core 75	
Alanine	$n = 112$
Alanine/Serine	$n = 1$
Alanine/Threonine	$n = 2$
Glutamine	$n = 1$
Serine	$n = 5$
Threonine	$n = 91$
Valine	$n = 1$
Core 91	
Leucine	$n = 162$
Methionine	$n = 51$

but substitutions of aa 70, aa 75 and aa 91 were frequently found, as previously reported. The distribution of mutations in the HCV core region at aa 70, aa 75 and aa 91 is shown in Table 2. The sequence of the HCVJ strain was defined as the consensus sequence, and the approach of counting the number of mutations to the chosen consensus sequence in ISDR was used to analyze the ISDR system. The number of NS5A-ISDR mutations was as follows: none ($n = 102$), 1 ($n = 63$), 2 ($n = 14$), 3 ($n = 8$), 4 ($n = 8$), 5 ($n = 7$), 6 ($n = 2$), 7 ($n = 4$) and 8 ($n = 5$). The relationships between substitutions of amino acids in the HCV core region and NS5A-ISDR are shown in Fig. 1. There were no significant relationships between the two regions. Thus, the HCV core region and the NS5A-ISDR were independent factors.

Virological response

Of 213 patients, 117 (54.9%) showed EVR, with HCV-negativity, at 12 weeks, and 76 became HCV-negative after 12 weeks; overall, 187 patients became HCV-negative at the end of treatment (87.8%). However, 85 patients continued to be HCV-positive after withdrawal of IFN treatment, and 102 of 213 (47.9%) patients were defined as achieving a SVR. Of 117 patients with EVR, 87 (74.4%) achieved SVR. Of 96 patients without EVR, 81 became non-SVR (84.4%). Thus, EVR was strongly associated with SVR.

Factors associated with early virologic response

The results of univariate analysis for factors predictive of EVR are shown in Table 3. The EVR rate according to amino acid substitutions of ISDR are shown in Table 4. The EVR rate of patients with more than two mutations in the ISDR (mutant-type) was 68.9%. Of 166 patients without glutamine (Gln) at aa 70 in the core region, 100 achieved EVR. The EVR rate of patients with Leu91 in the core region was 61.1%. The results of multivariate analysis for factors predictive of EVR are shown in Table 5. Factors related to EVR on multivariate analysis were non-Gln70, Leu91 and ISDR mutant-type.

Factors associated with sustained virologic response

The results of univariate analysis for factors predictive of SVR are shown in Table 6. The SVR rate according to amino acid substitutions of ISDR are shown in Table 4. SVR occurred more frequently in patients without Gln70 (55.4%) than in those with Gln70 (21.3%) (odds ratio, 0.217; 95% confidence interval (CI), 0.101–0.466; $P < 0.0001$). SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% with mutant-type ISDR (odds ratio, 0.465; 95% CI, 0.240–0.899; $P = 0.0227$). Factors related to SVR on multivariate analysis were non-Gln70 and ISDR mutant-type, as shown in Table 7.

The virological response according to amino acid substitutions in the 70 core region and ISDR

The SVR and EVR rates according to amino acid substitutions in the 70 core region and ISDR are shown in Table 8. The best response for both SVR and EVR was achieved in patients with non-Gln70 and mutant-type ISDR, and the

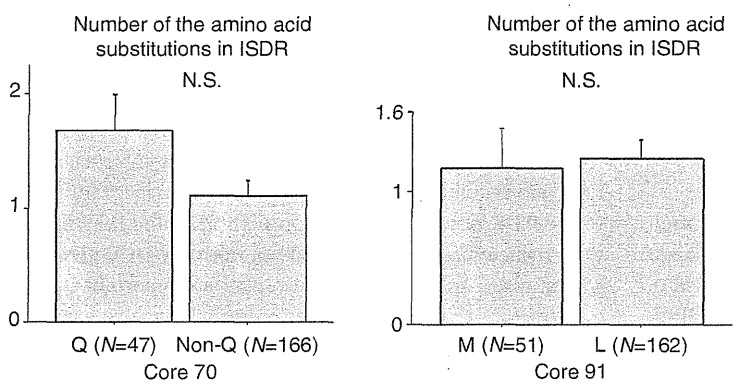

Fig. 1 The association between amino acid substitutions in core region and ISDR. ISDR, interferon sensitivity-determining region; Q, glutamine; L, leucine; M, methionine; NS, not significant.