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Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: Amino acid substitutions in the core region and low-density lipoprotein cholesterol levels

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***Background/Aims*: We showed previously that amino acid (aa) substitutions in the HCV core region (HCV-CR) are predictors of non-virological response (NVR) to peginterferon (PEG-IFN) plus ribavirin (RBV) therapy. Here, we determined the predictive factors of sustained virological response (SVR) and early virologic response (EVR) to this treatment.**

***Methods*: We evaluated the response to 48-week PEG-IFN-RBV therapy in 114 Japanese adults infected with HCV genotype 1b and determined the predictors of EVR and SVR.**

***Results*: EVR was achieved by 70% and SVR by 45% of patients. 64% of patients who achieved EVR also showed SVR, while none of non-EVR achieved SVR. Multivariate analysis identified low-density lipoprotein cholesterol (LDL-C) ($\geqslant$86 mg/dl), aa substitutions in HCV-CR (double-wild-type; arginine at aa 70/leucine at aa 91), gamma-glutamyl transpeptidase (GGT) (<109 IU/l), RBV dose ($\geqslant$11.0 mg/kg), and leukocyte count ($\geqslant$4500/mm^3) as significant determinants of EVR, and aa substitutions in HCV-CR (double-wild-type), LDL-C ($\geqslant$86 mg/dl), male gender, ICG R15 (<10%), GGT (<109 IU/l), and RBV dose ($\geqslant$11.0 mg/kg) as determinants of SVR. Prediction of response to therapy based on combination of these factors had high sensitivity, specificity, positive, and negative predictive values.**

***Conclusions*: Our study identified aa substitutions in the core region and serum LDL-C as predictors of response to PEG-IFN-RBV therapy in Japanese patients infected with HCV genotype 1b.**

***Keywords*: HCV; Core region; LDL cholesterol; Peginterferon; Ribavirin; Early virologic response; Sustained virological response; Mutation-specific primer; Double-wild type; ICG R15**

1. Introduction

For chronic hepatitis C virus (HCV) infection, the early virologic response (EVR) at 12 weeks after the completion of 48-week treatment with peginterferon (PEG-IFN) plus ribavirin (RBV) is an important predictor of the sustained virological response (SVR) [1]. The observation that patients lacking EVR following PEG-IFN-α-2a-RBV combination therapy are highly unlikely to develop SVR was adopted as an assessment criterion by the National Institutes of Health Consensus Development Conference [2]. The predictive potential of EVR was also confirmed in patients treated with PEG-IFN-α-2b-RBV [3]. The underlying mechanisms of the

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different virological responses to treatment are still unclear.

We studied previously determinants of the response to the IFN-RBV therapy in patients with high titers of genotype 1b (≥100 kiloIU (KIU)/ml), which is dominant in Japan [4,5]. Our results identified substitutions of amino acid (aa) 70 and/or 91 in the HCV core region as an independent and significant pretreatment factor associated with non-virologic response (NVR), i.e., patients who do not achieve HCV-RNA negativity, as determined by PCR. Especially, substitutions of arginine by glutamine at aa 70 and/or leucine by methionine at aa 91 were significantly more common in NVR patients. Furthermore, we also showed that the falls in HCV-RNA levels during treatment in patients with specific substitutions in the core region (HCV-CR) were significantly less than in those without such substitutions [4,5]. Whether aa substitutions in HCV core region are also useful as a predictor of EVR and SVR await, further investigation.

Recent studies have shown that various host factors, such as body mass index (BMI), fasting blood sugar (FBS), total cholesterol (TC), triglycerides (TG), and hepatocyte steatosis, are significant predictors of efficacy of IFN monotherapy and PEG-IFN-RBV dual therapy [6–9]. However, more studies that implement multivariate analysis are required to confirm the predictive values of these factors for the efficacy of PEG-IFN-RBV dual therapy, especially where these factors are analyzed with other factors, including viral and host factors.

The aims of the present study were to analyze the response to 48-week PEG-IFN-RBV therapy in Japanese patients with HCV genotype 1b. Specifically, the study was designed to (1) identify the pretreatment predictive factors associated with EVR and SVR and (2) determine the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the EVR and SVR predictive factors.

2. Patients and methods

2.1. Study population

A total of 201 HCV-infected Japanese patients were consecutively recruited into the study protocol between December of 2001 and June of 2005 at Toranomon Hospital, Tokyo. Among these, 114 patients were selected based on the following criteria. (1) Negativity for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positivity for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, CA), and positivity for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostic Systems, CA). (2) Infection with HCV genotype 1b alone. (3) A high viral load (≥100 KIU/ml) by quantitative analysis of HCV RNA with PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche) within the preceding 2 months of enrolment. (4) No HCC. (5) Body weight >40 kg. 6) Lack of coinfection with human immunodeficiency virus. (7) No previous treatment with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (8) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg. (9) None had other forms of hepatitis, such as hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (10) None of the females was pregnant or lactating mother. (11) All accepted treatment for ≥24 weeks as outlined in the study protocol, as well as repeated evaluation of HCV-RNA levels during treatment (at least once every month). (12) All patients have completed 24 weeks after cessation of treatment, and SVR could be evaluated. (13) Each signed a consent form of the study protocol that had been approved by the Human Ethics Review Committee of Toranomon Hospital.

Patients received PEG-IFNα-2b at a median dose of 1.5 μg/kg (range, 0.8–1.8 μg/kg) subcutaneously each week-oral RBV at a median dose of 10.9 mg/kg (range, 3.4–14.2 mg/kg) daily for 48 weeks. The RBV dose was adjusted according to body weight (600 mg for ≤60 kg, 800 mg for >60 kg and ≤80 kg, and 1000 mg for >80 kg), except for 27 patients who started at a reduction dose according to low pretreatment levels of hemoglobin (Hb). In 35 patients, the dose of RBV was reduced during treatment due to falls in Hb concentration.

Table 1 summarizes the profiles of the patients. They included 75 men and 39 women. The median duration of treatment was 48 weeks (range, 24–48 weeks). Patients who achieved HCV-RNA negativity based on HCV-RNA qualitative PCR analysis at 24 weeks after cessation of combination therapy were defined as SVR. Patients who achieved >2 $\log_{10}$ falls in HCV-RNA level compared with baseline based on HCV-RNA quantitative PCR analysis or HCV-RNA negativity based on HCV-RNA qualitative PCR analysis at 12 weeks of combination therapy were defined as EVR.

2.2. Laboratory tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for alanine aminotransferase (ALT) and HCV-RNA levels. The serum samples were frozen at −80 °C within 4 h of collection and then thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of NS5 region [10]. HCV-RNA level was measured quantitatively by PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche) before, during, and after therapy. The lower limit of the assay was 5 KIU/ml. Samples collected during and after therapy that had undetectable levels of HCV-RNA (<5 KIU/ml) were checked also by qualitative PCR (Amplicor, Roche), which has a higher sensitivity than quantitative analysis, and the results were labeled as positive or negative. The lower limit of the assay was 50 IU/ml. For evaluation of EVR, we used the $\log_{10}$ of the cut-off value (5 KIU/ml) for HCV-RNA values below the limit of detection.

2.3. Histopathological examination

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens contained six or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on the histological scoring system of Desmet et al. [11]. Hepatocyte steatosis was graded as none (absent), mild (<33% of hepatocytes involved), moderate (>33% but <66% of hepatocytes involved), or severe (>67% of hepatocytes involved) [12].

2.4. Detection of amino acid substitutions in core region

We developed a simple and low-cost PCR method for detecting substitutions of aa 70 or aa 91 in HCV-CR of genotype 1b using mutation-specific primer, as an alternative to the direct sequencing method. The major protein type was determined based on the relative intensity of the bands for wild (aa 70: arginine, aa 91: leucine) and mutant

Table 1
Profile and laboratory data of participating patients infected with HCV genotype 1b at commencement of 48-week peginterferon-ribavirin combination therapy

Demographic data	
Number	114
Gender (M/F)	75 / 39
Age (years)*	54 (30–70)
History of blood transfusion	38 (33.3%)
Family history of liver disease	35 (30.7%)
Body mass index (kg/m^2)*	23.2 (17.6–30.3)
Laboratory data*	
Serum aspartate aminotransferase (IU/l)	60 (17–266)
Serum alanine aminotransferase (IU/l)	81 (25–504)
Serum albumin (g/dl)	3.7 (3.0–4.5)
γ-Glutamyl transpeptidase (IU/l)	67 (15–393)
Leukocytes (/mm^3)	4800 (2300–8800)
Hemoglobin (g/dl)	14.6 (10.6–17.6)
Platelets (× 10^4/mm^3)	17.6 (6.6–30.9)
ICG R15 (%)	15 (4–49)
Serum iron (μg/dl)	147 (18–308)
Serum ferritin (μg/l)	150 (<10–927)
Creatinine clearance (ml/min)	100 (53–146)
Viremia level (KIU/ml)	2000 (67–>5000)
Total cholesterol (mg/dl)	169 (100–236)
High-density lipoprotein cholesterol (mg/dl)	45 (10–83)
Low-density lipoprotein cholesterol (mg/dl)	100 (53–162)
Triglycerides (mg/dl)	100 (33–362)
Uric acid (mg/dl)	5.6 (2.3–8.8)
Fasting blood sugar (mg/dl)	96 (75–257)
Histological findings	
Stage (F1/F2/F3/F4/ND)	51/28/16/1/18
Hepatocyte steatosis (none to mild/moderate to severe/ND)	86/8/20
Treatment	
PEG-IFNα-2b dose (μg/kg)	1.5 (0.8–1.8)
Ribavirin dose (mg/kg)	10.9 (3.4–14.2)
Amino acid substitutions in the core region**	
aa 70 (wild/non-wild/ND)	54/38/8
aa 91 (wild/non-wild/ND)	58/40/2
aa 70 and aa 91 (double-wild/non-double-wild/ND)	35/61/4

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.

** Amino acid substitutions were evaluated in pretreatment serum samples of 100 patients by PCR with mutation-specific primers.

Two patterns of mutant and competitive were labeled as non-wild. Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type, while the other patterns were considered non-double-wild-type. ND, not determined.

(aa 70: glutamine/histidine, aa 91: methionine) in agarose gel electrophoresis. If the intensities of the bands were similar, the case was regarded as competitive. The detection rate was 94.4%, the sensitivity was 10 KIU/ml, the reproducibility was high, and consistency with direct sequencing was 97.1% in positive cases [13]. In this study, the pattern of arginine (wild) at aa 70 and leucine (wild) at aa 91 was evaluated as double-wild-type, while the other patterns were non-double-wild-type. The mutation in this study refers to substitution from consensus sequence. In previous studies, HCV-J was considered as a prototype and the aa substitution was evaluated by comparison with the consensus sequence prepared from 50 clinical trial samples [4,14]. In the present study, the PCR genotyping could be performed in 100 patients; the remaining 14 patients could not be analyzed due to the lack of adequate serum samples obtained before treatment.

HCV-RNA was extracted from the serum samples and cDNA was prepared by reverse transcription using MMLV Superscript II reverse transcriptase. The obtained cDNA was amplified by PCR using the following primers: the first PCR was performed using cc11 (sense, 5′-GCC ATG GTG GTC TGC GGA AC-3′: 125–144) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′: 933–953) primers. In the second PCR, for aa 70 the wild-type-specific reaction was performed using 70W2 (sense, 5′-TAT CCC CAA GGC TCG CCG-3′: 521–538) and e14, and the mutant-specific reaction was performed using 70M2 (sense, 5′-TAT CCC CAA GGC TCG CCA-3′: 521–538) and e14. For aa 91, the wild-type-specific reaction was performed using cc9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′: 237–253) and 91W (antisense, 5′-CAT CCT GCC CAC CCC AR-3′, R = A or G: 600–616), and the mutant-specific reaction was performed using cc9 and 91M (5′-CAT CCT GCC CAC CCC AT-3′: 600–616) [13].

The cycle conditions were 94 °C for 4 min + (94 °C for 30 s, 64 °C for 30 s, and 72 °C for 1 min) × 20 cycles + 72 °C for 7 min in the first PCR; and 94 °C for 1 min + (94 °C for 30 s and 72 °C for 1.5 min) × 23 cycles + 72 °C for 7 min for aa 70, and 94 °C for 1 min + (94 °C for 30 s and 68 °C for 1.5 min) × 21 cycles + 72 °C for 7 min for aa 91 in the second PCR. Two microliters of cDNA was used in the first PCR and 1 μl of the first PCR product was used in the second PCR. For detection, 5 μl of the second PCR product was electrophoresed for 30 min on 3.0% agarose gel. The final concentration of all primers was 0.2 pmol/μl [13].

To avoid false-positive results, the procedures recommended by Kwok and Higuchi [15] to prevent contamination were strictly applied to these PCR assays. No false positive results were observed in this study.

2.5. Statistical analysis

SVR was analyzed on an intention to treat basis. Non-parametric tests were used to compare variables between groups (Mann–Whitney U test, χ^2 test and Fisher's exact probability test). Univariate and multivariate logistic regression analyses were used to determine the predictors of SVR and EVR. We also calculated the odds ratios and 95% confidence intervals (95%CI). All P values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with SVR and EVR included the following variables: sex, age, history of blood transfusion, familial history of liver disease, BMI, aspartate aminotransferase (AST), ALT, albumin, γ-glutamyl transpeptidase (GGT), leukocyte count, Hb, platelets, indocyanine green retention rate at 15 min (ICG R15), serum iron, serum ferritin, creatinine clearance, viremia level, TC, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), TG, uric acid (UA), FBS, hepatocyte steatosis, pathological staging, PEG-IFN dose/body weight, RBV dose/body weight, and aa substitutions in HCV-CR. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL). Sensitivity, specificity, PPV, and NPV were also calculated to determine the reliability of predictors of the response to therapy.

3. Results

3.1. Response to therapy

EVR and SVR were evaluated in all 114 patients. EVR was achieved by 80 of 114 (70.2%) patients, and SVR by 51 of 114 (44.7%) patients. 44.7% (51/114 patients) achieved both EVR and SVR, 29.8% (34/114) were considered non-EVR and non-SVR, 25.4% (29/

Table 2
Factors associated with early virologic response to 48-week peginterferon-ribavirin combination therapy in patients infected with HCV genotype 1b, identified by multivariate analysis

Factor	Category	Odds ratio (95% confidence interval)	*P*
LDL cholesterol (mg/dl)	1: <86	1	
	2: ⩾86	30.29 (4.855–189.0)	<0.001
Amino acid substitution in core region	1: double-wild-type[a]	1	
	2: non-double wild-type	0.046 (0.006–0.346)	0.003
γ-Glutamyl transpeptidase (IU/l)	1: <109	1	
	2: ⩾109	0.166 (0.035–0.782)	0.023
Ribavirin dose (mg/kg)	1: <11.0	1	
	2: ⩾11.0	4.341 (1.075–17.53)	0.039
Leukocyte count (/mm^3)	1: <4500	1	
	2: ⩾4500	4.209 (1.061–16.70)	0.041

Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.
Normal range for LDL cholesterol: 86–135 mg/dl.
[a] Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type, and the other patterns were considered non-double-wild-type.

114) were EVR and non-SVR, and 0% (0/114) as non-EVR and SVR. Thus, 63.8% (51/80) of those who achieved EVR also achieved SVR, and none of non-EVR could achieve SVR.

3.2. Predictors of EVR as determined by univariate and multivariate analyses

Univariate analysis identified nine parameters that influenced the EVR: LDL-C (⩾86 mg/dl; $P < 0.001$), aa substitutions of HCV-CR (double-wild-type; $P = 0.001$), leukocyte count (⩾4500/mm^3; $P = 0.003$), GGT (<109 IU/l; $P = 0.008$), TC (⩾170 mg/dl; $P = 0.038$), RBV dose/body weight (⩾11.0 mg/kg; $P = 0.042$), PEG-IFN dose/body weight (⩾1.25 μg/kg; $P = 0.055$), TG (<100 mg/dl; $P = 0.059$), and AST (<60 IU/l; $P = 0.065$). Multivariate analysis that included the above variables identified five parameters that independently influenced the EVR: LDL-C (⩾86 mg/dl; $P < 0.001$), aa substitutions of HCV-CR (double-wild-type; $P = 0.003$), GGT (<109 IU/l; $P = 0.023$), RBV dose/body weight (⩾11.0 mg/kg; $P = 0.039$), and leukocyte count (⩾4500/mm^3; $P = 0.041$). Especially, LDL-C (⩾86 mg/dl) and aa substitutions of HCV-CR (double-wild-type) of five parameters increased chances for EVR 20-fold or more (Table 2).

3.3. Assessment of amino acid substitutions and LDL-cholesterol as predictors of EVR

EVR rates of patients with double-wild-type of HCV-CR or high-serum LDL-C levels (⩾86 mg/dl) were defined as PPV (prediction of EVR). Non-EVR rates of patients with non-double-wild-type of HCV-CR or low-serum LDL-C levels (<86 mg/dl) were defined as NPV (prediction of non-EVR).

In patients with double-wild-type of HCV-CR, the sensitivity, specificity, PPV, and NPV for prediction of EVR were 46.4%, 88.9%, 91.4%, and 39.6%, respectively. In patients with high serum LDL-C levels, the sensitivity, specificity, PPV, and NPV were 81.0%, 56.3%, 82.1%, and 54.5%, respectively. Thus, evaluation of aa substitutions in HCV-CR indicated high specificity and PPV, while that of serum LDL-C level indicated high sensitivity and PPV for prediction of EVR. Furthermore, when both predictors were used, the sensitivity, specificity, PPV, and NPV were 32.4%, 100%, 100%, and 37.0%, respectively. When one or two predictors

Table 3
Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for prediction of early virologic response (EVR) to dual therapy, based on the combination of amino acid substitutions in the core region and low-density lipoprotein cholesterol (LDL-C) level

	Sensitivity	Specificity	PPV[b]	NPV[c]
(A) Double-wild-type of core region[a]	46.4 (32/69)	88.9 (24/27)	91.4 (32/35)	39.3 (24/61)
(B) High level of LDL-C	81.0 (64/79)	56.3 (18/32)	82.1 (64/78)	54.5 (18/33)
(A) and (B)	32.4 (22/68)	100 (27/27)	100 (22/22)	37.0 (27/73)
(A) and/or (B)	97.4 (74/76)	39.3 (11/28)	81.3 (74/91)	84.6 (11/13)

Data in parentheses represent the numbers used for determining the sensitivity, specificity, PPV and NPV.
[a] Wild at aa 70 and wild at aa 91 were evaluated as double-wild type, and the other patterns were considered non-double-wild-type.
[b] PPV; EVR rates for patients with a combination of double-wild-type of the core region, or high levels (⩾86 mg/dl) of LDL-C (prediction of EVR).
[c] NPV; non-EVR rates for patients with non-double-wild-type of the core region, or low levels (<86 mg/dl) of LDL-C (prediction of non-EVR).

Table 4
Factors associated with sustained virological response to 48-week peginterferon-ribavirin combination therapy in patients infected with HCV genotype 1b, identified by multivariate analysis

Factor	Category	Odds ratio (95% confidence interval)	*P*
Amino acid substitution in core region	1: double-wild-type[a]	1	
	2: non-double wild-type	0.102 (0.022–0.474)	0.004
LDL cholesterol (mg/dl)	1: <86	1	
	2: ⩾86	12.87 (2.177–76.09)	0.005
Gender	1: male	1	
	2: female	0.091 (0.017–0.486)	0.005
ICG R15 (%)	1: <10	1	
	2: ⩾10	0.107 (0.017–0.678)	0.018
γ-Glutamyl transpeptidase (IU/l)	1: <109	1	
	2: ⩾109	0.096 (0.011–0.819)	0.032
Ribavirin dose (mg/kg)	1: <11.0	1	
	2: ⩾11.0	5.173 (1.152–23.22)	0.032

Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.
Normal range for LDL cholesterol: 86–135 mg/dl.
[a] Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type, and the other patterns were considered non-double-wild-type.

were used, the sensitivity, specificity, PPV, and NPV were 97.4%, 39.3%, 81.3%, and 84.6%, respectively. Thus, prediction of EVR by the combination of aa substitutions in HCV-CR and serum LDL-C level had high sensitivity, specificity, PPV, and NPV (Table 3).

3.4. Predictors of SVR as determined by univariate and multivariate analyses

Univariate analysis identified 12 parameters that influenced SVR: histopathological staging of liver fibrosis (F1; $P = 0.002$), leukocyte count (⩾4500/mm^3; $P = 0.004$), aa substitutions of HCV-CR (double-wild-type; $P = 0.005$), PEG-IFN dose/body weight (⩾1.25 μg/kg; $P = 0.006$), gender (male; $P = 0.007$), age (<55 years; $P = 0.009$), RBV dose/body weight (⩾11.0 mg/kg; $P = 0.009$), GGT (<109 IU/l; $P = 0.019$), ICG R15 (<10%; $P = 0.029$), LDL-C (⩾86 mg/dl; $P = 0.063$), Hb (⩾14.0 g/dl; $P = 0.064$), and AST (<60 IU/l; $P = 0.064$). Multivariate analysis identified six parameters that independently influenced the SVR: aa substitutions of HCV-CR (double-wild-type; $P = 0.004$), LDL-C (⩾86 mg/dl; $P = 0.005$), gender (male; $P = 0.005$), ICG R15 (<10%; $P = 0.018$), GGT (<109 IU/l; $P = 0.032$), and RBV dose/body weight (⩾11.0 mg/kg; $P = 0.032$) (Table 4). These results indicate that aa substitutions of HCV-CR and LDL-C levels are significant and independent predictors of both EVR and SVR, especially.

3.5. Assessment of amino acid substitutions and LDL cholesterol as predictors of SVR

Finally, we evaluated the ability to predict SVR by aa substitutions of HCV-CR and serum LDL-C level (each, $P < 0.01$). The SVR rates of patients with double-wild-type of HCV-CR or high serum levels of LDL-C were defined as PPV (prediction of SVR). The non-SVR rates of patients with non-double-wild-type of HCV-CR or low serum levels of LDL-C were defined as NPV (prediction of non-SVR).

In patients with double-wild-type of HCV-CR, the sensitivity, specificity, PPV, and NPV for SVR were 52.4%, 75.9%, 62.9%, and 67.2%, respectively. Thus, aa substitutions in HCV-CR have a high specificity for prediction of SVR. In patients with high-serum levels

Table 5
Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for prediction of sustained virological response (SVR), based on a combination of amino acid substitutions in the core region and low-density lipoprotein cholesterol (LDL-C) levels

	Sensitivity	Specificity	PPV[b]	NPV[c]
(A) Double-wild-type of core region[a]	52.4 (22/42)	75.9 (41/54)	62.9 (22/35)	67.2 (41/61)
(B) High level of LDL-C	80.0 (40/50)	37.7 (23/61)	51.3 (40/78)	69.7 (23/33)
(A) and (B)	33.3 (15/45)	87.9 (51/58)	68.2 (15/22)	63.0 (51/81)
(A) and/or (B)	100 (47/47)	22.8 (13/57)	51.6 (47/91)	100 (13/13)

Data in parentheses represent the numbers used for determining the sensitivity, specificity, PPV, and NPV.
[a] Wild at aa 70 and wild at aa 91 were evaluated as double-wild type, and the other patterns were considered non-double-wild-type.
[b] PPV, EVR rates for patients with a combination of double-wild-type of the core region or high levels (⩾86 mg/dl) of LDL-C (prediction of EVR).
[c] NPV, Non-EVR rates for patients with non-double-wild-type of the core region or low levels (<86 mg/dl) of LDL-C (prediction of non-EVR).

of LDL-C, the sensitivity, specificity, PPV, and NPV were 80.0%, 37.7%, 51.3%, and 69.7%, respectively. Thus, serum LDL-C level has high sensitivity in predicting SVR. Furthermore, when both predictors were used, the sensitivity, specificity, PPV, and NPV were 33.3%, 87.9%, 68.2%, and 63.0%, respectively. When one or more of the two predictors were used, the sensitivity, specificity, PPV, and NPV were 100%, 22.8%, 51.6%, and 100%, respectively. These results indicate that the use of the combination of the above two predictors has high sensitivity, specificity, and NPV for prediction of SVR (Table 5).

4. Discussion

We reported previously that substitutions of aa 70 and/or 91 in the HCV core region are an independent and significant predictor of NVR [4,5]. Based on a larger number of patients, the present study also identified aa substitutions in HCV-CR as a predictor of EVR and SVR in patients on 48-week PEG-IFN-RBV dual therapy. Previous studies reported that the HCV core region might be associated with resistance to IFN monotherapy involving the Jak-STAT signaling cascade [16–19]. Our result could be also interpreted to mean that aa substitutions in HCV-CR are associated with those proteins involved in resistance to IFN monotherapy, such as SOCS proteins known to inhibit IFN-α-induced activation of the Jak-STAT pathway and expression of the antiviral proteins 2′,5′-OAS and MxA [20]. Furthermore, our result also indicates that aa substitutions in HCV-CR might serve as a surrogate marker for other proteins associated with resistance to the antiviral actions of IFN. Further studies that examine the structural and functional impact of aa substitutions during combination therapy should be conducted to confirm the above finding.

Importantly, our study also identified serum LDL-C levels as a predictor of the response to PEG-IFN-RBV therapy, and we agree with the recent findings of Gopal et al. [21]. Previous studies reported that endocytosis of HCV via the LDL receptor(s) is mediated by the formation of a complex between HCV and VLDL or LDL [22,23]. Furthermore, there is evidence that intracellular cholesterol level modulates LDLr expression, and thus a high LDL-C could downregulate LDLr and diminish the spread of hepatocyte HCV infection. Thus, the correlation between treatment efficacy and LDL-C may be explained by the role of LDL-C in transporting the HCV-LDL complex into the hepatocyte. It should be noted, however, that other *in vitro* studies also showed that statins, which upregulate LDLr, might decrease HCV replication [24–26]. Other mechanisms could also explain the role of LDL-C and the response to PEG-IFN-RBV therapy. For example, high-LDL-C levels could act by modulating cytokine release [27] and antiviral cellular immune response [28,29]. On the other hand, it is also reported that apolipoprotein E4 allele is associated with high LDL-C levels [30], and with poor response to treatment in patients with genotype 1 HCV [31]. The discrepancy between our results and such findings may be explained by the small number of patients in our study, differences in host factors including race [32–34], and/or differences in vial factors, such as the distribution of genotype 1a or 1b, and geographic diversities of genotype 1b [35]. Further studies of large number of patients matched for race and HCV genotype are required to explore the relationship between serum LDL-C level and the response to PEG-IFN-RBV therapy.

Our results also showed that a high ICG R15 value was a negative predictor of SVR to PEG-IFN-RBV therapy. Previous data indicated that absence of advanced liver fibrosis is a predictor of SVR to IFN monotherapy and IFN-RBV dual therapy [36–38], and that advanced liver fibrosis is usually associated with high rates of ICG R15 [39]. However, our study showed that a milder form of liver fibrosis was not a predictor of response to dual treatment, whereas a low level of ICG R15 was. This discrepant finding may be due to the fact that estimates of liver dysfunction assessed by the degree of liver fibrosis (which is evaluated using only four stages (F1, F2, F3, F4), in contrast to ICG R15), are less sensitive to those by ICG R15. It is also possible that the above discrepancy is related to our exclusion of patients with cirrhosis (F4) (the exclusion was because the Japanese Government Health Insurance system does not provide cover for combination therapy for patients with cirrhosis). Further studies are required to explore the relationship between the severity of histopathological changes in the liver and response to dual therapy especially in patients with cirrhosis.

Our results should be interpreted with caution since we did not include patients of other races or other HCV genotypes. Any generalization of the results should await confirmation by studies of patients of other races infected with other HCV genotypes.

Pretreatment prediction of the response to PEG-IFN-RBV therapy is still incomplete. So far, viral factors (e.g., aa substitutions in HCV-CR), host factors (e.g., LDL-C [21], gender [40], ICG R15, and GGT [41]), and treatment-related factors (e.g., RBV dose [5,42]) have been confirmed to influence the response to such treatment in Japanese patients infected with HCV genotype 1b. Furthermore, evaluation using a combination of predictors indicates the high-sensitivity, specificity, PPV, and NPV of such prediction. We conclude that the response to PEG-IFN-RBV therapy seems to be based on a dynamic tripartite interaction of virus, host, and treatment regimen. Further understanding of the

complex interaction between these factors should facilitate the development of more effective therapeutic regimens.

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References

[1] Fried MW, Shiffman ML, Reddy R, Smith C, Marinos G, Gonçales FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347: 975–982.

[2] National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C: 2002-June 10-12, 2002. Hepatology 2002; 36: S3-20.

[3] Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology 2003;38:645–652.

[4] Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48:372–380.

[5] Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype1b and high viral load. J Med Virol 2006;78:83–90.

[6] D'Souza R, Sabin CA, Foster GR. Insulin resistance plays a significant role in liver fibrosis in chronic hepatitis C and in the response to antiviral therapy. Am J Gastroenterol 2005;100: 1509–1515.

[7] Martinez-Bauer E, Crespo J, Romero-Gomez M, Moreno-Otero R, Sola R, Tesei N, et al. Development and validation of two models for early prediction of response to therapy in genotype 1 chronic hepatitis C. Hepatology 2006;43:72–80.

[8] Poynard T, Ratziu V, McHutchison J, Manns M, Goodman Z, Zeuzem S, et al. Effect of treatment with peginterferon or interferon alfa-2b and ribavirin on steatosis in patients infected with hepatitis C. Hepatology 2003;38:75–85.

[9] Akuta N, Suzuki F, Tsubota A, Suzuki Y, Someya T, Kobayashi M, et al. Efficacy of interferon monotherapy to 394 consecutive naive cases infected with hepatitis C virus genotype 2a in Japan: therapy efficacy as consequence of tripartite interaction of viral, host and interferon treatment-related factors. J Hepatol 2002;37:831–836.

[10] Chayama K, Tsubota A, Arase Y, Saitoh S, Koida I, Ikeda K, et al. Genotypic subtyping of hepatitis C virus. J Gastroenterol Hepatol 1993;8:150–156.

[11] Desmet VJ, Gerber M, Hoofnagle JH, Manna M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513–1520.

[12] D'Alessandro AM, Kalayouglu M, Sollinger HW, Hoffmann RM, Reed A, Knechtle SJ, et al. The predictive value of donor liver biopsies for the development of primary nonfunction after orthotopic liver transplantation. Transplantation 1991;51: 157–163.

[13] Okamoto K, Akuta N, Kumada H, Kobayashi M, Matsuo Y, Tazawa H. A nucleotide sequence variation detection system for the core region of hepatitis C virus-1b. J Virol Methods (in press).

[14] Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 1990;87:9524–9528.

[15] Kwok S, Higuchi R. Avoiding false positives with PCR. Nature 1989;339:237–238.

[16] Blindenbacher A, Duong FH, Hunziker L, Stutvoet ST, Wang X, Terracciano L, et al. Expression of hepatitis C virus proteins inhibits interferon α signaling in the liver of transgenic mice. Gastroenterology 2003;124:1465–1475.

[17] Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, et al. IFN-α antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J 2003;17:488–490.

[18] Melén K, Fagerlund R, Nyqvist M, Keskinen P, Julkunen I. Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. J Med Virol 2004;73:536–547.

[19] de Lucas S, Bartolome J, Carreno V. Hepatitis C virus core protein down-regulates transcription of interferon-induced antiviral genes. J Infect Dis 2005;191:93–99.

[20] Voltides G, Sorensen AS, Kopp F, Zitzmann K, Cengic N, Brand S, et al. SOCS-1 and SOCS-3 inhibit IFN-alpha-induced expression of the antiviral proteins 2,5-OAS and MxA. Biochem Biophys Res Commun 2004;30:1007–1014.

[21] Gopal K, Johnson TC, Gopal S, Walfish A, Bang CT, Suwandhi P, et al. Correlation between beta-lipoprotein levels and outcome of hepatitis C treatment. Hepatology 2006;44:335–340.

[22] Agnello V, Abel G, Elfahal M, Knight GB, Zhang QX. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc Natl Acad Sci USA 1999;96:12766–12771.

[23] Andre P, Komurian-Pradel F, Deforges S, Perret M, Berland JL, Sodoyer M, et al. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J Virol 2002;76:6919–6928.

[24] Ye J, Wang C, Sumpter R, Brown MS, Goldstein JL, Gale M. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. Proc Natl Acad Sci USA 2003;100:15865–15870.

[25] Kapadia SB, Chisari FV. Hepatitis C virus replication is regulated by host geranylgeranylation and fatty acids. Proc Natl Acad Sci USA 2005;102:2561–2566.

[26] Ikeda M, Abe K, Yamada M, Dansako H, Naka K, Kato N. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. Hepatology 2006;44:117–125.

[27] Netea MG, Demacker PNM, Kullberg BJ, Boerman OC, Verschueren I, Stalenhoef AFH, et al. Low-density lipoprotein receptor-deficient mice are protected against lethal endotoxemia and severe gram-negative infections. J Clin Invest 1996;97:1366–1372.

[28] Ludewig B, Jaggi M, Dumrese T, Brduscha-Riem K, Odermatt B, Hengartner H, et al. Hypercholesterolemia exacerbates virus-induced immunopathologic liver disease via suppression of antiviral cytotoxic T cell responses. J Immunol 2001;166:3369–3376.

[29] Muldoon MF, Marsland A, Flory JD, Rabin BS, Whiteside TL, Manuck SB. Immune system differences in men with hypo- or hypercholesterolemia. Clin Immunol Immunopathol 1997;84:145–149.

[30] Schaefer EJ, Lamon-Fava S, Johnson S, Ordovas JM, Schaefer MM, Castelli WP, et al. Effects of gender and menopausal status on the association of apolipoprotein E phenotype with plasma lipoprotein levels. Results from the Framingham Offspring Study. Arterioscler Thromb 1994;14:1105–1113.

[31] Mueller T, Gessner R, Sarrazin C, Graf C, Halangk J, Witt H, et al. Apolipoprotein E4 allele is associated with poor treatment response in hepatitis C virus (HCV) genotype 1. Hepatology 2003:1592 (Correspondence).

[32] McHutchison JG, Poynard T, Pianko S, Gordon SC, Reid AE, Dienstag J, et al. The impact of interferon plus ribavirin on response to therapy in black patients with chronic hepatitis C. The International Hepatitis Interventional Therapy Group. Gastroenterology 2000;119:1317–1323.
[33] Kaplan DE, Sugimoto K, Ikeda F, Stadanlick J, Valiga M, Shetty K, et al. T-cell response relative to genotype and ethnicity during antiviral therapy for chronic hepatitis C. Hepatology 2005;41:1365–1375.
[34] Borenstein AR, Mortimer JA, Wu Y, Jureidini-Webb FM, Fallin MD, Small BJ, et al. Apolipoprotein E and cognition in community-based samples of African Americans and Caucasians. Ethn Dis 2006;16:9–15.
[35] Nakano I, Fukuda Y, Katano Y, Nakano S, Kumada T, Hayakawa T. Why is the interferon sensitivity-determining region (ISDR) system useful in Japan?. J Hepatol 1999;30:1014–1022.
[36] Jouet P, Roudot-Thoraval F, Dhumeaux D, Metreau JM. Comparative efficacy of interferon alfa in cirrhotic and noncirrhotic patients with non-A, non-B, C hepatitis. Gastroenterology 1994;106:686–690.
[37] Poynard T, McHutchinson J, Goodman Z, Ling MH, Albrecht J. Is an "a la carte" combination interferon alfa-2b plus ribavirin regimen possible for the first line treatment in patients with chronic hepatitis C? The ALGOVIRC Group. Hepatology 2000;31: 211–218.
[38] Bruno S, Camma C, Di Marco V, Rumi M, Vinci M, Camozzi M, et al. Peginterferon alfa-2b plus ribavirin for naïve patients with genotype 1 chronic hepatitis C: a randomized controlled trial. J Hepatol 2004;41:474–481.
[39] Stremmel W, Wojdat R, Groteguth R, Zoedler M, Ebener T, Niederau C, et al. Liver function tests in a clinical comparison. Gastroenterology 1992;30:784–790.
[40] Conjeevaram HS, Fried MW, Jeffers LJ, Terraults NA, Wiley-Lucas TE, Afdhal N, et al. Peginterferon and ribavirin treatment in African American and Caucasian American patients with hepatitis C genotype 1. Gastroenterology 2006;131:147–470.
[41] Berg T, Sarrazin C, Herrmann E, Hinrichsen H, Gerlach T, Zachoval R, et al. Prediction of treatment outcome in patients with chronic hepatitis C: significance of baseline parameters and viral dynamics during therapy. Hepatology 2003;37:600–609.
[42] Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet 2001;358:958–965.

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Long-Term Presence of HBV in the Sera of Chronic Hepatitis B Patients with HBsAg Seroclearance

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Key Words
Chronic hepatitis B · Hepatitis B virus DNA · Seroclearance, hepatitis B surface antigen

Abstract
Objects: The aim of this study was to elucidate the presence of serum hepatitis B virus (HBV) DNA at a prolonged time after seroclearance of hepatitis B surface antigen (HBsAg). ***Methods:*** Seventy Japanese patients who had been observed for >5 years after HBsAg seroclearance were included in this study. Anti-HBs, anti-HBe and anti-HBc antibodies were measured 0, 5 and 10 years after HBsAg seroclearance. Serum HBV DNA was measured using nested polymerase chain reaction (PCR) at 0, 5 and 10 years after HBsAg seroclearance. The PCR detection of serum HBV DNA using the X gene and core gene primers was done. The HBV DNA was regarded as positive when PCR detection of HBV DNA using either or both the X gene and core gene primers was positive. A multivariate regression analysis was used to assess the factors contributing to the positivity of serum HBV DNA 5 years after HBsAg seroclearance: the factors examined included age, gender, histological findings, HBV genotype, aminotransferase, total protein and interferon administration. ***Results:*** The titers of 200-fold diluted serum anti-HBc were 6.5 ± 4.0 at 0 year after HBsAg seroclearance, 1.8 ± 1.4 at 5 years and 0.9 ± 0.7 at 10 years. The titers of 200-fold diluted serum anti-HBc decreased 5 and 10 years after HBsAg seroclearance with statistical significance. The positive rate of HBV DNA by the nested PCR was 71.4% (50/70) at 0 year after HBsAg seroclearance, 21.4% (15/70) at 5 years and 14.3% (3/21) at 10 years. However, there were no significant factors contributing to the positivity of serum HBV DNA 5 years after HBsAg seroclearance. ***Conclusion:*** Our results suggest that serum HBV DNA disappears with an incidence of 10–20% 5 and 10 years after HBsAg seroclearance.

Introduction

Chronic hepatitis B is a serious liver disease with significant mortality. In patients with chronic hepatitis B virus (HBV) infection, persistent viral replication is associated with ongoing necroinflammation in the liver and progressive liver damage [1–3]. However, in patients with seroclearance of hepatitis B envelope antigen (HBeAg) and marked reduction of HBV DNA, the prognosis of the disease is generally improved [4–6]. Moreover, hepatitis B surface antigen (HBsAg) seroclearance has probably been associated with a good prognosis [7–12].

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An explosion of papers argue that some patients with seroclearance of HBsAg showed positive HBV DNA at the time of HBsAg seroclearance or within 1 year of HBsAg seroclearance [13–17]. However, it is not clear how long serum HBV DNA could be detected after prolonged observation after HBsAg seroclearance. Moreover, it is still a question whether the patients with seroclearance of HBsAg could be really cleared of serum HBV DNA or not. To further investigate these issues, we performed the present study on the long-term virological outcome after HBsAg seroclearance in Japanese patients.

Materials and Methods

Patients

From 1972 to 2002, a total of 5,055 chronic HBsAg carriers, who were known to be seropositive for HBsAg for at least 6 months, were studied in Toranomon Hospital in Tokyo, Japan. After a mean follow-up period of 4 years (range 0.5–30 years), 231 patients were noted to have delayed HBsAg seroclearance, which is defined as persistent absence of HBsAg antigenemia by radioimmunoassay for at least 1 year until the last examination. Of these 70 patients had the following criteria: (1) laparoscopy and liver biopsy taken before HBsAg seroclearance showed histological features of chronic active hepatitis or liver cirrhosis; (2) the follow-up period was more than 5 years after seroclearance of HBsAg.

We excluded from the study all the patients: (1) with concurrent hepatitis C virus and hepatitis D virus; (2) with a history of alcohol abuse or autoimmune liver disease; (3) with clinical evidence of hepatocellular carcinoma at entry into the study on the basis of ultrasonography, α-fetoprotein levels (<200 ng/ml) and/or histology; (4) with a history or clinical evidence of complications of decompensated cirrhosis at enrollment (that is ascites, encephalopathy or icterus).

Thirty-seven of 70 patients had spontaneous seroclearance of HBsAg, 20 patients had been given interferon (IFN) therapy for 1–16 months, 9 had been given steroid withdrawal monotherapy and 4 had been treated with combination therapy of steroid + IFN. The total median dose of IFN therapy was 336 mega units (range, 168–624 mega units). The patients treated with steroids were generally given prednisolone for 4 weeks, in a single dose of 40 mg/day for 1 week, 30 mg/day for 1 week, 20 mg/day for 1 week and then 10 mg/day for 1 week until it was abruptly withdrawn (total dose 700 mg).

Methods

The serums were stored at −80° until enzyme assays and measurement of HBV DNA level by the nested PCR method could be performed on all the samples for 70 patients at one time. Serum samples had been conserved at 0, 5 and 10 years after seroclearance of serum HBsAg. Serum HBV DNA was determined using the nested PCR independently by an experienced technician (J.S.), who had no clinical information or knowledge of each patient. The sensitivity of HBV DNA according to the manufacturer is about 50–100 copies/ml in the nested PCR method. Two kinds of primers in the core and X gene of HBV were used in the nested PCR method. First of all, primers used for the detection of HBV were Cof1 (sense, 5′-CTGCCTTACTTTTGGAGAGA-3′) and Cer1 (antisense, 5′-ACTTTACTGGGCTTTATTA-3′) for the first PCR and core sense (sense, 5′-GAGTGTGGATTCGCACTCCTC-3′) and anticore (5′-GATTGAGATCTTCTGCGACGC-3′) for the second PCR in the core gene. Second, primers used for detection of HBV were P2 (sense, 5′-GTCCCGTCGGCGCTGAATCCC-3′) and Br102 (antisense, 5′-GCAGATGAGAAGGCACAGAC-3′) for the first PCR and X sense (sense, 5′-CTGGATCCTGCGCGG GACGTCCTT-3′) and anti-X (5′-GTTCACGGTGGTCTCCAT-3′) for the second PCR in the X gene. In the first PCR and the second PCR, amplification was performed over 35 cycles (94 for 1 s; 55° for 1 s; 72° for 1 s) after initial denaturing at 94° for 4 min and a final extension at 72° for 7 min. Negative and positive controls confirmed the HBV DNA band in parallel. Ten healthy volunteers without HBsAg and anti-HCV were selected for negative HBV DNA controls. Ten patients with chronic hepatitis B and with HBsAg were selected for positive controls. The HBV DNA was considered positive when PCR detection of HBV DNA using either or both the X gene and core gene primers showed positivity. On the other hand, the HBV DNA was considered negative when PCR detection of HBV DNA using both the X gene and core primers showed negativity.

When serum samples showed positive HBV DNA by the nested PCR, we also examined the serum HBV DNA level. It was measured by a transcription-mediated amplification and hybridization-protection assay (Chugai Diagnostics, Tokyo, Japan), and the results were expressed as log genome equivalents (LGE) per milliliter. The lower detection limit of this assay is 3.7 LGE/ml, which is equivalent to 5,000 copies/ml.

HBsAg, anti-HBs, HBeAg, anti-HBe and antibody to HDV were all assayed using commercially available radioimmunoassay kits. Anti-HBc was assayed by chemiluminescent enzyme immunoassay. Antibody against HCV was detected with a third-generation enzyme-linked immunoassay (Ortho Diagnostic Japan, Tokyo). The HBV genotype was determined using a previously reported method [18]. Biochemical tests were made using routine automated techniques and carried out in the laboratories of Toranomon Hospital. This study was approved by the institutional review board of our hospital. The physicians in charge explained the purpose and method of this clinical trial to each patient, who gave their informed consent for participation.

Liver Histology

Liver biopsy specimens were obtained percutaneously under the observation by laparoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan).

Statistical Analysis

We used Fisher's exact test (two-tailed) or the Wilcoxon rank sum test to compare differences between groups. Moreover, we used univariate analysis and multivariate analysis (multiple logistic regression analysis) to establish which factors contributed to the positivity of HBV DNA 5 years after HBsAg seroclearance. Results for each variable were transformed into categorical data consisting of two simple original numbers for univariate and multivariate analyses. Variables that achieved statistical significance

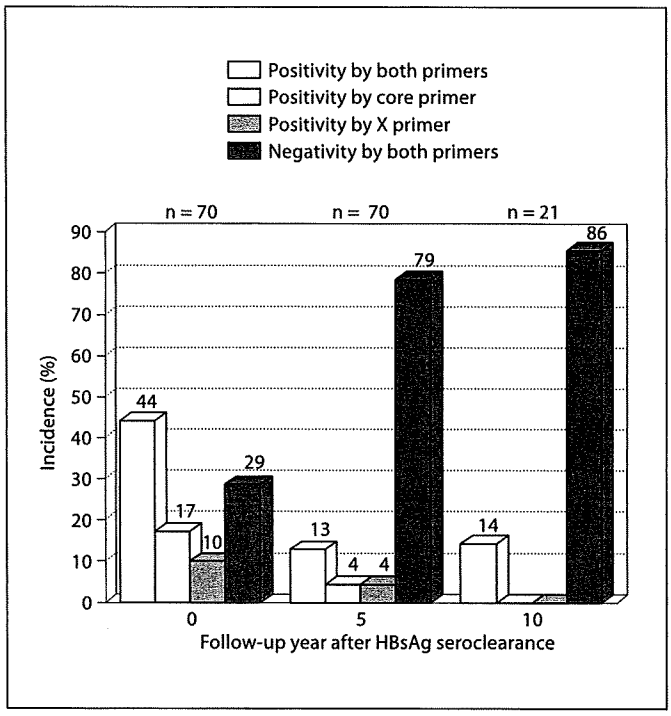

Fig. 1. Changes of detection pattern of serum HBV DNA after seroclearance of HBsAg. Negative controls: 10 healthy volunteers; positive controls: 10 patients with chronic hepatitis B; core (X): positivity indicates positive HBV DNA by nested PCR using core (X) gene primers, negativity indicates negative HBV DNA by nested PCR using core (X) gene primers.

($p < 0.05$) were subjected to multiple logistic regressions to identify significant independent predictors. The SPSS software package (SPSS 10.0 for Windows; SPSS Inc., Chicago, Ill., USA) was used for analyses.

Table 1. Characteristics of subjects at the time of seroclearance of HBsAg

Number	70
Sex (male/female)	55/15
Age, years	53 (30–82)
HBV genotype (A/B/C/D/F)	3/7/45/2/6
US (non-LC/LC)	40/30
Total protein, g/dl	7.4 (6.6–8.8)
Albumin, g/dl	4.2 (3.4–5.1)
Total bilirubin, g/dl	0.7 (0.1–1.7)
AST, IU/l	21 (11–71)
ALT, IU/l	16 (6–101)
Hb, g/dl	15.2 (12.9–17.1)
Platelets, $\times 10^4/mm^3$	17.3 (8.4–32.5)
Follow-up period after disappearance of HBs antigen, years	8.3 (5.3–23.6)

Data are numbers of patients or medians, with ranges in parentheses. ALT = Alanine aminotransferase; AST = aspartate aminotransferase; Hb = hemoglobin; US = ultrasonographic findings; LC = liver cirrhosis.

Table 2. Change of anti-HBc antibody after HBsAg seroclearance

	Follow-up year of HBsAg seroclearance		
	0	5	10
Anti-HBc antibody	14.2 ± 2.7	13.9 ± 2.2	13.3 ± 3.6
Anti-HBc antibody (200-fold dilution)	6.5 ± 4.0	1.8 ± 1.4	0.9 ± 0.7

The serum was diluted 1:200 with saline. The titer of anti-HBc antibody was determined by the chemiluminescent immunoassay method.

Results

Clinical Profiles

Table 1 shows the characteristics of the 70 patients who had seroclearance of HBsAg. The median age of the 70 patients (male 55, female 15) was 53 years. Thirty-seven patients had spontaneously cleared HBsAg. At the time of HBsAg seroclearance, 30 patients showed liver cirrhosis.

Sixty-three of 70 (87.9%) patients had normal alanine aminotransferase levels after HBsAg seroclearance. Seven patients with elevated alanine aminotransferase had 4 fatty infiltrations of the liver and 3 cases of alcohol abuse.

Changes of Anti-HBs, Anti-HBe and Anti-HBc

Table 2 shows the titers of serum anti-HBc. As regards the titer of nondiluted anti-HBc, there was no difference between the time of HBsAg seroclearance, 5 years and 10 years after HBsAg seroclearance. The titers of 200-fold diluted serum anti-HBc decreased 5 and 10 years after HBsAg seroclearance with statistical significance.

Serum HBV DNA after HBsAg Seroclearance

The detection pattern of serum HBV DNA based on the difference of HBV primers by the nested PCR is shown in figure 1. The negative controls of healthy volunteers showed negative HBV DNA with both primers. On the

Table 3. Predictive factors for the positivity of HBV DNA 5 years after HBsAg seroclearance

Factor	Category	Odds ratio	95% CI	p value
IFN therapy	−/+	1/0.58	0.14–2.32	0.438
Age, years	<60/≥60	1/1.99	0.56–7.07	0.287
Total protein, g/dl	<8/≥8	1/1.84	0.90–3.76	0.096
Liver histology	non-LC/LC	1/2.00	0.28–5.48	0.781
HBV genotype	B/C	1/0.254	0.05–1.36	0.109
Sex	male/female	1/0.23	0.03–1.88	0.169
AST, IU/l	≥38/<38	1/1.59	0.57–4.43	0.375
Platelets, $\times 10^4/mm^3$	≤20/>20	1/1.20	0.700–2.06	0.504
ALT, IU/l	≥50/<50	1/1.28	0.46–3.55	0.634

ALT = Alanine aminotransferase; AST = aspartate aminotransferase; CI = confidence interval; LC = liver cirrhosis.

other hand, the positive controls with chronic hepatitis B showed positive HBV DNA with both primers. The consistent rate of PCR detection of HBV DNA using both the X gene and core primers was 84.5% (136/161) in all. The positive rate of HBV DNA was 71.4% (50/70) at year 0, 21.4% (15/70) at 5 years and 14.3% (3/21) at 10 years by using both the X gene and core primers.

A multivariate regression analysis was used to assess the factors contributing to the positivity of serum HBV DNA: the factors examined included age, gender, histological findings, HBV genotype and IFN administration. However, there were no significant factors contributing to the positivity of serum HBV DNA (table 3).

In total 68 serum samples which showed positive HBV DNA by the nested PCR were examined by the transcription-mediated amplification and hybridization-protection assay. All the samples showed serum HBV DNA of less than 3.7 LGE/ml.

Discussion

In the present study, the detection rate of serum HBV by the nested PCR after HBsAg seroclearance was about 70% at the time of HBsAg seroclearance and about 10–20% at 5 and 10 years after seroclearance of HBsAg. The positive rate of HBV DNA decreased 5 and 10 years after HBsAg seroclearance compared to the time of HBsAg seroclearance. Moreover, the titer of anti-HBc antibody by the 200-fold dilution gradually decreased after HBsAg seroclearance. This suggests that HBV may ultimately be cleared from the serum after a long time of more than 10 years. However, this present study showed that about 10–20% of patients had serum HBV DNA levels of 50–100 copies/ml at 5 and 10 years after seroclearance of HBsAg. The remaining patients might have low levels of HBV DNA of <50–100 copies/ml. Yuen et al. [14] have reported that HBV remains in the liver even if serum HBV is shown to be negative in some patients. These findings mean that a trace of HBV remains for a prolonged period after HBsAg seroclearance.

HBV DNA replications sometimes occur after administration of steroids and/or immunosuppressive agents in patients with a small amount of residual HBV [19–21]. Our previous study suggests that steroid withdrawal therapy for HBeAg-positive patients with chronic hepatitis induces an elevation of serum HBV DNA and acute exacerbation of liver function [10]. Five of 230 HBeAg-positive patients treated with steroid withdrawal therapy showed acute exacerbation of liver function and icterus. Therefore, when the patients with serum HBsAg are given steroids and/or immunosuppressive agents, they should be carefully followed up by monitoring serum levels of HBV DNA and liver function. The present study shows that a trace of HBV remains during a prolonged period after HBsAg seroclearance. This suggests the following point: when patients with HBsAg seroclearance are treated with steroids and/or immunosuppressive agents, they should be carefully followed up by monitoring the serum level of HBV DNA and/or liver function to prevent acute exacerbation of liver impairment.

Seventy patients enrolled in the present study were not treated with steroids and/or immunosuppressive agents, so they did not show acute exacerbation during the follow-up. Moreover, these 70 patients did not show progression to decompensated liver cirrhosis and/or death due to hepatocellular carcinoma. Thus, our results suggest that even if patients with HBsAg seroclearance have a trace of

HBV DNA, they have generally a good prognosis concerning liver function.

In conclusion, as some patients also had a trace of serum HBV DNA 5 and/or 10 years after seroclearance of HBsAg, they should be carefully followed concerning administration of steroids and/or immunosuppressive agents.

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References

1 Liaw YF, Tai DI, Chu CM, Chen TJ: The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. Hepatology 1988;8:493–496.

2 De Franchis R, Meucci G, Vecchi M, Tatarella M, Colombo M, Del Ninno E, Rumi MG, Donato MF, Ronchi G: The natural history of asymptomatic hepatitis B surface antigen carriers. Ann Intern Med 1993;118:191–194.

3 Viola LA, Barison IG, Coleman JC, Paradinas FJ, Fluker JL, Evans BA, Murray-Lyon IM: Natural history of liver disease in chronic hepatitis B surface antigen carriers. Lancet 1981;2:1156–1159.

4 Wong DK, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J: Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B: a meta-analysis. Ann Intern Med 1993;119:312–323.

5 Niederau C, Heintges T, Lange S, Goldmann G, Niederau CM, Mohr L, Haussinger D: Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. N Engl J Med 1996;334:1422–1427.

6 Hsu YS, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, Liaw YF: Long- term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. Hepatology 2002;35:1522–1527.

7 Lindsay KL, Redeker AG, Ashcavai M: Delayed HBsAg clearance in chronic hepatitis B viral infection. Hepatology 1981;1:586–589.

8 Liaw YF, Sheen IS, Chen TJ, Chu CM, Pao CC: Incidence, determinants and significance of delayed clearance of serum HBsAg in chronic hepatitis B infection: a prospective study. Hepatology 1991;13:627–631.

9 Chen YC, Sheen IS, Chu CM, Liaw YF: Prognosis following spontaneous HBsAg seroclearance in chronic hepatitis B with or without concurrent infection. Gastroenterology 2002;123:1084–1089.

10 Arase Y, Ikeda K, Murashima N, Chayama K, Tsubota A, Koida I, Suzuki Y, Saitoh S, Kobayashi M, Kobayashi M, Kumada H: Time course of histological changes in patients with a sustained biochemical and virological response to corticosteroid withdrawal therapy for chronic hepatitis B. Am J Gastroenterol 1999;94:3304–3309.

11 Perrillo RP, Brunt EM: Hepatic histologic and immunochemical changes in chronic hepatitis B after prolonged clearance of hepatitis B e antigen and hepatitis B surface antigen. Ann Intern Med 1991;115:113–115.

12 Chung HT, Lai CL, Lok ASF: Pathogenic role of hepatitis B virus in hepatitis B surface antigen-negative decompensated cirrhosis. Hepatology 1995;22:25–29.

13 Blum HE, Liang TJ, Galun E, Wands JR: Persistence of hepatitis B viral DNA after serological recovery from hepatitis B virus infection. Hepatology 1991;14:56–63.

14 Yuen MF, Ho Wong DKH, Sahlon E, Tse E, Ng IO, Yuan HJ, Sin CW, Sander TJ, Boume EJ, Hall JG, Condreay LD, Lai CL: HBsAg seroclearance in chronic hepatitis B in the Chinese: virological, histological, and clinical aspects. Hepatology 2004;39:1694–1701.

15 Ahn SH, Park YN, Park JY, Change HY, Lee JM, Shin JE, Han KH, Park C, Moor YM, Chon CY: Long-term clinical and histological outcomes in patients with spontaneous hepatitis B surface antigen seroclearance. J Hepatol 2005;42:188–194.

16 Yotsuyanagi H, Yasuda K, Iino S, Moriya K, Shintani Y, Fujie H, Tsutsumi T, Kimura S, Koike K: Persistent viremia after recovery from self-limited acute hepatitis B. Hepatology 1998;27:1377–1382.

17 Huo TI, Wu JC, Lee PC, Chan GY, Lui WY, Tsay SH, Ting LT, Change FY, Lee SD: Seroclearance of hepatitis B surface antigen in chronic carriers does not necessarily imply a good prognosis. Hepatology 1998;28:231–236.

18 Usuda S, Okamoto H, Iwanari H, Baba K, Usuda F, Miyakawa Y, Mayumi M: Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods 1999;80:97–112.

19 Hoofnagle JH, Davis GL, Pappas SC, Hanson RG, Peters M, Avian MI, Waggoner JG, Jones FA, Seeff LB: A short course of prednisolone in chronic type B hepatitis. Ann Intern Med 1986;104:12–17.

20 Lau JY, Bird GL, Gimson AE, Alexander GJ, Williams R: Treatment of HBV reactivation after withdrawal of immunosuppression. Lancet 1991;337:802.

21 Nakamura Y, Motokura T, Fujita A, Yamashita T, Ogata E: Severe hepatitis related to chemotherapy in hepatitis B virus carriers with hematologic malignancies. Cancer 1996;78:2210–2215.

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Factors associated with the virological response of lamivudine-resistant hepatitis B virus during combination therapy with adefovir dipivoxil plus lamivudine

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Background. The aim of this study was to investigate the factors associated with the response of lamivudine-resistant hepatitis B virus (HBV) during combination therapy with adefovir dipivoxil plus lamivudine. ***Methods.*** Sixty-three patients with breakthrough hepatitis received a 10-mg once-daily dose of oral adefovir dipivoxil. ***Results.*** The rates of undetectable serum HBV-DNA were 49.2% after 24 weeks, 61.9% after 48 weeks, and 67.2% after 72 weeks. The cumulative hepatitis B e antigen (HBeAg) loss rates in patients with alanine aminotransferase (ALT) levels of more than twice the upper limit of normal (ULN) were significantly higher than in patients with ALT less than twice the ULN ($P = 0.0145$). Multivariate analysis revealed that baseline ALT level ($P = 0.003$) and HBeAg status ($P = 0.049$) were associated with early virological response. ***Conclusions.*** Baseline ALT level was associated with HBeAg loss and seroconversion, and baseline ALT level and HBeAg status were associated with the virological response of lamivudine-resistant HBV during combination therapy with adefovir dipivoxil plus lamivudine.

Key words: adefovir dipivoxil, lamivudine-resistant mutant, hepatitis B virus, HBeAg seroconversion

Introduction

Chronic hepatitis B is a common disease and leads to progressive liver disease and hepatocellular carcinoma. Lamivudine, a nucleoside analog administered orally, inhibits the replication of hepatitis B virus (HBV), and is used worldwide for the treatment of chronic hepatitis B.[1–3] Lamivudine improves alanine aminotransferase (ALT) levels and liver histological findings, causes hepatitis B e antigen (HBeAg) loss and seroconversion, and is well tolerated.[4,5] However, the emergence of lamivudine-resistant HBV strains in patients on long-term lamivudine therapy has been observed; the mutation that results in such resistance occurs in the HBV DNA polymerase gene in the YMDD motif.[6–8] The emergence of such mutant viruses results in the reelevation of HBV DNA and ALT levels and causes clinical and histologic progression.[9] Resistance was recently reported to develop in 12.5% of patients after 1 year of lamivudine therapy, in 43.8% after 3 years, and in 62.5% after 5 years.[10]

Adefovir dipivoxil (ADV) is a nucleotide analog that selectively inhibits viral polymerases and reverse transcriptases.[11–14] ADV has antiviral activity against not only wild-type HBV but also lamivudine-resistant HBV mutants in vitro and in vivo.[15,16] Worldwide clinical trials of ADV monotherapy or ADV plus lamivudine combination therapy for lamivudine-resistant mutants have been recently reported in cases of compensated or decompensated liver disease and in liver transplant recipients.[17,18] These studies reported virological and biochemical improvements in lamivudine-resistant chronic hepatitis B after 48–52 weeks of ADV therapy: 20%–35% of patients showed a negative serum HBV DNA level by polymerase chain reaction (PCR) assay; 15%–17% showed negative HBeAg; and 53%–61% showed normalized serum ALT levels.[17,18] We also recently reported the result of a pilot study on the efficacy of combination therapy with ADV plus lamivudine for treating lamivudine-resistant chronic hepatitis B.[19] We found that by week 24, 55.6% of patients showed a negative serum HBV DNA level by PCR assay and 75% showed normalized serum ALT levels. Although these data are encouraging, clinical factors associated with the virological and biochemical responses to this treatment are unknown. In this study, we investigate the factors associated with the virological response of

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lamivudine-resistant HBV during combination therapy with ADV plus lamivudine.

Patients and methods

Patients

Adefovir dipivoxil was administered to 72 adult patients at Toranomon Hospital, Tokyo, Japan; the patients had received ongoing lamivudine treatment for chronic hepatitis B for more than 72 weeks since 2002. Serum HBV DNA and ALT levels increased again despite the continuation of lamivudine, indicating breakthrough hepatitis, in all patients, who then received ADV along with the lamivudine. Of the 72 patients treated with ADV, 63 were enrolled in this retrospective study. Enrollment in this study and the start of ADV treatment were determined by the following criteria: (1) Increase of serum HBV DNA levels was ≥1 log copies/ml during lamivudine treatment on at least two consecutive occasions, compared with the nadir of initial antiviral efficacy (≥1 log decrease in serum HBV DNA). (2) Serum aspartate transaminase (AST) and/or ALT levels were greater than the upper limit of normal (ULN) before the start of ADV treatment (ULN: AST = 38 IU/l, ALT = 50 IU/l). (3) Mutations of the YMDD motif were detected before the start of ADV treatment by the PCR-based method described later. (4) Other nucleoside analogs such as famciclovir and entecavir had not been previously administered. The exclusion criteria were as follows: (1) serum creatinine levels ≥1.5 mg/dl; (2) interferon (IFN) added to ADV and lamivudine to treat severe acute exacerbation; and (3) infection with hepatitis A, hepatitis C, delta viruses, or human immunodeficiency virus, or a history of other liver diseases such as autoimmune hepatitis, alcoholic liver disease, or metabolic liver disease.

Methods

Patients received a 10-mg once-daily dose of oral ADV. Lamivudine treatment was continued in all patients. Blood samples were obtained at least once every month before, during, and after treatment with ADV, and analyzed for virological markers, biochemical markers associated with liver function and renal function, and complete blood cell counts every visit. The diagnosis of cirrhosis was based on liver biopsy histology and/or on clinical criteria, including image studies and signs of portal hypertension. The primary efficacy measures of low hepatitis activity were undetectable HBV DNA level by PCR assay and normalization of the ALT level; the secondary efficacy measures were HBeAg loss and seroconversion. The rate of each measure was evaluated 24, 48, and 72 weeks after the start of treatment. Adverse effects were monitored clinically by careful interview and medical examination at least once every month. Patient compliance with treatment was evaluated by questionnaire.

Serum HBV DNA levels were evaluated by quantitative PCR assay (Amplicor HBV Monitor test, Roche Molecular Systems, Pleasanton, CA, USA). The detection range of this assay was 2.6–7.6 log copies/ml (400 to 4×10^7 copies/ml). Antibodies to hepatitis B s and e antigens were determined by commercially available radioimmunoassay systems (Abbott Japan, Tokyo, Japan). Confirmation of mutation in the HBV DNA polymerase gene (rtM204 of the YMDD motif) was determined using the PCR-based method of Chayama et al.[20] The HBV genotype was determined by enzyme-linked immunosorbent assay (HBV Genotype EIA, Institute of Immunology, Tokyo, Japan) based on the method of Usuda et al.[21]

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from every patient. This study was approved by the local ethics committee of Toranomon Hospital.

Statistical analysis

Analysis of efficacy was performed on an intention-to-treat basis. The χ-squared test was used to compare efficacies. The cumulative rate of HBeAg loss and seroconversion were calculated using the Kaplan-Meier method, and differences between the curves were tested using the log-rank test. Exploratory analyses with covariates of interest were used to evaluate potential predictors of early virological response. Early virological response was defined by an undetectable serum HBV DNA level using the Amplicor monitor assay at week 24. The baseline factors included were patient age, sex, ALT level, HBeAg status, HBV DNA level, YMDD mutant status, HBV genotype, and the presence of cirrhosis. Initially, univariate analyses were conducted using logistic regression analysis. Next, all factors found to be at least marginally associated with early virological response ($P < 0.15$) were tested by multivariate analysis using a stepwise logistic model. A P value of less than 0.05 was considered statistically significant. Changes in serum HBV DNA and ALT levels were plotted in a graph format using the median values. Statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

Results

Study population

The clinical and virological profiles of the 63 patients at the start of ADV treatment are shown in Table 1. At

baseline, 19 patients (30.2%) had cirrhosis, 42 patients (66.7%) were positive for HBeAg, and all 63 patients had detectable YMDD mutants. The median serum HBV DNA level at baseline was 7.3 log copies/ml, and the median serum ALT level was 3.9 × ULN.

Efficacy of ADV plus lamivudine treatment

Table 2 shows the point prevalence with respect to the efficacy measures in the intention-to-treat population analysis and summarizes the virological response 24, 48, and 72 weeks from the start of ADV treatment. The rates of undetectable serum HBV DNA levels (<2.6 log copies/ml) in the total study population were 49.2% (31/63) after 24 weeks, 61.9% (39/63) after 48 weeks, and 67.2% (39/58) after 72 weeks. The undetectable rates in the HBeAg-negative group were significantly higher than those in the HBeAg-positive group at week 24 ($P = 0.05$), week 48 ($P = 0.006$), and week 72 ($P = 0.001$).

Normalized rates of serum ALT levels are summarized in Table 2. The rates in the total study population were 73.0% (47/63) after 24 weeks, 81.0% (51/63) after 48 weeks, and 93.1% (54/58) after 72 weeks. The normalized rates of serum ALT levels in HBeAg-positive and HBeAg-negative groups were similar. No patient had a virological relapse of serum HBV DNA elevation of more than 2 log copies/ml from the lowest values or a biochemical relapse of serum ALT elevation over 2 × ULN.

Table 1. Baseline characteristics at commencement of adefovir dipivoxil ($n = 63$)

Treatment period (weeks)[a]	108 (73–165)
Age[a]	48 (26–73)
Sex (Male : Female)	52 : 11
Presence of cirrhosis (%)	30.2
HBV genotype (A : B : C)	4 : 3 : 56
HBeAg positive (%)	66.7
HBV DNA (log copies/ml)[a]	7.3 (4.0 to >7.6)
rtM204 mutant (I : V : I + V)[b]	28 : 10 : 25
AST (IU/l)[a]	116 (42–331)
ALT (IU/l)[a]	188 (24–892)
ALT / ULN[a]	3.8 (0.5–17.8)
Total bilirubin (mg/dl)[a]	0.8 (0.3–15.5)
Serum creatinine (mg/dl)[a]	0.8 (0.5–1.2)

HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; AST, aspartate transaminase; ALT, alanine aminotransferase; ULN, upper limit of normal; T-bil, total bilirubin
[a]Median (range)
[b]I → YIDD, V → YVDD, I + V → YIDD + YVDD mix

Cumulative rate of HBeAg loss and seroconversion

At baseline, 42 of 63 patients (66.7%) were HBeAg-positive. Among the 42 HBeAg-positive patients, the cumulative rates of HBeAg loss were 7.3% after 24 weeks, 19.5% after 48 weeks, and 29.7% after 72 weeks (Fig. 1). In this same group, the cumulative rates of HBeAg seroconversion (HBeAg-negative to anti-HBe-positive) were 4.9% after 24 weeks, 9.8% after 48 weeks, and 14.8% after 72 weeks (Fig. 2).

Patients were categorized into two groups by serum ALT levels at baseline: ALT levels ≥ 2 × ULN in one group and ALT levels < 2 × ULN in the other. The rates of HBe loss in the ALT ≥ 2 × ULN group were significantly higher than those in the ALT < 2 × ULN group ($P = 0.0145$) (Fig. 1). The rates of HBeAg seroconversion in the ALT ≥ 2 × ULN group were higher than those in the ALT < 2 × ULN group ($P = 0.1065$) (Fig. 2). No patient experienced a reappearance of HBeAg or reverse seroconversion to HBeAg-positive status. Patients who achieved HBeAg loss or seroconversion had a sustained response during this treatment.

Factors associated with early virological response after 24 weeks

Univariate analysis of individual baseline factors showed that the baseline ALT level ($P = 0.001$) and HBeAg status ($P = 0.040$) were each predictive factors of early virological response. There was no association with the other factors: patient age, sex, HBV DNA level, YMDD mutant status, HBV genotype, or the presence of cirrhosis. As shown in Table 3, multivariate analysis revealed that baseline ALT level ($P = 0.003$) and HBeAg status ($P = 0.049$) were associated with early virological response.

Table 2. Undetectable rate of HBV-DNA by Amplicor monitor assay and normalized rates of ALT levels

		24 weeks	48 weeks	72 weeks
Undetectable rate of HBV-DNA	HBeAg-positive	40.5% (17/42)*	50.0% (21/42)**	51.3% (19/37)***
	HBeAg-negative	66.7% (14/21)*	85.7% (18/21)**	95.2% (20/21)***
	Overall	49.2% (31/63)	61.9% (39/63)	67.2% (39/58)
Normalized rates of ALT	HBeAg-positive	73.8% (31/42)	76.2% (32/42)	89.2% (33/37)
	HBeAg-negative	76.2% (16/21)	90.5% (19/21)	100% (21/21)
	Overall	73.0% (47/63)	81.0% (51/63)	93.1% (54/58)

*$P = 0.032$
**$P = 0.006$
***$P < 0.001$

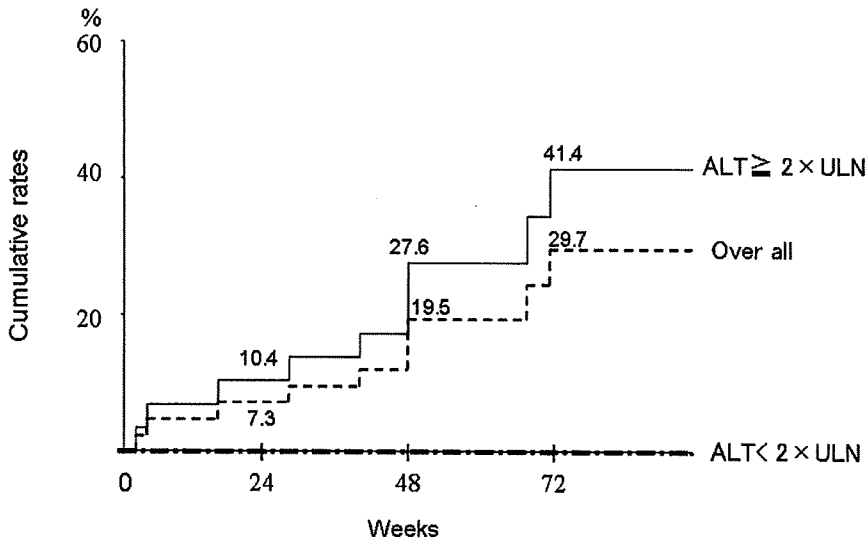

Fig. 1. Overall cumulative rate of hepatitis B e antigen loss during combination therapy with adefovir dipivoxil plus lamivudine: overall (*broken line*), alanine aminotransferase (*ALT*) greater than or equal to twice the upper limit of normal (*ULN*; *thick line*), and ALT less than twice the ULN (*dotted line*)

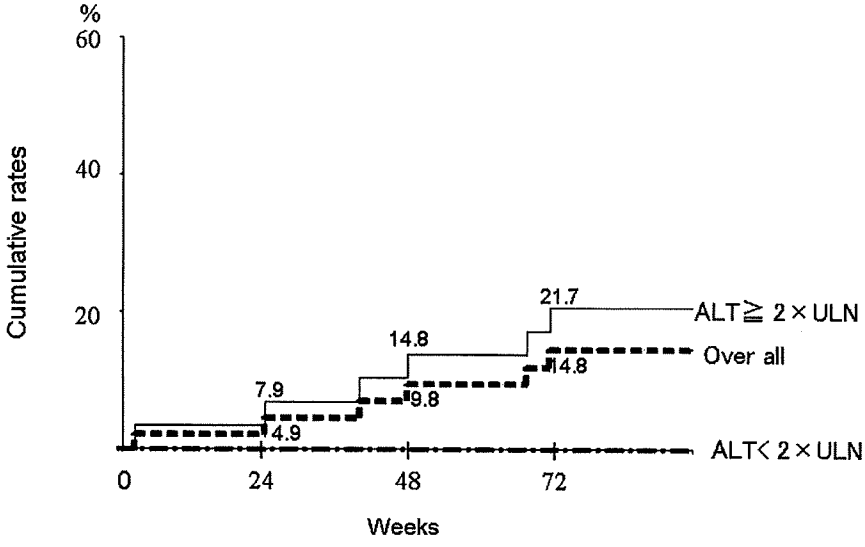

Fig. 2. Cumulative rate of hepatitis B e antigen seroconversion during combination therapy with adefovir dipivoxil plus lamivudine: overall (*broken line*), ALT greater than or equal to twice the ULN (*thick line*), and ALT less than twice the ULN (*dotted line*)

Table 3. Multivariate analysis of factors associated with early virological response[a] at week 24

Factors	Category	Odds ratio	95% CI	P^b
Baseline ALT	1: <2 × ULN	1		
	2: ≥ 2 × ULN	8.924	2.131–37.38	0.003
Baseline HBeAg status	1: negative	1		
	2: positive	0.300	0.091–0.993	0.049

CI, confidence interval
[a] Early virologic response: undetectable by Amplicor monitor assay
[b] *P* value by logistic regression analysis

Figure 3 shows the change in median serum HBV DNA level by baseline ALT level and HBeAg status. Patients were categorized into four groups: group A, ALT < 2 × ULN and HBeAg+; group B, ALT < 2 × ULN and HBeAg–; group C, ALT ≥ 2 × ULN and HBeAg+; and group D, ALT ≥ 2 × ULN and HBeAg–. Median values of the baseline HBV DNA level were 7.4 log copies/ml in group A, 7.4 log copies/ml in group B, 7.3 log copies/ml in group C, and 7.1 log copies/ml in group D. Although the HBV DNA level in group A declined similarly to levels in the other three groups during the first 4 weeks, the HBV DNA level in group

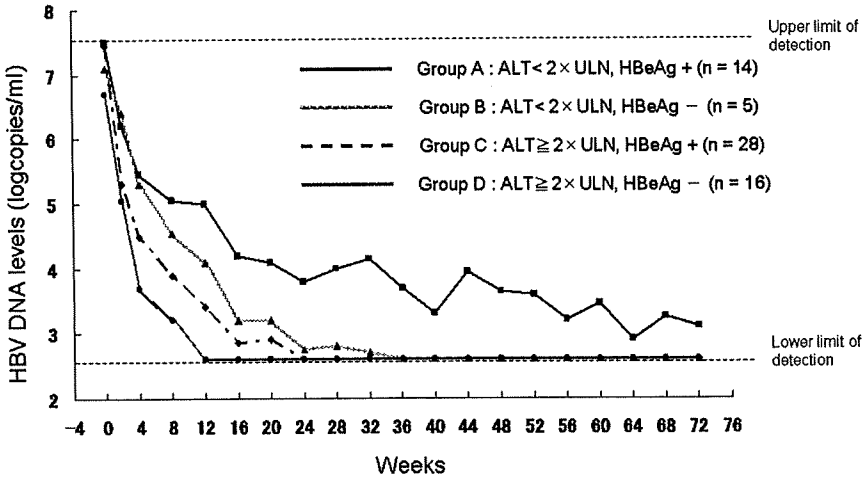

Fig. 3. Median values of hepatitis B virus DNA level by baseline ALT level and hepatitis B e antigen (*HBeAg*) status: group A (*thick line*), group B (*light gray line*), group C (*broken line*), and group D (*dark gray line*)

A decreased more slowly than in the other three groups over the first 4 weeks. The median values of the HBV DNA levels after 12 weeks were 5.0 log copies/ml in group A, 4.1 log copies/ml in group B, 3.4 log copies/ml in group C, and 2.6 log copies/ml in group D. The median values of the HBV DNA levels after 24 weeks were 3.8 log copies/ml in group A, 2.8 log copies/ml in groups B, and 2.6 log copies/ml in groups C and D.

Biochemical response

The median values of serum ALT level at baseline were 170 IU/l (3.4 × ULN) in HBeAg-positive patients and 184 IU/l (3.7 × ULN) in HBeAg-negative patients. In the first 12 weeks, there was an initial rapid reduction in serum ALT level of 124 IU/l in HBeAg-positive patients and 141 IU/l in HBeAg-negative patients. The median values of serum ALT level declined to less than the ULN after 12 weeks in HBeAg-positive patients and after 8 weeks in HBeAg-negative patients. These values remained less than the ULN thereafter.

Discussion

Previous studies have shown that HBeAg seroconversion induced by interferon therapy is associated with pretherapy ALT levels.[22] Some previous lamivudine studies also showed that an initially high ALT level was a predictor of sustained HBeAg seroconversion and/or HBV DNA suppression.[3,23–25] In this combination therapy of ADV plus lamivudine for the treatment of breakthrough hepatitis resulting from lamivudine-resistant mutants, our results also indicated that the pretherapy ALT level was associated with HBeAg seroconversion and serum HBV DNA suppression. In particular, rates of early virological response in the first 24 weeks showed a direct relation with baseline serum ALT levels. This observation may suggest that the host's endogenous immune response and the potent direct antiviral activity of ADV also caused the marked decline in serum HBV DNA level against lamivudine-resistant HBV.

The current study showed encouraging results regarding the efficacy of combination therapy with ADV plus lamivudine for the treatment of lamivudine-resistant HBV. However, two previous studies reported that the rates of HBV DNA negativity demonstrated by PCR assay were 20%–35% and serum ALT level normalization rates were 37%–61% after 48–52 weeks.[17,18] However, in this study, the overall rate of HBV DNA negativity was 61.9% and that of serum ALT level normalization was 81.0% after 48 weeks. The differences in these results seem to have been caused by a difference in baseline ALT levels; the median value of baseline serum ALT level in this study (188 IU/l) was higher than those in previous studies (74–94.5 IU/l). On the other hand, the rates of HBeAg loss were 19.5% in this study and 15–17% in previous studies.[17,18] With respect to the rates of HBeAg loss, our results are similar to previous results. Although baseline serum ALT level was associated with HBeAg loss and marginally associated with HBeAg seroconversion by the current univariate analysis (Fig. 2), there was no independent factor associated with HBeAg loss or seroconversion, including baseline serum ALT level, by multivariate analysis (data not shown). We considered that baseline ALT levels were more associated with a decrease of HBV DNA levels than with HBeAg loss or seroconversion because the rates of HBeAg loss were similar but the rates of HBV DNA negativity and ALT normalization were different between this study and previous studies.

On the basis of the current results, we consider that the favorable timing of ADV added to ongoing lamivu-

dine treatment for lamivudine-resistance is ALT elevation of more than 2 × ULN in patients without cirrhosis. Although starting ADV when serum ALT level is <2 × ULN is not too favorable in terms of subsequent sustained HBV DNA suppression and HBeAg seroconversion, ADV treatment should be begun when low-grade elevation of ALT levels is continuous. However, if decompensation is present and bilirubin is elevated, treatment should be begun immediately. In patients with cirrhosis, the timing of the start of ADV should be earlier than in those without cirrhosis because decompensation is sometimes present before ALT levels elevate to >2 × ULN. This concept supports the therapeutic recommendations of several consensus panels for the treatment of chronic hepatitis B.[26–28]

It was recently reported in multicenter studies from Asia and Japan that prolonged lamivudine treatment might delay disease progression and reduce the risk of hepatocellular carcinoma in cases of chronic hepatitis B.[29,30] Our colleagues indicated that persistently low HBV DNA levels might save patients from hepatocellular carcinogenesis in cases of HBV-related cirrhosis.[31] Some previous reports indicated that long-term lamivudine treatment led to a favorable prognosis if YMDD mutants did not emerge.[24,32,33] These reports support the idea that sustained HBV DNA suppression and serum ALT level normalization benefit patients. At present, many patients with chronic hepatitis B worldwide are taking lamivudine. Although YMDD mutants may emerge and cause biochemical relapse in patients who receive ongoing prolonged lamivudine, starting ADV at the earliest indication of virological and biochemical relapse and continuing to suppress HBV replication may extend the favorable prognosis in patients with YMDD mutants. The current results suggested the optimal timing of ADV addition to ongoing lamivudine treatment.

Combination therapy of ADV plus lamivudine was well tolerated in the 72 weeks of the current study. Other studies have already shown that 10 mg/day ADV is safe for a 1-year treatment period.[17,18] In the present study, no patient discontinued this treatment because of adverse events. It was recently reported that nephrotoxicity, as defined by an increase in serum creatinine level of at least 0.5 mg/dl from baseline or a serum phosphorus value of less than 1.5 mg/dl on two consecutive occasions, was not observed in patients treated with 10 mg/day ADV for a median follow-up period of approximately 64 weeks, but mild nephrotoxicity was demonstrated with the dose of 30 mg/day.[34] A major advantage of ADV is the very low emergence rate of drug-resistant mutants.[35] In the current study, drug resistance was not observed. The remaining issue regarding ADV is its long-term safety.

In summary, the current study shows that the baseline serum ALT level is associated with HBeAg loss and seroconversion and that the baseline serum ALT level and HBeAg status are associated with the virological response of lamivudine-resistant HBV during combination therapy with ADV plus lamivudine. The current findings may enhance the treatment of virological and biochemical relapse resulting from the emergence of YMDD mutants during prolonged lamivudine treatment.

References

1. Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HWL, Goodman Z, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1998;339:61–8.
2. Doong SL, Tsia CH, Schinazi RF, Liotta DC, Cheng YC. Inhibition of the replication of hepatitis B virus in vitro by 2′,3′-dideoxy-3′thiacytidine and related analogues. Proc Natl Acad Sci USA 1991;88:8495–9.
3. Liaw YF, Leung NWY, Chang TT, Guan R, Tai DI, Ng KY, et al. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Gastroenterology 2000;119:172–80.
4. Dienstage JL, Goldin RD, Heathcote EJ, Hann HW, Woessner M, Stephenson SL, et al. Histological outcome during long-term lamivudine therapy. Gastroenterology 2003;124:105–17.
5. Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, et al. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J Hepatol 1999;30:743–8.
6. Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM, et al. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. Hepatology 1996;3:711–3.
7. Nierters HG, Honkoop P, Haagsma EB, De Man RA, Schalm SW, Osterhaus AD. Identification of more than one mutation in the hepatitis B virus polymerase gene arising during prolonged lamivudine therapy. J Infect Dis 1998;177:1382–5.
8. Tipples GA, Ma MM, Fischer KP, Bain VG, Keneteman NM, Tyrrell DL. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 1996;3: 714–7.
9. Nafa S, Ahmed S, Tavan D, Pichouc C, Breby F, Stuyver L, et al. Early detection of viral resistance by determination of hepatitis B virus polymerase mutations in patients treated by lamivudine for chronic hepatitis B. Hepatology 2000;32:1078–88.
10. Suzuki F, Suzuki Y, Tsubota A, Akuta N, Someya T, Kobayashi M, et al. Mutations of polymerase, precore and core promoter gene in hepatitis B virus during 5-year lamivudine therapy. J Hepatol 2002;37:824–30.
11. Balzarini J, Naesens L, Herdewijn P, Rosenberg I, Holy A, Pouwels R, et al. Marked in vivo antiretrovirus activity of 9-(2-phosphonyl-methoxyethyl)adenine, a selective anti-human immunodeficiency virus agent. Proc Natl Acad Sci USA 1989; 86:332–6.
12. De Clercq E. New acquisitions in the development of anti-HIV agents. Antiviral Res 1989;12:1–19.
13. De Clercq E. Therapeutic potential of phosphonylmethoxyalkylpirines and pyrimidines as antiviral agents. Drug Exp Clin Res 1990;16:319–26.
14. Lin JC, De Clercq E, Pagano JS. Novel acyclic adenosine analogs inhibit Epstein-Barr virus replication. Antimicrob Agents Chemother 1987;31:1431–3.
15. Xiong K, Flores C, Yang H, Toole JJ, Gibbs CS. Mutations in hepatitis B DNA polymerase associated with resistance to lamivudine do not confer resistance to adefovir in vitro. Hepatology 1998;28:1669–73.

16. Gilson RJC, Chopra KB, Newell AM, Murray-Lyon IM, Nelson MR, Rice SJ, et al. A placebo-controlled phase I/II study of adefovir dipivoxil in patients with chronic hepatitis B virus infection. J Viral Hepatitis 1999;6:387–95.
17. Perrillo R, Hann HW, Mutimer D, Willems B, Leung N, Lee WM, et al. Adefovir dipivoxil added to ongoing lamivudine in chronic hepatitis B with YMDD mutant hepatitis B virus. Gastroenterology 2004;126:81–90.
18. Peters MG, Hann HW, Martin P, Heathcote EJ, Buggisch P, Rubin R, et al. Adefovir dipivoxil alone or in combination with in patients with lamivudine-resistant chronic hepatitis B. Gastroenterology 2004;126:91–101.
19. Hosaka T, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, Someya T, et al. Adefovir dipivoxil for treatment of breakthrough hepatitis caused by lamivudine-resistant mutants of hepatitis B virus. Intervirology 2004;47:362–9.
20. Chayama K, Suzuki Y, Kobayashi M, Kobayashi M, Tsubota A, Hashimoto M, et al. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild-type after cessation of therapy. Hepatology 1998;27:1711–6.
21. Usuda S, Okamoto H, Imawari H, Baba K, Tsuda F, Miyakawa Y, et al. Serological detection of hepatitis B virus genotype by ELIZA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Method 1999;80:97–112.
22. Hoofnagle JH, Di Bisceglie AM. The treatment of chronic viral hepatitis. N Engl J Med 1997;336:347–56.
23. Chien RN, Liaw YF, Atkins M, for the Asian Hepatitis Lamivudine Trial Group. Pretherapy alanine transaminase level as a determinant for hepatitis B e antigen seroconversion during lamivudine therapy in patients with chronic hepatitis B. Hepatology 1999;30:770–4.
24. Leung NWY, Lai CL, Chang TT, Guan R, Lee CM, Ng KY, et al., on behalf of the Asia Hepatitis Lamivudine Study Group. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology 2001;33:1527–32.
25. Perrillo RP, Lai CL, Liaw YF, Dienstag JL, Schiff ER, Schalm SW, et al. Predictors of HBeAg loss after lamivudine treatment for chronic hepatitis B. Hepatology 2002;36:186–94.
26. De Franchis R, Hadengue A, Lau G, Lavanchy D, Lok A, McIntyre N, et al. EASL international consensus conference on hepatitis B. J Hepatol 2003;39 Suppl 1:S3–25.
27. Liaw YF, Leung N, Guan R, Lau GK, Merican I. Asia-Pacific consensus statement on the management of chronic hepatitis B: an update. J Gastroenterol Hepatol 2003;18:239–45.
28. Lok ASF, McMahon BJ. Chronic hepatitis B: update of recommendations. Hepatology 2004;39:857–61.
29. Liaw YF, Sung JJY, Chow WC, Farrell G, Lee CZ, Yuen H, et al. Cirrhosis Asian Lamivuidne Multicentre Study Group. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 2004;51:1521–31.
30. Matsumoto A, Tanaka E, Rokuhara A, Kiyosawa K, Kumada H, Omata M, et al. Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: A multicenter retrospective study of 2795 patients. Hepatol Res 2005;32:173–84.
31. Ikeda K, Arase Y, Kobayashi M, Someya T, Saitoh S, Suzuki Y, et al. Consistently low hepatitis B virus DNA saves patients from hepatocellular carcinogenesis in HBV-related cirrhosis. Intervirology 2003;46:96–104.
32. Akuta N, Suzuki F, Suzuki Y, Sezaki H, Hosaka T, Someya T, et al. Favorable efficacy of long-term lamivudine therapy in patients with chronic hepatitis B: a 8-year follow-up study. J Med Virol 2005;75:491–8.
33. Lok ASF, Lai CL, Leung N, Yao GB, Cui ZY, Schiff ER, et al. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. Gastroenterology 2003;125:1714–22.
34. Izzedine H, Hulot JS, Launay-Vacher V, Marcellini P, Hadziyannis SJ, Currie G, et al. Adefovir Dipivoxil International 437 Study Group. Renal safety of adefovir dipivoxil in patients with chronic hepatitis B: two double-blind, randomized, placebo-controlled studies. Kidney Int 2004;66:1153–8.
35. Angus P, Vaughan R, Xiong S, Yang H, Delaney W, Gibbs C, et al. Resistance to adefovir dipivoxil therapy associated with selection of a novel mutation in the HBV polymerase. Gastroenterology 2003;125:292–7.