Table 3 Univariate analysis: Factors predictive of EVR

Factors	EVR (n = 117)	Non-EVR (n = 96)	P-value
Age (years)	54.7 ± 11.3	55.9 ± 9.7	0.4511
Gender: male/female	63/54	57/39	0.7830
ALT (IU/L)	69.6 ± 64.8	61.5 ± 36.2	0.3002
AST (IU/L)	59.4 ± 40.9	57.3 ± 33.5	0.7026
PLT ($\times 10^4/mm^3$)	17.4 ± 5.1	16.9 ± 5.18	0.4955
HCV RNA level (KIU/mL)	2051.3 ± 1373.4	2006.1 ± 1462.7	0.8216
Core 70:non-Q/Q	100/17	66/30	0.0046
Core 75: A/non-A	58/59	54/42	0.3387
Core 91: L/M	99/18	63/33	0.0020
ISDR: wild/mutant	84/33	81/15	0.0327

EVR, early virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; Q, glutamine; A, alanine; L, leucine; M, methionine; ISDR, interferon sensitivity-determining region

Table 4 Amino acid substitutions of ISDR and virologic response

ISDR; number of the amino acid substitutions	0 N = 102	1 N = 63	2 N = 14	3 N = 8	4 N = 8	5 N = 7	6 N = 2	7 N = 4	8 N = 5
EVR rate (%)	51 (50.0)	33 (52.4)	10 (71.4)	4 (50.0)	7 (87.5)	4 (80.0)	0 (0)	3 (75.0)	5 (100)
SVR rate (%)	41 (40.2)	31 (49.2)	10 (71.4)	4 (50.0)	4 (50.0)	5 (71.4)	0 (0)	3 (75.0)	4 (80.0)

EVR, early virologic response; SVR, sustained virologic response.

Table 5 Multivariate analysis: Factors predictive of EVR

Factors	P-value	Risk ratio	95% CI	
Gender: male	0.3760	0.754	0.403	1.410
Age: <60 years	0.8247	0.915	0.416	2.012
AST: <60 IU/L	0.3301	1.525	0.652	3.569
ALT: <60 IU/L	0.2484	0.613	0.267	1.407
PLT: $<17 \times 10^4/mm^3$	0.0666	0.530	0.269	1.044
Core 70: nonQ	0.0242	2.406	1.121	5.165
Core 91: A	0.0022	3.409	1.557	7.463
Core 75: M	0.0683	1.863	0.954	3.635
ISDR: mutant	0.0085	0.338	0.151	0.759

EVR, early virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, Interferon sensitivity-determining region; Q, glutamine; A, alanine; L, leucine; M; methionine.

worst response was achieved in patients with Gln70 and wild type ISDR. The SVR rates according to amino acid substitutions in the 70 core region and ISDR and EVR are shown in Table 9. The positive predictive values for SVR and non-SVR improved to 88.9% and 90.9%, respectively, when EVR was considered with the 70 core region and ISDR.

DISCUSSION

Peginterferon and ribavirin combination therapy has been standard treatment for patients with chronic hepatitis C. However, the SVR rate was almost 50% for HCV genotype 1b, which is a refractory strain. The standard doses and duration of peginterferon plus ribavirin may be suboptimal for half of the patients; patients need a new approach for eradicating HCV. Peginterferon and ribavirin therapy has been a useful treatment, but cost and adverse events have been problems. To select patients who could attain cure from HCV by current standard treatment, it is necessary to predict the response before therapy. Current guidelines for HCV treatment recommend that the selection of IFN treatment regimen depends on HCV genotypes and viral loads. Several studies have focused on sequence variation of the HCV genome and response to IFN therapy, but prediction of IFN responsiveness has been less well characterized. NS5A-ISDR heterogeneity is an important factor that may affect response to IFN, especially in Asia [6,7,9]. The ISDR interacts with PKR and regulates replication of HCV *in vitro* [5]. Mutations in the ISDR affect the interaction with PKR and may inhibit viral replication. Therefore, ISDR of not only HCV genotype 1b but also 2a and 2b could also play an important role as a predictor of IFN responsiveness in clinical research of standard IFN or Peg-IFN monotherapy [15,16]. The differences in HCV 1b subtype and race affect the utility of ISDR

Table 6 Univariate analysis: factors predictive of SVR

Factors	SVR (n = 102)	Non-SVR (n = 111)	P-value
Age (years)	53.6 ± 10.8	56.7 ± 10.2	0.0319
Gender: male/female	57/45	63/48	0.7830
ALT (IU/L)	69.6 ± 66.7	62.6 ± 38.5	0.3606
AST (IU/L)	58.8 ± 40.9	58.3 ± 34.8	0.9469
PLT ($\times 10^4/mm^3$)	17.7 ± 5.1	16.7 ± 5.0	0.1563
HCV RNA level (KIU/mL)	2111.1 ± 1504.9	1956.4 ± 1319.8	0.4386
Core 70:non-Q/Q	92/10	74/37	0.0001
Core 75: A/non-A	50/52	62/49	0.3388
Core 91: L/M	82/20	80/31	0.1984
ISDR: wild/mutant	72/30	93/18	0.0227

SVR, sustained virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; Q, glutamine; A, alanine; L, leucine; M, methionine, ISDR, Interferon sensitivity-determining region.

Table 7 Multivariate analysis: factors predictive of SVR

Factors	P-value	Risk ratio	95% CI	
Age: <60 years	0.5219	0.770	0.346	1.714
Gender: male	0.6775	1.140	0.614	2.116
AST: <60 IU/L	0.1017	0.487	0.206	1.153
ALT: <60 IU/L	0.1690	1.799	0.779	4.157
PLT: $<17 \times 10^4/mm^3$	0.4067	1.324	0.682	2.573
HCV RNA levels: <106 IU/mL	0.6409	0.841	0.405	1.743
Core70: nonQ	0.0004	0.220	0.094	0.512
Core91: M	0.5643	0.799	0.373	1.711
Core75: A	0.3993	0.757	0.396	1.446
ISDR: mutant	0.0096	2.879	1.294	6.407

SVR, sustained virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count, HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; Q, glutamine; A, alanine; L, leucine; M, methionine.

Table 8 The SVR and EVR rate according to amino acid substitutions in 70 core region and ISDR

Core70/ISDR	SVR (n = 102)	EVR (n = 117)
Q/wild (n = 33)	6 (18.2%)	11 (33.3%)
Q/mutant (n = 14)	4 (28.6%)	6(42.9%)
Non-Q/wild (n = 132)	66 (50.0%)	73 (55.3%)
Non-Q/mutant (n = 34)	26 (76.5%)	27 (79.4%)

SVR, sustained virologic response; EVR, early virologic response; SDR, interferon sensitivity-determining region; Q, Glutamine; ISDR, interferon sensitivity-determining region.

sequences for predicting IFN responsiveness [7,17,18]. Thus, ISDR was found to be good for predicting IFN outcome of patients in Asian countries rather than of patients in Western countries. The approach of counting the number of mutations to the HCV-J strain in the ISDR was used in the original report by Enomoto *et al.*, [6] and they classified the mutations into three groups: wild type (no mutation), intermediate (1–3 mutations) and mutant-type (more than four mutations). SVR did not occur in any of the 30 patients with wild type ISDR in the original report using standard IFN monotherapy. In the present study, 41 of 102 patients (40.2%) with the wild type ISDR (no mutation) achieved SVR because of improvement of Peg-IFN plus RBV combination therapy. We examined the association between the number of mutations and SVR with adjustment for current standard treatment. We were unable to identify a significant relation between no mutation and one mutation in ISDR and SVR. Thus, sequences of the HCV-J strain and HCV-J strain with single substitutions were defined as the wild-type, and ISDR sequences with more than two mutations were defined as the mutant-type. SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% of patients with mutant-type ISDR in this study. ISDR alone was insufficient to predict IFN responsiveness in patients who received peginterferon plus ribavirin combination therapy. We speculated that the other region would explain differences in IFN sensitivity in patients infected with wild type ISDR. HCV core, E2-PePHD and NS5A-V3 regions were reported to be associated with IFN response [8,10,19,20]. The HCV core interacts with several cell factors and modulates numerous gene expressions, including down-regulating transcription of IFN-induced antiviral genes, and it affects the inhibition of the antiviral action of IFN. Several studies indicated that the HCV core region could predict IFN responsiveness [8,10]. Therefore, the utility of substitutions of amino acids in the HCV core region combined with NS5A-ISDR sequences for predicting

Table 9 The SVR rate according to EVR amino acid substitutions in 70 core region and ISDR

Core70/ISDR	SVR of patients with EVR (n = 87)	Non SVR of patients with EVR (n = 30)	SVR of patients without EVR (n = 15)	Non SVR of patients without EVR (n = 81)
Q/wild (n = 33)	4 (40%*)	7	2	20 (90.9%**)
Q/mutant (n = 14)	3 (50%*)	3	1	7 (87.5%**)
Non-Q/wild (n = 132)	56 (76.7%*)	17	10	49 (83.1%**)
Non-Q/mutant (n = 34)	24 (88.9%*)	3	2	5 (71.4%**)

*Positive predictive value for SVR. **Positive predictive value for non-SVR. SVR, sustained virologic response; EVR, early virologic response; ISDR, interferon sensitivity-determining region; Q, glutamine.

IFN responsiveness was investigated. The non-Gln70 amino acid substitution in the HCV core region was related to SVR on univariate and multivariate analysis. SVR occurred more frequently in patients without Gln70 (50.6%) than with Gln70 (14.3%). SVR was not associated with aa 75 and aa 91 in the core region. When core 70 was considered in the analysis of ISDR, the SVR rates varied widely according to amino acid substitutions in core region 70 and ISDR. For instance, only 18.1% of patients with Gln70 and wild type ISDR achieved SVR compared with 76.4% in those with non-Gln70 and mutant-type ISDR. Despite having genotype 1b, patients with non-Gln70 and mutant-type ISDR responded to IFN as well as those with genotypes 2 and 3. Pegylated-IFN-alpha 2b and ribavirin combination therapy was suitable for treatment of Japanese patients with HCV genotype 1b, particularly those with non-Gln70 and mutant-type ISDR. Optimal duration of IFN therapy in some patients with non-Gln70 and mutant-type ISDR could be shorter than 48 weeks; and in these patients, costs and side effects could be reduced without reducing the efficacy of IFN therapy by using a shorter regimen. On the other hand, patients with Gln70 and wild type ISDR resistant to pegylated-IFN-alpha 2b and ribavirin combination therapy should receive much more powerful treatment, such as triple therapy including the new protease inhibitor, peginterferon alfa and ribavirin as their first regimen [21,22]. This is an important consideration to achieve optimal therapy and avoid unnecessary treatment. The effects of amino acid substitutions in core 70 on gene expression and core protein function were unclear, and further studies are needed to determine their mechanism. Although the effects of amino acid substitutions of the core region and ISDR were unclear, the mutation at core 70 and the ISDR system could be clinically used as a simple diagnostic tool to predict SVR in patients infected with genotype 1b. It is not easier to routinely measure the HCV sequence to determine the core 70 and ISDR sequence. Virologic response, as rapid virologic response and EVR, could be easy to measure by commercial kits in clinical practice and would be useful for prediction of achieving SVR for chronic hepatitis C patients. The present study also confirmed that EVR has been associated with SVR, but virologic response cannot be assessed before treatment. HCV sequencing analysis will become a convenient method because of progression of sequencing technology and cost reduction. In this respect, the core region and ISDR were useful predictors of virologic response. Analysis of EVR in combination with the core region and ISDR revealed that 24 of 34 patients with non-Gln70 and mutant-type ISDR and EVR achieved SVR. EVR, core region and ISDR are considered strong indicators of SVR for patients with HCV genotype 1b. Although validation of these observations in larger cohorts is required, amino acid substitutions in the core region of HCV and ISDR were useful for predicting the response to pegylated-IFN-alpha 2b and ribavirin combination therapy in patients with chronic hepatitis C genotype 1b. Combining amino acid substitutions in the core region and ISDR could improve the predictive value of SVR in patients with genotype 1b, but the efficacy is still not satisfactory. The explanation for the lack of SVR in patients with non-Gln70 and mutant-type ISDR remains unclear. The other regions of HCV or host factors are candidates for a third factor for improving the prediction of SVR [23,24].

CONCLUSION

Amino acid substitutions in the 70 core region of HCV and ISDR were useful for predicting the response to pegylated-IFN-alpha 2b and ribavirin combination therapy in patients with chronic hepatitis C genotype 1b.

Data of this study were presented in part at the 59th annual meeting of the American association for the study of liver diseases (AASLD), October 31-November 4, 2008, San Francisco, CA, USA.

DISCLOSURE

All people have nothing to disclose.

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CLINICAL STUDIES

Association of interleukin 28B and mutations in the core and NS5A region of hepatitis C virus with response to peg-interferon and ribavirin therapy

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Keywords
core region – genotype 1b – hepatitis C virus – interleukin 28B – NS5A

Abbreviations
aa, amino acid; ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region; SVR, sustained virological response.

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Abstract

Background and aims: Mutations in the core and NS5A region of hepatitis C virus (HCV) genotype 1b have been associated with response to interferon (IFN) therapy. Genome-wide association studies have revealed that the single-nucleotide polymorphism (SNP) of interleukin 28B (IL28B) contributes to IFN response. The aim of this study was to investigate whether the SNP of IL28B (rs8099917) and amino acid substitutions in the core and NS5A region affect the response to IFN therapy. *Methods:* A total of 299 patients (157 men, 142 women; mean age, 55.9 ± 10.3 years) infected with HCV genotype 1b were studied. The fibrosis stage was diagnosed as F0 ($n=23$), F1 ($n=121$), F2 ($n=62$), F3 ($n=32$) and F4 ($n=7$) by liver biopsy. *Results:* Of the 299 patients, 138 achieved sustained virological response (SVR). On univariate analysis, predictors of SVR were age < 60 years, male gender, higher platelet count, lack of fibrosis, non-Q at core 70, mutant-type interferon sensitivity-determining region (ISDR) and IL28B genotype TT. The factors related to SVR on multivariate analysis were IL28B ($P=0.0001$), fibrosis ($P=0.0111$) and mutations in the core region70 ($P=0.0267$) and ISDR ($P=0.0408$). The best SVR was achieved in patients with non-Q70, mutant-type ISDR and T allele (74.5%), and the worst was achieved in patients with Q70, wild-type ISDR and G allele (8.1%). *Conclusions:* The SNP of IL28B and mutations in the core region and NS5A are associated with IFN responsiveness. Both host and viral factors might be useful for predicting IFN response.

It has been estimated that 170 million worldwide are infected with hepatitis C virus (HCV), which causes chronic hepatitis that can develop into potentially fatal cirrhosis and hepatocellular carcinoma (1). Therefore, HCV infection is a major global health problem. Pegylated-interferon (IFN)-α and ribavirin combination therapy is standard treatment for patients with chronic hepatitis C, but it eradicates HCV for only 50% of patients with genotype1 (2, 3). The difference in response was investigated, and several factors were identified, including age, liver fibrosis, HCV genotype, HCV RNA levels and race (4–7). Viral factors were frequently the focus for investigation of IFN responsiveness, and amino acid (aa) substitutions in the core and NS5A regions were reported as markers that could be used to predict the response to IFN therapy (8–14). However, these relationships were controversial (15, 16), and investigations were limited to viral factors alone to clarify IFN responsiveness. However, host genetic factors, as well as genetic heterogeneity in the HCV genome, contribute to IFN treatment outcomes. Therefore, several genome-wide association studies were performed to understand the host factors that were associated with IFN responsiveness; these revealed that interleukin 28B (IL28B) polymorphisms are strongly associated with response to IFN therapy (17–20). The single-nucleotide polymorphisms (SNPs) of IL28B, rs12979860 and rs8099917 genotypes are significantly associated with the outcome of IFN therapy. Although Caucasians and Hispanics have weak linkage-disequilibrium between these two SNPs, Japanese patients have strong linkage-disequilibrium, with no discrepancy between rs12979860 and rs8099917. Thus, rs8099917, which is strongly associated in Japanese reports, was selected for the present study (21). SNP of IL28B and mutations in the core and NS5A regions had different effects on IFN responsiveness, and their combined use might improve the ability to predict the response to IFN. However, the relationships between IL28B and viral factors such as mutations in the core and NS5A regions are little known. The aim of this study

was to investigate whether the SNP of IL28B and aa substitutions in the core and NS5A regions in patients with HCV genotype 1b affect the response to pegylated-IFN-α 2b and ribavirin combination therapy.

Methods

A total of 432 patients with chronic hepatitis C genotype1b and high viral load who were treated at Nagoya University Hospital, Fujita Health University Hospital and Ogaki Municipal Hospital were enrolled; 299 patients who completed IFN treatment for 48 weeks and had complete clinical data were selected for this study. Patients whose HCV RNA levels were < 100 KIU/ml were excluded. The patients' clinical characteristics are summarized in Table 1. The core region (aa 30–110) and interferon sensitivity-determining region (ISDR) (aa 2209–2248) were examined by direct sequencing. Identification of the SNP of IL28B (rs8099917) was performed by a real-time polymerase chain reaction (PCR) system. Liver biopsy was performed in 245 patients, and fibrosis stage was diagnosed according to the METAVIR criteria (22). Patients received subcutaneous injections of pegylated-IFN-α 2b (1.5 μg/kg) once each week plus oral ribavirin (600 mg for < 60 kg, 800 mg for 60–80 kg, 1000 mg for > 80 kg) daily for 48 weeks. Serum was stored at − 80 °C for virological examination at pretreatment. Patients who were persistently negative for serum HCV RNA at 24 weeks after withdrawal of IFN treatment were considered to have a sustained virological response (SVR). The other patients were considered to have non-SVR. This study was approved by each hospital's ethics committee. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki.

Virological analysis

The HCV-RNA quantitative viraemia load was determined by PCR. HCV was genotyped by direct sequencing of the 5′-untranslated region and/or E1 regions as described previously (23, 24). Genotypes were classified according to the nomenclature proposed by Simmonds *et al.* (25). Direct sequencing of the HCV core and NS5A-ISDR region was performed as reported previously (9, 14). In brief, RNA was extracted from 140 μl of serum with a commercial kit (QIAamp Viral RNA Kit, Qiagen, Valencia, CA, USA) and dissolved in 50 μl of diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligo and random hexamer primers using a commercial kit (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA). The 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 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, USA). Primers for the core region were sense, 5′-GGGAGGTCTCGTA GACCGTGCACCATG-3′ and antisense, 5′-GAGMGG KATRTACCCCATGAGRTCGGC-3′, and primers for the NS5A-ISDR were sense 5′-TGGATGGAGTGCGGTTGCA CAGGTA-3′ and antisense 5′-TCTTTCTCCGTGGAGG TGGTATTG-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 with 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. Baseline characteristics of the patients

Clinical characteristics	*N* = 299
Age (years)	55.9 ± 10.3
Sex: male/female	157/142
AST (IU/L)	58.7 ± 48.9
ALT (IU/L)	69.8 ± 66.9
Platelet count (10^4/μL)	16.6 ± 5.3
HCV RNA level (KIU/ml)	1760
The fibrosis stage	(100–7200)
F0, F1, F2, F3, F4	23,121, 62, 32, 7
Body weight (kg)	57.9 ± 12.7

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

Genomic analysis

Detection of the SNP of IL28B (rs8099917) was carried out by a real-time PCR system. In brief, genomic DNA was extracted from 150 μl of whole blood using a commercial kit (QIAamp DNA Blood mini Kit, Qiagen) and was dissolved in 50 μl of diethylpyrocarbonate-treated water. DNA (10 ng) was used for PCR with primers and probes from a commercial kit (Taqman SNP Genotyping Assays, Applied Biosystems). The SNP of IL28B (rs8099917) was amplified, and the results were analysed by real-time PCR in a thermal cycler (7300 Real-time PCR System, Applied Biosystems).

Statistical analysis

Data are expressed as means ± standard deviation. The paired *t*-test, the χ^2-test and Fisher's exact test were used

to analyse differences in variables. A P value < 0.05 was considered significant. Multiple logistic regression models were used to identify factors predictive of SVR. The statistical software used was SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Virological response

Of 299 patients, 35 (11.7%) showed a rapid virological response (RVR), with HCV negativity at 4 weeks, and 172 (57.5%) showed an early virological response (EVR), with HCV negativity at 12 weeks. Overall, 234 patients became HCV negative at the end of treatment (78.3%). However, 138 patients continued to be HCV negative after withdrawal of IFN treatment, and 138 of 299 (46.2%) patients were defined as achieving SVR. Of 35 patients with RVR, 33 (94.3%) achieved SVR. Of 172 patients with EVR, 126 (73.3%) achieved SVR. Of 127 patients without EVR, 115 became non-SVR (90.6%). Thus, RVR and EVR were associated with SVR ($P < 0.001$).

Genetic heterogeneity in NS5A-interferon sensitivity-determining region and response to interferon therapy

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 analyse the ISDR system as in previous reports (12–14). Seventy-one patients with more than two mutations in the ISDR were defined as mutant type, and the other 228 patients were wild type. SVR was achieved in 41.2% (95/228) of the patients with wild-type ISDR and in 60.6% (43/71) of the patients with mutant-type ISDR ($P = 0.0063$). ISDR was associated with SVR.

Amino acid substitutions in core regions of the hepatitis C virus genome and response to interferon therapy

Eighty-five patients with glutamine in core region 70 were defined as Q-type, and the other 214 patients were non-Q-type, as in previous reports (14). Overall, 118 of 214 patients with non-Q in the core region achieved SVR (55.1%). The SVR rate of patients with Q in core region 70 was 23.5% (20/85). Q70 in core region 70 was significantly associated with poor response to IFN therapy ($P < 0.0001$). The distribution of mutations in the HCV core region at aa 91 was leucine (L), 210 and methionine (M), 89. There were no significant differences between mutations in the HCV core region at aa 91 and SVR.

The prevalence of the single-nucleotide polymorphism of Interleukin28B (rs8099917) T (major allele) and G (minor allele) and response to interferon therapy

The frequencies of the IL28B genotypes were major homozygotes (TT), 219; heterozygotes (TG), 76; and minor homozygotes (GG), 4. The rates of SVR in the patients with TT, TG and GG were 57.9% (127/219), 14.5% (11/76) and 0% (0/4) respectively. The G allele of the IL28B genotype was significantly associated with poor response to IFN therapy ($P < 0.0001$).

Table 2. Association between interleukin 28B genotypes and amino acid substitutions in hepatitis C virus core region and interferon sensitivity-determining region

	ISDR	
	Mutant	Wild
TG/GG	12	68
TT	59	160
P value = 0.0324		
	HCV core region 70	
	Non-Q	Q
TG/GG	35	45
TT	179	40
P value < 0.0001		
	HCV core region 91	
	L	M
TG/GG	46	34
TT	164	55
P value = 0.0044		

The number is patients' number.
HCV, hepatitis C virus; IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region; L, leucine; Q, glutamine; M, methionine.

The relationships between substitutions of aa in the HCV core region, NS5A-ISDR and the SNP of IL28B are shown in Table 2. NS5A-ISDR and both mutations in the HCV core regions were associated with IL28B genotypes. ISDR wild-type and Q70, which were resistant strains to IFN therapy, were more frequently found in patients with resistant TG/GG allele than in those with sensitive TT allele.

Factors associated with sustained virological response

The results of univariate analysis for factors predictive of SVR are shown in Table 3. Patients with SVR were younger than those without SVR. Males were more frequent among SVR patients than non-SVR patients. SVR patients had higher platelet counts than non-SVR patients. SVR was achieved in 23.1% (9/39) of patients with advanced fibrosis and 50.5% (104/206) of patients without advanced fibrosis ($P = 0.0016$). SVR was achieved in 41.7% (95/228) of patients with wild-type ISDR and 60.6% (43/71) of patients with mutant-type ($P = 0.0063$). SVR occurred more frequently in patients without Q70 (55.1%; 118/214) than in those with Q70 (23.5%; 20/85; $P = 0.0001$). Achievement of SVR occurred more frequently in patients with TT allele (58%; 127/219) than in those with TG and GG alleles (13.8%; 11/80;

Table 3. Univariate analysis: factors predictive of sustained virological response

Factors	SVR ($n=138$)	Non-SVR ($n=161$)	*P* value
Age (years)	53.8 ± 11.5	57.9 ± 8.7	0.0005
Gender: male/female	82/56	75/86	0.0280
ALT (IU/L)	71.3 ± 76.7	68.4 ± 57.6	0.7110
AST (IU/L)	54.6 ± 46.7	62.1 ± 50.6	0.1983
PLT ($\times 10^4$/mm^3)	17.7 ± 5.5	15.6 ± 4.9	0.0008
Fibrosis: F0, 1, 2/3, 4	104/9	102/30	0.0016
HCV RNA level (KIU/ml)	2001.5 ± 1441.2	2168.3 ± 1432.4	0.3705
Core 70: non-Q/Q	118/20	96/65	0.0001
Core 91: L/M	104/106	34/55	0.0771
ISDR: wild/mutant	95/43	133/28	0.0063
IL28B: TT/TG+GG	127/11	92/69	0.0001

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

Table 4. Factors associated with sustained virological response by multivariate analysis

Factor	Category	Risk ratio	95% CI	*P* value
IL28B genotype	TT	0.106	0.043–0.259	0.0001
Fibrosis	F3, F4	3.550	1.335–9.440	0.0111
Core70	Q	2.496	1.111–5.604	0.0267
ISDR	Wild	2.206	1.034–4.710	0.0408

Only factor that achieved statistical significance ($P < 0.05$) on multivariate logistic regression analysis are shown.
IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region.

$P=0.0001$). Age, sex, platelet count, liver fibrosis, core 70, ISDR and IL28B were associated with SVR. The same 11 factors used in univariate analysis were used in multivariate analysis. The factors related to SVR on multivariate analysis were IL28B genotype, liver fibrosis, core 70 and ISDR, as shown in Table 4. The other factors were not significant.

The virological response according to interleukin28B genotypes and amino acid substitutions in the 70 core region and interferon sensitivity-determining region

The SVR rates according to IL28B genotypes and aa substitutions in the 70 core region and ISDR are shown in Table 5. The best SVR rate was achieved in patients with non-Q70, mutant-type ISDR and T allele, and the worst response was achieved in patients with Q70, wild-type ISDR and G allele.

Discussion

Viral factors associated with SVR have been the most frequently studied, and several regions, including 5′UTR, core, E2, NS5A and NS5B, have been suggested to play important roles in IFN responsiveness (8–16, 26–31). The aa substitutions in the HCV core and NS5A region would be two major viral factors that have strong associations with IFN response. The ISDR located in the

Table 5. The sustained virological response rate according to interleukin 28B and amino acid substitutions in 70 core region and interferon sensitivity-determining region

Core70/ISDR	IL28B; TT 58% (127/219)	IL28B; GT/GG 13.8% (11/80)
Q/wild 20% (13/65)	35.7% (10/28)	8.1% (3/37)
Q/mutant 35% (7/20)	50% (6/12)	12.5% (1/8)
non-Q/wild 50.3% (82/163)	57.6% (76/132)	19.4% (6/31)
non-Q/mutant 70.6% (36/51)	74.5% (35/47)	25% (1/4)

P-value = 0.0001 by Cochran–Armitage test.
IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region; Q, glutamine.

NS5A region was originally reported in 1996 by Enomoto *et al.* (8) and confirmed by several Asian studies (9, 12–14), but controversial results were reported by Western studies (15, 16). Meta-analysis showed the relationships between ISDR and SVR and suggested that unidentified factors have an effect on IFN responsiveness (32). The ISDR interacts with protein kinase R (PKR) and inactivates replication of HCV *in vitro* (33). Therefore, ISDR heterogeneity plays an important role that may affect response to IFN. However, some reports have not confirmed the interaction between PKR and NS5A (34, 35), and they suggested the PKR-independent effects of NS5A (36, 37). Thus, the effects of aa substitutions of the ISDR are unclear, and investigators searched for other viral factors. The aa substitutions at 70 and 91 in the HCV core region were reported as factors that could predict IFN responsiveness (10). Thus, several studies have reported that combining aa substitutions in the HCV core region and NS5A region could improve the predictive value of SVR in patients with genotype 1b (12–14). These results were useful to develop individualized treatment strategies for chronic hepatitis C patients.

For instance, in this study, only 20% of patients with Q70 and wild-type ISDR achieved SVR, compared with 70.6% of those with non-Q70 and mutant-type ISDR. However, the majority of patients were classified into those with non-Q70 and wild-type (50.3%), and another factor for improving SVR prediction was considered necessary. Three genome-wide association studies of SVR to pegylated-IFN-α and ribavirin combination therapy for chronic hepatitis C patients with genotype 1 from Japan, the USA and Australia identified SNPs of IL28B associated with IFN responsiveness (17–19). SVR was achieved in 13.8% of patients with IL28B minor allele (TG and GG) and in 58% with IL28B major allele (TT) in this study, and the SNP of IL28B was associated with the response to IFN in patients with HCV genotype 1b, as in previous reports. The effects of both host and viral factors on IFN responsiveness would affect the IFN treatment outcome. Thus, the SNP of IL28B was considered in the analysis of aa substitutions in the HCV core and the NS5A region for improving the prediction of SVR. The strain with the worst SVR outcome was Q70 and wild-type ISDR, with an SVR of 20%. When IL28B was considered in the analysis of patients with Q70 and wild-type ISDR, 8.1% of patients with TG/GG for IL28B achieved SVR compared with 35.7% of those with TT for IL28B. These results indicate the effects of both host and viral factors on IFN responsiveness. The best responders were 47 patients who simultaneously had non-Q70, mutant-type ISDR and TT allele; 35 (74.5%) achieved SVR. The clear suggestion of a correlation between the combination of the SNP of IL28B and aa substitutions in the core region70 and ISDR with IFN responsiveness would not be supported in the non-Q/mutant/G allele and the Q/mutant/G allele groups because of the small number of patients. Both mutations in core region 70 and ISDR were strongly associated with IL28B genotype. Thus, the prevalence of patients with core 70 non-Q, ISDR mutant, and IL28B genotype G was rare, and it was difficult to find these combinations. Patients infected with IFN-resistant strains Q70 and wild-type ISDR could be clearly identified as non-responders to IFN therapy (8.1%) by the IL28B genotype; the positive predictive value for non-SVR was 91.9%. Meanwhile, patients infected with IFN-sensitive strains non-Q70 and mutant-type ISDR showed that the positive predictive value for SVR was 74.5%. Montes-Cano *et al.* (38) reported that the influence of IL28B would be stronger among patients infected with an IFN-resistant genotype (HCV genotype 1) than in those infected with an IFN-sensitive genotype (HCV genotype non-1). The SNP of IL28B would be strongly associated with the response to IFN, especially for poor responders.

Interleukin28B genotype was associated with spontaneous viral clearance, as well as IFN responsiveness (17, 20). A Spanish study found that the prevalence of HCV genotype depends on IL28B genotype and speculated that IL28B would be a candidate to explain HCV genotype differences in the IFN response (38). The IFN-resistant strain (Q70) was detected more frequently in patients with the IL28B minor allele (TG and GG) (56.3%) than in those with the IL28B major allele (TT) (18.3%). The present study showed similar results: patients with IL28B G allele, which is associated with poor response to IFN, seemed to more frequently have the IFN-resistant strain (Q70). Further study is needed to clarify the effect of the IL28B gene on differences in IFN response between each HCV genotype and subgenotype. The IL28B polymorphism might regulate the expression of hepatic interferon-stimulated genes and cause the difference in IFN responsiveness (39). The association between IL28B genotypes and IL28B gene expression is controversial (18, 19, 39). The effects of the SNP of IL28B on gene expression and mechanisms against HCV infection are still under debate. Although the effect of the SNP of IL28B was unclear, as were the aa substitutions of the core region and ISDR, these factors could be used to predict SVR in patients infected with genotype 1b. The SNP of IL28B plays an important role in choosing optimal therapy and avoiding unnecessary treatment.

In conclusion, the SNP of IL28B and aa substitutions in the core region and ISDR were associated with response to IFN in patients with HCV genotype 1b. Combined use of both host and viral factors could improve prediction of the IFN response.

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Four-year study of lamivudine and adefovir combination therapy in lamivudine-resistant hepatitis B patients: influence of hepatitis B virus genotype and resistance mutation pattern

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SUMMARY. To investigate the efficacy of long-term lamivudine (3TC) and adefovir dipivoxil (ADV) combination therapy in 3TC-resistant chronic hepatitis B virus (HBV) infected patients, we analysed 28 3TC-resistant patients treated with the combination therapy during 47 months (range, 9–75). At 12, 24, 36, and 48 months, the rates of virological response with undetectable HBV DNA (≤2.6 log copies/mL) were 56, 80, 86, and 92%, respectively. Among 17 hepatitis B e antigen (HBeAg)-positive patients, HBeAg disappeared in 24% at 12 months, 25% at 24 months, 62% at 36 months, and 88% at 48 months. When HBV genotypes were compared, patients with genotype B achieved virological response significantly more rapidly than those with genotype C ($P = 0.0496$). One patient developed virological breakthrough after 54 months, and sequence analysis of HBV obtained from the patient was performed. An rtA200V mutation was present in the majority of HBV clones, in addition to the 3TC-resistant mutations of rtL180M+M204V. The rtN236T ADV-resistant mutation was observed in only 25% clones. *In vitro* analysis showed that the rtA200V mutation recovered the impaired replication capacity of the clone with the rtL180M+M204V mutations and induced resistance to ADV. Moreover, rtT184S and rtS202C, which are known entecavir-resistant mutations, emerged in some rtL180M+M204V clones without rtA200V or rtN236T. In conclusion, 3TC+ADV combination therapy was effective for most 3TC-resistant patients, especially with genotype B HBV, but the risk of emergence of multiple drug-resistant strains with long-term therapy should be considered. The mutation rtA200V with rtL180M+M204V may be sufficient for failure of 3TC+ADV therapy.

Keywords: chronic hepatitis B, drug resistance, HBV, rtA200V.

INTRODUCTION

Hepatitis B virus (HBV) causes acute and chronic infection, and chronic hepatitis often leads to liver cirrhosis and hepatocellular carcinoma (HCC) [1]. HBV contains a small (3.2 kb), circular, partially double-stranded DNA genome, and nucleoside or nucleotide analogues inhibit HBV replication by interfering with reverse transcriptase/DNA polymerase of the virus [2]. Although therapy with these drugs results in virological, biochemical, and histological improvement in most patients [3], the effect is often transient because of the emergence of drug-resistant HBV mutants [4].

Lamivudine (3TC), a nucleoside analogue of L-deoxycytidine, is associated with highly frequent emergence of drug-resistant mutants: the cumulative rate is about 20% per year [5,6]. Mutations that result in the replacement of methionine at amino acid 204 to valine or isoleucine (rtM204V/I) within the tyrosine-methionine-aspartate-aspartate (YMDD) motif in the reverse transcriptase (RT) region of HBV polymerase are found in most of the 3TC-resistant isolates [7]. Compensatory mutations rtV173L and rtL180M, which restore the replication capacity of the YMDD mutant *in vitro*, are observed frequently together with the YMDD mutation [8,9]. Adefovir dipivoxil (ADV) is a phosphonate nucleotide analogue of adenosine monophosphate, and ADV-resistance rates are lower than those of 3TC [10]. Two mutations, rtA181V/T and rtN236T, are associated with resistance to ADV [11–14], and the cumulative 5-year occurrence of genotypic resistance is reported to be 29% [15]. *In vitro* studies showed that these mutations confer a weaker

Abbreviations: ADV, adefovir dipivoxil; ALT, alanine aminotransferase; eGFR, estimated glomerular filtration rate; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; RT, reverse transcriptase; TDF, tenofovir disoproxil fumarate.

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decrease in the susceptibility to ADV, in comparison with the greater decrease in 3TC susceptibility because of the YMDD mutant [11,16]. This finding may explain the lower rate of the emergence of ADV resistance.

Although the number of approved drugs has increased in recent years, the treatment of chronic HBV infection remains a clinical challenge. Especially, how to manage drug-resistant patients including 3TC-resistant patients is a major problem. Continuation of 3TC monotherapy or retreatment with 3TC after its temporary discontinuation is ineffective options for 3TC-resistant patients [17]: the lack of any further benefit and the possibility of rapid re-emergence of resistant HBV have been reported [18]. Against 3TC-resistant HBV, ADV and entecavir (ETV) have a suppressive effect *in vivo* and *in vitro* [19–21]. Combination therapy of ADV and 3TC is effective for 3TC-refractory patients and has a low frequency of viral breakthrough [22]: the 3-year cumulative rate of *de novo* resistant mutants was 4% with no development of viral breakthrough in 3TC-resistant patients. However, further longer-term efficacy of the combination therapy remains unknown. ETV is a potent drug with infrequent development of resistance for treatment-naïve patients [23]. ETV monotherapy was shown to be effective during the first year of therapy in 3TC-resistant patients [20], but pre-existing 3TC-resistant mutants are favourable for the emergence of ETV resistance [21], and a comparatively high rate of the emergence of ETV-resistant strains has been reported in long-term studies [23]. Therefore, ETV monotherapy seems to be a less attractive option for the long-term treatment of 3TC-resistant patients.

Several previous reports have described the differences in the responses to antiviral therapy between HBV genotypes. A case–control study of 3TC treatment for genotypes B and C showed that the responses were not different, but the emergence of the YMDD mutation was more frequent in genotype C [24]. It was also reported that the YMDD mutation and breakthrough hepatitis developed more often in patients with genotype A than in patients with genotype B or C [25]. However, the impact of the genotype on the efficacy to ADV is uncertain.

Here, we studied the long-term efficacy of 28 3TC-resistant patients treated with the combination of 3TC and ADV and compared the response between HBV genotypes. Sequence analysis of HBV from a patient with resistance to the combination therapy was performed, and *in vitro* drug susceptibility of the mutant HBV clones was assessed to clarify the mechanism of the emergence of resistance.

MATERIALS AND METHODS

Patients

A total of 28 consecutive Japanese patients with chronic HBV infection who were treated with 3TC+ADV at Tohoku University Hospital from June 2003 to August 2009 for more than 6 months were enrolled in this study. All patients developed virological breakthrough during 3TC monotherapy, and ADV was added in. Virological breakthrough was defined as an increase in the serum HBV DNA level of ≥1 log copies/mL, which was determined using the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan), at two or more consecutive examinations in comparison with the lowest level after treatment. To evaluate renal function, the estimated glomerular filtration rate (eGFR) level using the Cockcroft-Gault formula [(140 - age) × (weight in kilograms) × (0.85 if female)/(72 × serum creatinine)] [26] was calculated. No patients were infected with HCV, nor had a history of other liver diseases. The patients were evaluated for the rate of virological response (undetectable HBV DNA: <2.6 log copies/mL), biochemical response [alanine aminotransferase (ALT) normalization: ≤35 IU/L], hepatitis B e antigen (HBeAg) loss, and virological breakthrough.

Antiviral treatment

Adefovir dipivoxil was administered at a dosage of 10 mg/day in all but one patient in addition to 3TC at a dosage of 100 mg/day. One patient received 10 mg of ADV on alternate days and 50 mg/day of 3TC daily because of reduced eGFR at the start of treatment. This occurred when the eGFR level dropped to <50 mL/min.

Determination of HBV genotype

The HBV genotype was determined as described previously [27] with minor modifications. Briefly, total DNA was extracted from 50 μL of serum sample by QIAamp Blood Mini kit (QIAGEN GmbH, Hilden, Germany) and subjected to nested polymerase chain reaction (PCR) with high fidelity polymerase (PrimeSTAR HS DNA polymerase; TaKaRa Bio Inc., Shiga, Japan), to amplify a 396-nt sequence in the S gene. The amplification products were sequenced on both strands directly using the BigDye Terminator v3.1 Cycle Sequencing kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using Genetyx-Mac (Version 12.2.7; Genetyx Corp., Tokyo, Japan). The genotype of HBV was determined by phylogenetic analysis with HBV isolates whose genotype was known.

Sequencing analysis of HBV reverse transcriptase region

Total DNA extracted from 50 μL of serum sample was subjected to nested PCR to amplify the 1148-nt sequence [nt 52 to 1199, the nucleotide numbers are in accordance with a genotype C HBV isolate of 3,215 nt (AB033550)] including the RT region of HBV polymerase. The first-round PCR was carried out with primers B026 [5′-TCA TCC WCA GGC CAT GCA GTG GA-3′ (W = A or T)] and B025 (5′-CTA GGA GTT CCG CAG TAT GGA TCG-3′), and the second round with

primers B011 [5′-YTT YCC TGC TGG TGG CTC CAG TTC-3′ (Y = C or T)] and B024 (5′-GGG GTT GCG TCA GCA AAC ACT TG-3′). The amplification products were sequenced on both strands directly or after cloning into pUC118. Sequencing analysis after cloning was performed at nt 497-1161.

Construction of plasmid

A cloned mutant sequence including the RT region from a sample obtained after the development of 3TC and ADV resistance was digested with BlnI (TaKaRa Bio Inc.) and EcoT22I (TaKaRa Bio Inc.). The digested fragment (nt 179-1068) was ligated into the BlnI-EcoT22I site of pBFH2R, which contained a 1.3-fold HBV genome [28]. Quick Change II-E Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to introduce nucleotide substitutions into the plasmid. Each mutation found in the RT region, rtL180M [C to A at nt 667 (C667A)], rtT184S (A679T), rtA200V (C728T), rtS202C (A733T), rtM204V (A739G), and rtN236T (A836C), was converted into the wild type or another mutant nucleotide. To construct plasmids with combined nucleotide substitutions, these converted plasmids were used next as templates. As a result, variant constructs harbouring rtM204I, rtL180M+M204V, rtL180M+T184S+M204V, rtL180M+A200V+M204V, rtL180M+S202C+M204V, rtL180M+M204V+N236T, and rtL180M+A200V+M204V+N236T were composed, and all constructs were sequenced to confirm the nucleotide substitutions.

Cell culture and transfection

Human hepatoma HepG2 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% bovine serum at 37 °C and 5% CO_2. Cells were seeded in 24-well plates at 1.25×10^5 cells/well. On the next day, 375 ng of plasmid DNA were transfected into these cells using TransIT LT-1 Transfection Reagent (Mirus, Madison, WI, USA), and cells were washed twice with phosphate-buffered saline after 4 h. Five hundred microliter of the medium and various amounts of adefovir (Toronto Research Chemicals Inc., Ontario, Canada) were added, and the culture supernatant was collected 4 days later. Experiments were performed at least in triplicate.

Real-time PCR and determination of IC_{50}

HBV DNA in the culture supernatant was quantified by real-time PCR as described previously [28] to determine the 50% inhibitory concentration (IC50) for ADV of each mutant HBV clone. Briefly, to digest the input plasmid DNA in the culture supernatant, 5 μL of the supernatant were treated with 5 units of DNase I (TaKaRa Bio Inc.) at 37 °C for 2 h, and the reaction was stopped with EDTA. Then, total DNA was extracted with a QIAamp DNA Blood Mini kit, and 10 μL of 200 μL DNA solution were subjected to real-time PCR using a LightCycler (Roche Diagnostics). Dose–response curves were plotted to determine the ADV IC50.

Statistical analysis

Statistical analyses were performed using Fisher's exact probability test for comparison of proportions between two groups and Mann–Whitney *U* test for comparison of continuous variables between two groups. The cumulative rate of undetectable HBV DNA or ALT normalization was calculated using the Kaplan–Meier method, and differences between the curves were tested using Log-rank test. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Study profile

The demographic and clinical profiles of the 28 patients [20 men and 8 women, median age 53.5 years (range 18–72)] at commencement of 3TC+ADV therapy are shown in Table 1. One (3.6%), 7 (25.0%), and 19 (67.9%) patients had HBV of genotypes A, B, and C, respectively. Eight (28.6%) patients had cirrhosis, 7 (25.0%) had HCC, and 17 (60.7%) patients were HBeAg positive. The mutations of the YMDD motif were determined by direct sequencing, and the YIDD, YVDD, and YIDD+YVDD mixed pattern were found in 14 (50%), 11 (39%), and 2 (7%)of the patients, respectively. Only one (4%) patient had no mutation in the YMDD motif. There were no significant differences in the profiles between patients with genotype B and those with genotype C.

Response to lamivudine and adefovir dipivoxil combination therapy

The 3TC-resistant patients treated with the combination therapy were followed up for a median of 47 months (range, 9–75). All patients continued to be treated with 3TC and ADV until virological breakthrough. The 6-, 12-, 24-, 36-, and 48-month rates of virological response with HBV DNA ≤ 2.6 log copies/mL were 39, 56, 80, 86, and 92%, respectively (Table 2). The ALT normalization rates were 57% at 6 months, 70% at 12 months, 84% at 24 months, 82% at 36 months, and 77% at 48 months. When compared between genotype B and C, the results of patients with genotype B tended to be favourable for both virological and biochemical response (Figs 1a,b). The cumulative probability of undetectable HBV DNA was significantly higher in genotype B than in genotype C ($P = 0.0496$), whereas there was no significant difference in that of ALT normalization. Notably, patients with genotype B achieved early virological response (HBV DNA < 2.6 log copies/mL at 6 months) significantly more frequently than those with genotype C

Table 1 Demographic and clinical characteristics of the 28 lamivudine-resistant patients at the start of adefovir addition to the treatment

	Overall (n = 28)*	Genotype B (n = 7)	Genotype C (n = 20)
Age (years), median (range)	53.5 (18–72)	51.0 (18–72)	53.5 (35–68)
Male patients, no. (%)	20 (71.4)	5 (71.4)	14 (70.0)
Patients with cirrhosis, no. (%)	8 (28.6)	1 (14.3)	7 (35.0)
Patients with HCC, no. (%)	7 (25.0)	0 (0)	7 (35.0)
HBeAg positive, no. (%)	17 (60.7)	3 (42.9)	13 (65.0)
HBV DNA (log copies/mL), median (range)	7.6 (4.3 to >7.6)	7.2 (5.3 to >7.6)	7.6 (4.3 to >7.6)
Patients with rtM204 mutation (M:I:V:I/V, no.)	1:14:11:2	1:3:2:1	0:11:8:1
ALT (IU/L), median (range)	86.5 (29–1027)	314.0 (47–760)	78.5 (29–1027)
T. Bil (mg/dL), median (range)	1.1 (0.5–4.5)	1.1 (0.5–1.5)	1.1 (0.5–4.5)
Albumin (g/dL), median (range)	4.1 (2.7–4.8)	4.2 (3.8–4.8)	4.0 (2.7–4.6)
Serum creatinine (mg/dL), median (range)	0.7 (0.4–1.2)	0.7 (0.6–1.2)	0.7 (0.4–1.2)
Prior lamivudine therapy (month), median (range)	28.6 (2–76)	36.5 (2–76)	28.6 (5–65)

HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; T. Bil, total bilirubin. *One patient had genotype A HBV.

Table 2 Virological and biochemical response to lamivudine and adefovir combination therapy during a median of 47 months

	Months of treatment						
Response	0 (n = 28)	6 (n = 28)	12 (n = 27)	24 (n = 25)	36 (n = 22)	48 (n = 13)	60 (n = 7)
HBV DNA < 2.6	0 (0)	11 (39.3)	15 (55.6)	20 (80.0)	19 (86.4)	12 (92.3)	6 (85.7)
HBV DNA 2.6 to <5.0	1 (3.6)	15 (53.6)	11 (40.7)	5 (20.0)	3 (13.6)	1 (7.7)	1 (14.3)
HBV DNA ≥ 5.0	27 (96.4)	2 (7.1)	1 (3.7)	0 (0)	0 (0)	0 (0)	0 (0)
ALT normalization*	NA	16 (57.1)	19 (70.4)	21 (84.0)	18 (81.8)	10 (76.9)	6 (85.7)
HBeAg disappearance†	NA	1/17 (5.9)	4/17 (23.5)	4/16 (25.0)	8/13 (61.5)	7/8 (87.5)	4/5 (80.0)
Virological breakthrough	NA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (14.3)

Values are shown as numbers of patients followed by percentages in parentheses. NA, not applicable. *ALT ≤ 35 IU/L. †Values are shown as numbers of patients/total followed by percentages in parentheses.

[5/7 (71%) vs. 5/20 (25%), P = 0.0427]. Although the status of HBeAg at the start of ADV seemed to influence the response, the difference was not significant (Figs 1c,d). Among 17 HBeAg-positive patients, HBeAg disappeared in 6% at 6 months, 24% at 12 months, 25% at 24 months, 62% at 36 months, and 88% at 48 months. There was no patient with hepatitis B surface antigen (HBsAg) loss during follow-up in this study.

Three of 22 patients who were treated for more than 36 months did not achieve virological response. One of them developed virological breakthrough after 54 months of combination therapy. The other patients had 2.8 and 3.5 log copies/mL of serum HBV DNA at 36 months of therapy but did not develop breakthrough. None of the patients experienced biochemical breakthrough. One patient with HCC died of HCC progression at 9 months after ADV. None of the 21 patients without HCC at the start of ADV developed HCC during follow-up.

The renal toxicity with a ≥0.3 mg/dL increase in serum creatinine level was observed in five of the 28 patients. Two of them had a ≥0.5 mg/dL increase: the serum creatinine levels were increased from 0.8 to 1.4 mg/dL after 31 months in a patient, and from 0.9 to 1.7 mg/dL after 34 months in another patient. As their eGFR levels were lowered to 39 and 29 mL/min, the dosage of ADV was reduced to alternate-day administration. After the reduction of ADV, their serum creatinine and eGFR recovered.

Profile of a patient with lamivudine and adefovir dipivoxil resistance

He was a 53-year-old Japanese man with HBeAg-positive liver cirrhosis at the start of 3TC monotherapy in April 2002. The genotype of HBV was found to be genotype C. His clinical course is shown in Fig. 2. He developed breakthrough hepatitis with serum HBV DNA of >7.6 log copies/mL and alanine aminotransferase (ALT) of 236 IU/L in March 2003. ADV was added to the ongoing 3TC therapy in June 2003, and HBV DNA was gradually reduced reaching <2.6 log copies/mL 3 years later. However, virological

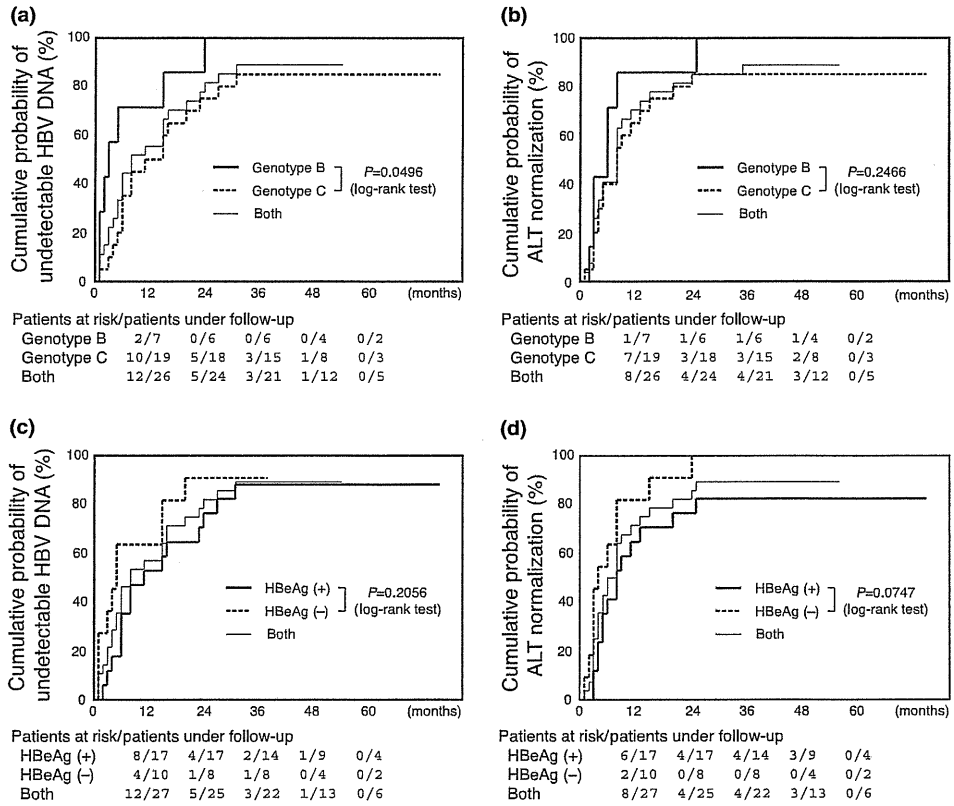


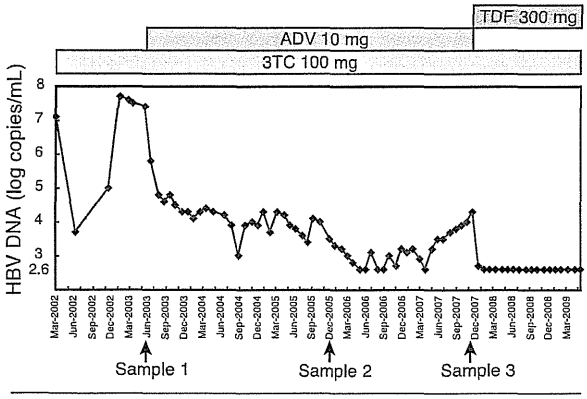
Fig. 1 Cumulative probability of virological or biochemical response during lamivudine (3TC) and adefovir dipivoxil (ADV) combination therapy. (a) Cumulative probability of undetectable HBV DNA (<2.6 log copies/mL) in patients with genotype B and those with genotype C. (b) Cumulative probability of ALT normalization (≤35 IU/L) in patients with genotype B and those with genotype C. (c) Cumulative probability of undetectable HBV DNA in HBeAg-positive patients and HBeAg-negative patients. (d) Cumulative probability of ALT normalization in HBeAg-positive patients and HBeAg-negative patients.

breakthrough was observed at 4 years after starting ADV, and his HBV DNA reached 4.3 log copies/mL in December 2007. Because his liver was cirrhotic and the hepatic functional reserve was impaired, combination therapy of tenofovir disoproxil fumarate (TDF) and 3TC was started before ALT flair. Two months later, his HBV DNA was suppressed to <2.6 log copies/mL, and viral breakthrough has not been observed to date (20 months later).

Mutations found in the HBV reverse transcriptase region of the lamivudine and adefovir dipivoxil-resistant patient

To investigate the mutations responsible for the viral breakthrough during the 3TC and ADV combination therapy, nucleotide sequences of the HBV RT region of the patient were compared between 3 time points: at the beginning of ADV treatment, at 30 months after ADV therapy, and at the time of viral breakthrough (54 months after ADV therapy). Direct sequencing analysis showed 10 amino acid changes during the clinical course (Fig. 2). The 3TC-resistant mutation of rtM204I changed to rtM204V after ADV treatment. Along with the change, the mixed mutation of rtL180L/M changed to rtL180M, which was reported to emerge with rtM204V during 3TC therapy [9]. The rtN236T mutation, which is a known ADV-resistance mutation [11], emerged as a mixed mutation with wild type (rtN236N/T) after viral breakthrough. Notably, rtA200V, which has never been reported as an ADV-resistant mutation, emerged also after viral breakthrough as a mixed mutation (rtA200V/A). Meanwhile, no specific mutation was found in the 2 patients without virological breakthrough who did not achieve virological response after 3 years of the combination therapy.

Clonal analysis was performed to examine the significance of these mutations of the RT region (Table S1). Several minor mutations were found during the 3TC and ADV therapy. After viral breakthrough, rtA200V was found in 63% of the clones, while rtN236T was found in only 25% of the clones. Therefore, rtA200V seemed to be responsible for the treatment failure of ADV. Moreover, rtT184S and S202C, which were reported as ETV resistance–associated mutations [29], were found as a minor population.



RT mutation	L80	P109	D134	R153	L180	A200	M204	V214	L229	N236
Sample 1	I/L	P/Q	G/D	R/Q	L/M	–	I	–	L/W	–
Sample 2	–	–	–	–	M	–	V	A	–	–
Sample 3	–	–	G/D	–	M	V/A	V	–	–	N/T

Fig. 2 Clinical course of a lamivudine (3TC)-resistant patient who developed virological breakthrough during 3TC and adefovir dipivoxil (ADV) combination therapy, and changes of amino acids in the reverse transcriptase (RT) region detected by direct sequencing analysis. After breakthrough, therapy was switched to 3TC plus tenofovir disoproxil fumarate (TDF) combination. The arrows indicate the time point when serum samples were obtained for sequencing analysis. Sample 1, 2, and 3 were obtained at the start of ADV, 30 months after ADV, and 54 months after ADV, respectively.

To investigate further the mutant populations, the combinations of these mutations and 3TC-resistant mutations were analysed (Fig. 3). At 30 months after ADV therapy, 100% of clones had mutations rtL180M+M204V. Subsequently, the mutations of rtT184S, A200V, S202C, and N236T emerged in the rtL180M+M204V clones after viral breakthrough. Of note, rtN236T was not found in clones without rtA200V.

Replication capacity and drug susceptibility of HBV mutants

We analysed the replication capacity of HBV clones with combined mutations as shown in Fig. 3. A clone with rtL180M+M204V+N236T mutations, which was not found in the patient, was also included for comparison. Consistent with a previous report [30], 3TC-resistant mutations of rtM204I or rtL180M+M204V lowered the replication capacity significantly in comparison with the wild-type clone (Table 3). From additional mutations to rtL180M+M204V found in the patient, only rtA200V restored the impaired replication capacity significantly. The ETV-resistant mutation of rtT184S and rtS202C did not seem to have such an effect. The ADV-resistant mutation, rtN236T, lowered the replication capacity further, and rtA200V did not restore the lowered capacity caused by rtN236T.

The 7 HBV clones with mutations in the RT region were analysed for their susceptibility to ADV. The IC_{50} of each clone is shown in Table 3. The clones with the 3TC-resistant mutations of rtM204I or rtL180M+M204V showed moderate resistance to ADV. In comparison with the clone with rtL180M+M204V, clones with additional mutations of rtT184S, A200V, or S202C showed significantly higher resistance to ADV. An additional mutation of rtN236T led to much greater resistance to ADV. Taking into account the results from the clonal analysis of serum samples and the replication capacity of each clone, rtA200V may be responsible for the treatment failure of 3TC+ADV therapy when it presents with 3TC-resistant mutations such as rtL180M+M204V. The mutations of rtT184S or S202C with rtL180M+M204V also confer ADV resistance, but the clones

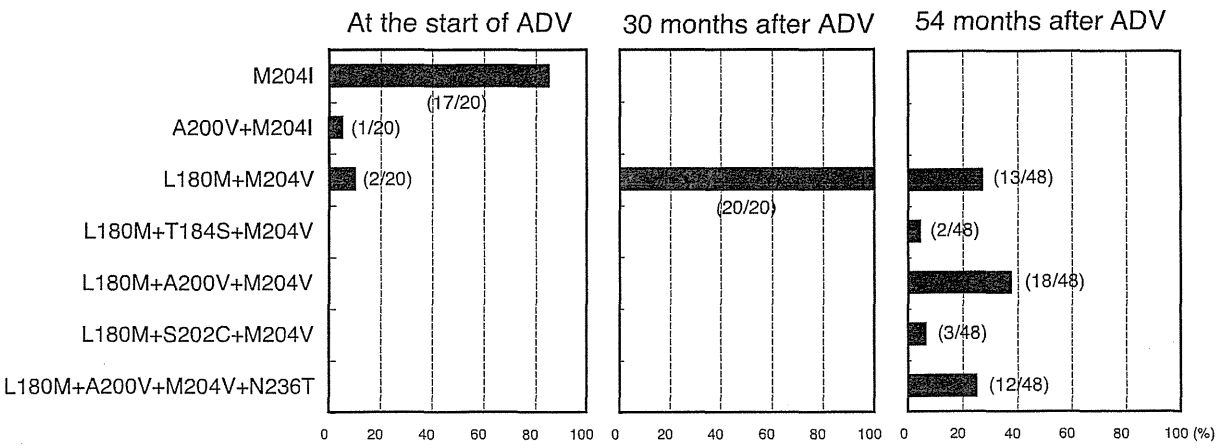


Fig. 3 Clonal analysis of HBV obtained from the patient with 3TC and ADV resistance. The serum samples were collected at the time points indicated in Fig. 2. The percentages (no. of clones/total in parentheses) of the clones with the combined mutations in the RT region are shown.

Table 3 Replication capacity and susceptibility to adefovir of the HBV mutants

HBV mutants	HBV DNA ($\times 10^7$ log copies/mL)*	Fold replication†	IC_{50} (μM)*	Fold resistance†
Wild type	13.60 ± 3.50	1	0.42 ± 0.06	1
M204I	2.17 ± 0.38	0.16	0.87 ± 0.2	2.07
L180M+M204V	4.38 ± 0.77	0.32	0.73 ± 0.06	1.74
L180M+T184S+M204V	5.98 ± 0.80	0.44	0.91 ± 0.04	2.17‡
L180M+A200V+M204V	8.90 ± 0.56	0.65‡	1.09 ± 0.12	2.60‡
L180M+S202C+M204V	4.86 ± 0.19	0.36	2.19 ± 0.63	5.21‡
L180M+M204V+N236T	0.88 ± 0.68	0.07‡	>10	>25
L180M+A200V+M204V+N236T	0.54 ± 0.38	0.04‡	>10	>25

*Values are expressed as means ± SD of experiments performed in triplicate. †(Mean value of the mutant)/(mean value of the wild type). ‡$P < 0.05$ in comparison with the clone with rtL180M+M204V.

with these mutations were not major, because they had no effect in enhancing the replication capacity of HBV.

DISCUSSION

As clinical and histological improvement accompanies reductions in HBV replication, therapies that reduce HBV replication are expected to limit the progression of liver disease and improve the natural history of chronic HBV infection [10]. Currently, the management of hepatitis B patients with drug resistance is one of the major problems in clinical practice for hepatitis B. A substantial part of 3TC-treated patients has mutant HBV with the YMDD mutation, and several clinical trials to treat 3TC-resistant hepatitis B have been performed. It has been reported first that with ADV alone and in combination with 3TC, the viral and biochemical responses were the same for 3TC-resistant patients in a 1 year study [31]. However, several studies of longer term treatment have shown that adding ADV was superior to switching to ADV monotherapy for patients with 3TC resistance [32–34]. In this study, we demonstrated that the add-on ADV therapy for 3TC-resistant hepatitis B patients effectively suppressed serum HBV DNA for a median of 47 months. Moreover, the biochemical response of ALT normalization was achieved in 77% patients and HBeAg loss in 88% of the HBeAg-positive patients at 48 months. The undetectability of HBV DNA was assessed by the Amplicor HBV monitor test, but recently, this can be assessed by a more sensitive real-time assay such as the Cobas TaqMan HBV test (Roche Diagnostics). The treatment duration to achieve HBV DNA undetectability might be longer if a more sensitive assay was used.

The influence of HBV genotype on the response or resistance to ADV has not been clarified, whereas the efficacy to 3TC was reported to be different between HBV genotypes [24,25]. This study showed that the virological response to 3TC+ADV was significantly earlier in genotype B than in C. However, there were several limitations of the results: the patients with genotype B were fewer, and no multivariate analysis was performed. In addition, all patients with HCC were genotype C, and ALT levels of genotype B tended to be higher, although there were no significant differences. The effect of genotype on the response to 3TC±ADV should be confirmed in larger studies. The baseline HBeAg status in 3TC+ADV combination therapy in 3TC-resistant patients was reported to influence the viral response: HBeAg-negative patients showed better virological and biological response [35]. In this study, the same tendency was observed, but the difference was not significant.

Initial virological suppression by ADV monotherapy was reported to be a good prognostic factor for the treatment of both naïve patients [36] and 3TC-resistant patients [37]. Taking into account the results of this study and previous reports, it is suggested that patients with genotype B HBV might develop resistance to 3TC+ADV less frequently than those with genotype C. In fact, the 3TC+ADV-resistant patient in this study was infected with genotype C HBV. Because the development of resistance to 3TC+ADV combination therapy is rare [22,35], it is difficult to evaluate whether the early virological response or genotype B is associated with the lower frequency of resistance to 3TC+ADV combination therapy. Further long-term study is needed to clarify this issue.

Although the emergence of resistance in this study was rare during the combination therapy as previously reported [22,35], one patient developed virological breakthrough after 4.5 years. We identified a characteristic mutation pattern of HBV in this patient. The mutation of rtA200V rescued the *in vitro* replication capacity that was impaired by rtL180M+M204V and reduced the susceptibility to ADV. In previous reports, rtA200V emerged as an additional mutation with the 3TC-resistant mutation in patients under 3TC monotherapy [38,39]. The effect of this mutation is not as strong as the effect of rtM204I/V±L180M on 3TC susceptibility *in vitro*, which showed >1000-fold resistance [40]. However, the clinical dose of ADV is comparatively low

because of renal toxicity [41], and the weakly resistant profile *in vitro* can explain the great clinical impact. Villet *et al.* reported that rtA200V was observed in a patient with 3TC monotherapy, and it was no longer detected after the combination therapy with ADV and 3TC [39]. The difference of results between the previous study and our study may be because of the emergence of mutations with a potent effect on ADV resistance, such as rtV173L and rtA181V, in the previous study. Because these mutations may have a greater effect on ADV resistance than rtA200V, the HBV clones with rtA200V seemed to disappear in the previous study case.

The known ADV-resistant mutation of rtN236T was found in only 25% clones, exclusively with rtA200V. This may indicate that rtN236T appeared after the emergence of rtA200V. In the active replication of the clones with rtA200V, which restored the replication capacity and enhanced ADV resistance, other mutations including rtN236T might occur more readily.

The rtA200V mutation is the result of nucleotide substitution C728T. This change in the overlapping S region results in an amino acid substitution affecting HBsAg: Leu to Phe at aa192 (sL192F). There is a possibility that sL192F may affect the replication capacity of HBV, but the actual mechanism is unknown.

Interestingly, the ETV-resistant mutations of rtT184S and rtS202C were also detected during 3TC+ADV combination therapy by clonal analysis. These mutations confer ETV resistance in the presence of the 3TC-resistant mutations of rtM204I/V±L180M [21]. This study showed that these mutations also have an ADV-resistance profile. These mutations may not cause viral breakthrough, because the population of these mutants in the patient was minor (4% and 6%, respectively), and their replication capacity was lower than that with rtA200V *in vitro*. The emergence of these mutations suggested that long-term 3TC+ADV therapy has the possibility of leading to multiple drug resistance including ETV resistance.

The combination therapy of 3TC and ADV is very effective with little frequency of viral breakthrough for 3TC-refractory patients. However, some patients do not achieve complete viral suppression of serum HBV DNA to under 2.6 log copies/mL. It was considered that the incomplete suppression of viral replication might favour further selection of drug-resistant mutants [42]. Although there have been a few reports of cases that showed resistance to 3TC+ADV therapy to date, the number of resistant cases will increase along with the increase in cases with long-term therapy. The 3TC- and ADV-resistant patient in this study was treated with 3TC and TDF after the virological breakthrough, and HBV DNA was promptly suppressed. Although TDF was reported to show cross-resistance with ADV *in vitro* [16,40,43], there are several reports that showed the effectiveness of TDF for ADV-refractory patients [44–46]. It is thought that the potency of TDF might result from its higher clinical dose compared to that of ADV [47].

In conclusion, this study showed that the combination therapy of 3TC and ADV effectively suppressed HBV replication in 3TC-resistant patients with chronic HBV infection for 4 years. Especially, patients with genotype B achieved earlier virological response than those with genotype C. However, one of the 28 patients developed virological breakthrough during the combination therapy over 4 years, and the HBV mutation of rtA200V, in addition to 3TC-resistant mutations, was demonstrated to contribute to the ADV resistance. Moreover, ETV-resistant mutations emerged coincidentally in minor HBV clones. The risk of emergence of multiple drug-resistant mutant should be considered in cases with long-term therapy with nucleos(t)ide analogues, especially when serum HBV DNA cannot be suppressed completely. Potent antiviral agents should be administered in such cases to prevent the emergence of multiple drug-resistant HBV mutants that are difficult to treat.

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