

TABLE III. Factors Associated With Rapid Virological Response to 48-Week Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV genotype1b, Identified by Multivariate Analysis

| Factor | Category | Odds ratio (95% CI) | P |
|---|--------------------------|---------------------|-------|
| Amino acid substitution in core region ^a | (1) Non-double wild-type | 1 | 0.001 |
| | (2) Double wild-type | 7.692 (2.421–24.39) | |
| Total cholesterol (mg/dl) | (1) <170 | 1 | 0.003 |
| | (2) ≥170 | 5.459 (1.768–16.86) | |
| Age (years) | (1) ≥55 | 1 | 0.042 |
| | (2) <55 | 3.165 (1.044–9.615) | |

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

^aThe pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild-type, and the other patterns as non-double wild-type.

gender (male sex; $P < 0.001$), AFP ($<11 \mu\text{g/L}$; $P < 0.001$), stage of fibrosis (F1; $P = 0.001$), leukocyte count ($\geq 4,500/\text{mm}^3$; $P = 0.001$), age (<55 years; $P = 0.002$), aa substitutions of the HCV core region (double wild-type; $P = 0.003$), PEG-IFN dose/body weight ($\geq 1.25 \mu\text{g/kg}$; $P = 0.003$), AST ($<60 \text{ IU/L}$; $P = 0.006$), platelet count ($\geq 15 \times 10^4/\text{mm}^3$; $P = 0.012$), UA ($\geq 7.0 \text{ mg/dl}$; $P = 0.029$), Hb ($\geq 14.0 \text{ g/dl}$; $P = 0.032$), serum albumin ($\geq 3.9 \text{ g/dl}$; $P = 0.056$), RBV dose/body weight ($\geq 11.0 \text{ mg/kg}$; $P = 0.059$), and viremia level ($<1,000 \text{ KIU/ml}$; $P = 0.084$). Multivariate analysis that included the above variables identified six parameters that independently influenced the sustained virological response: aa substitutions of the HCV core region (double-wild-type; $P = 0.001$), gender (male sex; $P = 0.002$), AFP ($<11 \mu\text{g/L}$; $P = 0.005$), leukocytes ($\geq 4,500/\text{mm}^3$; $P = 0.011$), ribavirin dose ($\geq 11.0 \text{ mg/kg}$; $P = 0.029$), and age (<55 years; $P = 0.030$). Especially, aa substitutions of the HCV core region (double-wild-type), gender (male sex), and AFP ($<11 \mu\text{g/L}$) were three parameters the increased the likelihood of sustained virological response fivefold or more (Table VI).

Predictors of Sustained Virological Response in Patients Who Achieved Early Virological Response as Determined by Univariate and Multivariate Analyses

Univariate analysis identified ten parameters that influenced the sustained virological response in patients who were able to achieve early virological response: gender (male sex; $P < 0.001$), stage of fibrosis (F1; $P = 0.002$), AST ($<60 \text{ IU/L}$; $P = 0.014$), age (<55 years; $P = 0.015$), leukocyte count ($\geq 4,500/\text{mm}^3$; $P = 0.020$), PEG-IFN dose/body weight ($\geq 1.25 \mu\text{g/kg}$; $P = 0.025$),

viremia level ($<1,000 \text{ KIU/ml}$; $P = 0.027$), AFP ($<11 \mu\text{g/L}$; $P = 0.057$), and Hb ($\geq 14.0 \text{ g/dl}$; $P = 0.058$). Multivariate analysis that included the above variables identified four parameters that independently influenced the sustained virological response of patients who achieved early virological response: gender (male sex; $P = 0.001$), stage of fibrosis (F1; $P = 0.002$), leukocyte count ($\geq 4,500/\text{mm}^3$; $P = 0.020$), and aa substitutions of the HCV core region (double wild-type; $P = 0.025$). Especially, gender (male sex) and stage of fibrosis (F1) were the two parameters that increased fivefold or more the chance for sustained virological response among the patients who achieved early virological response (Table VII).

Comparison of Factors Associated With Each of Treatment Efficacy Identified by Multivariate Analysis

Table VIII shows the variables that achieved excellent statistical significance ($P < 0.01$ and Odds ratio >5.0) on multivariate logistic regression for each evaluation of treatment efficacy. With regard to viral factors, the HCV core region was the most important predictor of rapid and early virological responses, end-of treatment response, and sustained virological response. For the host factors, lipid metabolic factors including LDL-C and TC were the two most important predictors of rapid and early virological responses as well as end-of treatment response. Thus, the HCV core region and lipid metabolic factors were important predictors of viral kinetics and treatment efficacy. Furthermore, sex and AFP were also identified as other important predictors of sustained virological response, in addition to viral and lipid metabolic factors.

TABLE IV. Factors Associated With Early Virological Response to 48-Week Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV Genotype1b, Identified by Multivariate Analysis

| Factor | Category | Odds ratio (95% CI) | P |
|---|--------------------------|---------------------|-------|
| Amino acid substitution in core region ^a | (1) Non-double wild-type | 1 | 0.001 |
| | (2) Double wild-type | 10.20 (2.674–38.46) | |
| LDL cholesterol (mg/dl) | (1) <86 | 1 | 0.002 |
| | (2) ≥86 | 5.844 (1.911–17.87) | |
| Level of viremia (KIU/ml) | (1) <2,000 | 1 | 0.027 |
| | (2) ≥2,000 | 3.359 (1.147–9.833) | |

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

^aThe pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild-type, and the other patterns as non-double wild-type.

TABLE V. Factors Associated With End-of Treatment Response to 48-Week Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV Genotype1b, Identified by Multivariate Analysis

| Factor | Category | Odds ratio (95% CI) | P |
|---|--------------------------|---------------------|--------|
| Amino acid substitution in core region ^a | (1) Non-double wild-type | 1 | <0.001 |
| | (2) Double wild-type | 17.86 (4.132–76.92) | |
| LDL cholesterol (mg/dl) | (1) <86 | 1 | 0.004 |
| | (2) ≥86 | 6.803 (1.859–25.00) | |
| Alpha-fetoprotein (μg/L) | (1) ≥11 | 1 | 0.007 |
| | (2) <11 | 5.525 (1.608–18.87) | |
| Level of viremia (KIU/ml) | (1) <2,000 | 1 | 0.012 |
| | (2) ≥2,000 | 4.098 (1.359–12.35) | |
| Uric acid (mg/dl) | (1) <7.0 | 1 | 0.020 |
| | (2) ≥7.0 | 9.259 (1.414–58.82) | |

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

^aThe pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild-type, and the other patterns as non-double wild-type.

DISCUSSION

Using multivariate analysis, the present study identified viral- (HCV core region) and host-related factors (lipid metabolism and sex) that influenced the rapid and early virological responses to PEG-IFN-RBV combination therapy. The same analysis also identified male sex, stage of fibrosis, leukocyte count, and aa substitutions of the HCV core region as determinants of sustained virological response of patients who achieved early virological response. Identification of these viral and host factors in the early period of PEG-IFN-RBV therapy (4–12 weeks) should help design better therapeutic regimens for those patients who are less likely to achieve sustained virological response.

It was reported previously that substitutions of aa 70 and/or 91 in the HCV core region is an independent and significant predictor of the response to treatment [Akuta et al., 2005, 2006, 2007a,b]. Based on a larger number of patients, the present study also identified aa substitutions in the HCV core region as a predictor of the response to PEG-IFN-RBV therapy. Previous studies reported that the HCV core region might be associated with resistance to IFN monotherapy involving the Jak-STAT signaling cascade [Blindenbacher et al., 2003; Bode et al., 2003; Melén et al., 2004; de Lucas et al.,

2005]. The result could be also interpreted to mean that aa substitutions in the HCV core region is associated with those proteins involved in resistance to IFN monotherapy, such as SOCS proteins, which is known to inhibit IFN- α -induced activation of the Jak-STAT pathway and expression of the antiviral proteins 2',5'-OAS and MxA [Voltides et al., 2004]. Furthermore, the result also indicates that aa substitutions in the HCV core region 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.

It was shown previously that LDL-C is an independent and significant predictor of the response to PEG-IFN-RBV therapy [Akuta et al., 2007a]. The present study also identified lipid metabolic factors including LDL-C or TC as predictors of the treatment response, and is in agreement with similar findings of a recent study [Gopal et al., 2006]. 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 [Agnello et al., 1999; Andre et al., 2002]. Furthermore, there is evidence that intracellular cholesterol level modulates LDLr expression, and thus a

TABLE VI. Factors Associated With Sustained Virological Response to 48-Week Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV Genotype1b, Identified by Multivariate Analysis

| Factor | Category | Odds ratio (95% CI) | P |
|---|--------------------------|---------------------|-------|
| Amino acid substitution in core region ^a | (1) Non-double wild-type | 1 | 0.001 |
| | (2) Double wild-type | 5.988 (2.070–17.24) | |
| Sex | (1) Female | 1 | 0.002 |
| | (2) Male | 5.882 (1.901–18.18) | |
| Alpha-fetoprotein (μg/L) | (1) ≥11 | 1 | 0.005 |
| | (2) <11 | 7.576 (1.828–31.25) | |
| Leukocytes (/mm ³) | (1) <4,500 | 1 | 0.011 |
| | (2) ≥4,500 | 4.031 (1.374–11.83) | |
| Ribavirin dose (mg/kg) | (1) <11.0 | 1 | 0.029 |
| | (2) ≥11.0 | 3.156 (1.128–8.825) | |
| Age (years) | (1) ≥55 | 1 | 0.030 |
| | (2) <55 | 3.125 (1.120–8.696) | |

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

^aThe pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild-type, and the other patterns as non-double wild-type.

TABLE VII. Factors Associated With Sustained Virological Response in Patients Who Achieved Early Virological Response to 48-Week Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV Genotype1b, Identified by Multivariate Analysis

| Factor | Category | Odds ratio (95% CI) | P |
|---|--------------------------|---------------------|-------|
| Gender | (1) Female | 1 | 0.001 |
| | (2) Male | 7.576 (2.179–26.32) | |
| Stage of fibrosis | (1) F2,3,4 | 1 | 0.002 |
| | (2) F1 | 6.944 (2.058–23.26) | |
| Leukocytes (/mm ³) | (1) <4,500 | 1 | 0.020 |
| | (2) ≥4,500 | 4.432 (1.260–15.58) | |
| Amino acid substitution in core region ^a | (1) Non-double wild-type | 1 | 0.025 |
| | (2) Double wild-type | 4.000 (1.190–13.51) | |

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

^aThe pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild-type, and the other patterns as non-double wild-type.

high LDL-C could downregulate LDLr and diminishes the spread of hepatocyte HCV infection. Thus, the correlation between treatment efficacy and lipid metabolic factors may be explained by the role of LDL-C in transporting the HCV-LDL complex into the hepatocyte. 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 [Netea et al., 1996] and antiviral cellular immune response [Muldoon et al., 1997; Ludewig et al., 2001]. Further studies of a large number of patients are required to explore the relationship between various lipid metabolic factors and the response to PEG-IFN-RBV therapy.

The results showed that higher levels of AFP are a negative predictor of the response to PEG-IFN-RBV therapy. Previous data indicated that absence of advanced liver fibrosis is a positive predictor of sustained virological response to IFN monotherapy and IFN-RBV dual therapy [Jouet et al., 1994; Poynard et al., 2000; Bruno et al., 2004], and that advanced liver fibrosis is usually associated with elevation of serum AFP [Bayati et al., 1998; Chu et al., 2001; Hu et al., 2004]. We showed previously that high rates of ICG R15 or low levels of serum albumin are also associated with advanced liver fibrosis, and that they are independent and significant negative predictors of the response to PEG-IFN-RBV [Akuta et al., 2005, 2007a]. To our knowledge, this is the first report that describes the relationship between serum AFP levels and the response to PEG-IFN-RBV therapy. Further studies of

large number of patients are required to explore the most important indicator of histopathological changes in the liver (including stage of fibrosis, ICG R15, albumin, and AFP), and to investigate the relationship between severity of histopathological changes and response to the combination therapy.

Similar to recent findings [Akuta et al., 2007a], the present study also identified female sex as a negative predictor of sustained virological response to PEG-IFN-RBV therapy. It should be noted, however, that other studies also showed that male sex is also a negative predictor of sustained virological response to combination therapy [Poynard et al., 1998; Conjeevaram et al., 2006]. The discrepancy between our results and such findings may be explained by differences of age by sex. The age of the female patients in the present study (median; 60 years) was higher than that of the males (median; 51 years). Other mechanisms could be also explained by the small number of patients in our study, differences in host factors including sex [Schott et al., 2007] and race [McHutchison et al., 2000; Kaplan et al., 2005], and/or differences in viral factors, such as the distribution of genotype 1a or 1b, and geographic diversities of genotype 1b [Nakano et al., 1999]. Further studies of larger number of patients matched for age, race, and HCV genotype are required to explore the relationship between sex and the response to PEG-IFN-RBV therapy.

In this study, multivariate analysis identified sex and stage of fibrosis as parameters that independently influenced the sustained virological response of patients

TABLE VIII. Comparison of Factors Associated With Efficacy of 48-Week Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV Genotype1b, Identified by Multivariate Analysis

| Factor | Rapid virological response (at 4 weeks) | Early virological response (at 12 weeks) | End-of treatment response (at 48 weeks) | Sustained virological response |
|------------------|--|--|--|---|
| Virus | Core region $P = 0.001, 7.69 (2.42–24.4)^a$ | Core region $P = 0.001, 10.2 (2.67–38.5)^a$ | Core region $P < 0.001, 17.9 (4.13–76.9)^a$ | Core region $P = 0.001, 5.99 (2.07–17.2)^a$ |
| Lipid metabolism | Total cholesterol $P = 0.003, 5.46 (1.77–16.9)^a$ | LDL cholesterol $P = 0.002, 5.84 (1.91–17.9)^a$ | LDL cholesterol $P = 0.004, 6.80 (1.86–25.0)^a$ | |
| Others | | | Alpha-fetoprotein $P = 0.007, 5.53 (1.61–18.9)^a$ | Alpha-fetoprotein $P = 0.005, 7.58 (1.83–31.3)^a$ Gender $P = 0.002, 5.88 (1.90–18.2)^a$ |

Only variables that achieved excellent statistical significance ($P < 0.01$ and Odds ratio > 5.0) on multivariate logistic regression are shown.

^aOdds ratio (95% confidence interval).

who achieved early virological response, but these two factors were not identified as significant predictors of early HCV-RNA negativity during treatment. Interestingly, sex was not a predictor of rapid and early virological responses, end-of treatment response, or early HCV-RNA negativity when evaluated for treatment efficacy based on viral kinetics, but was remarkable significant predictor of sustained virological response to PEG-IFN-RBV. Thus, the underlying mechanisms of failure to develop a sustained virological response in those patients who show HCV-RNA negativity remain unclear. Further prospective studies of a large number of patients should be performed to determine whether it would be worth to extend the Peg-IFN-RBV therapy to 72 weeks in those patients who do not achieve sustained virological responses in spite of early virological response by 48 weeks therapy [Buti et al., 2003; Berg et al., 2006].

Pretreatment prediction of a sustained virological response to PEG-IFN-RBV therapy is still incomplete, and evaluation of treatment response based on viral kinetics is useful as early predictor of treatment efficacy. Especially, in this study, viral factors (e.g., aa substitutions in the HCV core region) and host factors (e.g., lipid metabolic factors, sex, and AFP) were important predictors of response to PEG-IFN-RBV therapy in Japanese patients infected with HCV genotype 1b, to say nothing of treatment-related factors (e.g., RBV dose) [Manns et al., 2001; Akuta et al., 2006, 2007a]. One limitation of our study was that we did not examine amino acid substitutions in areas other than the core region of HCV genome, such as the interferon sensitivity determining region (ISDR) and the interferon/ribavirin resistance determining lesion (IRRDR) of NS5A region, although they should be investigated in future studies [Enomoto et al., 1995, 1996; El-Shamy et al., 2007]. It is concluded that viral kinetics 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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Prediction of Response to Pegylated Interferon and Ribavirin in Hepatitis C by Polymorphisms in the Viral Core Protein and Very Early Dynamics of Viremia

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Key Words

Chronic hepatitis · Hepatitis C virus · HCV core ·
Pegylated interferon · Virological responses · Ribavirin

Abstract

Objective: To evaluate power of amino acid polymorphisms in core protein of hepatitis C virus (HCV) for predicting sustained virological response (SVR) to pegylated interferon (Peg-IFN)/ribavirin, when they were combined with virological response. **Methods:** Peg-IFN/ribavirin was given to 118 patients infected with HCV genotype 1b in high viral loads. Amino acid polymorphisms (Arg70 vs. Gln70 and Leu91 vs. Met91) in combination with on-treatment virological responses were correlated with SVR. **Results:** End-of-treatment response (ETR) was achieved in 71% and SVR in 47% of the 118 patients. In multivariate analysis, Arg70 and Leu91, and higher ribavirin dose were independently associated with ETR. In patients with Gln70 and/or Met91, SVR was more frequent in those with than without prompt virological response (PVR) for a decrease in viral load ≥ 1.0 log by 48 h.

Specificity in predicting patients without ETR and SVR, in combination with core polymorphisms, was not different between PVR and early virological response at 12 weeks. **Conclusion:** Core polymorphisms combined with PVR would be useful in promptly identifying the patients who will not respond to Peg-IFN/ribavirin, thereby avoiding unrewarding side effects and high costs. Copyright © 2007 S. Karger AG, Basel

Introduction

Worldwide, an estimated 300 million people are persistently infected with hepatitis C virus (HCV), and decompensated cirrhosis or hepatocellular carcinoma develop in 20–30% of them during the lifetime [1]. HCV is classified into six major genotypes named 1–6 that break down into many subtypes designated by lower-case letters [2]. Treatments based on interferon (IFN) have been developed to prevent morbidity and mortality caused by persistent HCV infection. Monotherapy with IFN started in 1986 [3], was followed by combined IFN and ribavirin [4, 5], and more recently, pegylated (Peg)-IFN plus ribavirin [6, 7]; they have achieved increasing efficacy in treatment of patients with chronic hepatitis C [8].

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HCV genotypes influence the response to IFN, of which genotype 1, as well as genotypes 4 and 6, is more resistant than genotypes 2 or 3 to antiviral treatments based on IFN [9, 10]. Response to IFN is influenced by viral loads, also [9, 10]. Thus, hepatitis C patients infected with genotype 1 in high viral loads are most resistant to IFN-based treatments. In Japan, more than half the patients with chronic hepatitis C are infected with HCV-1b ≥ 100 KIU/ml. Sustained virological response (SVR), defined by the loss of detectable HCV RNA 6 months after the treatment, to IFN monotherapy was dismal at merely 6%, but it increased to 20% with combined IFN and ribavirin [11]. Even by treatment with Peg-IFN and ribavirin, however, SVR has been achieved in 42–48% of the patients infected with HCV-1 in large-scale multicenter studies [6, 7]; the response would be lower in patients with HCV-1 in high viral loads [10].

Along with a high SVR, combined Peg-IFN and ribavirin accompany severe side effects and entail high costs. Hence, the patients with high-titer HCV-1 who do not achieve SVR need to be identified as early as possible, in order to free them of unnecessary side effects and high costs. Davis et al. [12] have proposed early virological response (EVR) at 12 weeks after treatment, defined by a reduction of HCV RNA by ≥ 2 logs, for predicting the lack of SVR to combined Peg-IFN and ribavirin. We have reported that polymorphisms of amino acid (aa) 70 of arginine or glutamine and aa 91 of leucine or methionine in the core protein are significantly associated with the lack of response to treatment with the standard or Peg-IFN combined with ribavirin [13–15]. An attempt was made in the present study for an earlier prediction of SVR in patients with HCV-1b ≥ 100 KIU/ml based on virological responses 48 h, 4 weeks and 12 weeks after the start of Peg-IFN plus ribavirin, when they were combined with Arg70 and Leu91 in the core protein.

Materials and Methods

Patients

During December 2001 through July 2005, 225 consecutive patients with chronic hepatitis C received combination therapy with Peg-IFN and ribavirin at the Department of Hepatology in Toranomon Hospital. The following inclusion criteria were met by 118 (52%) of them. They were: (1) positive for antibody to HCV (anti-HCV) and HCV RNA of genotype 1b by the qualitative method and not coinfecting with HCV of other genotypes; (2) negative for hepatitis B surface antigen (HBsAg) or antibody to human immunodeficiency virus type-1 (HIV-1); (3) confirmed for HCV RNA ≥ 100 KIU/ml within the past 2 months; (4) with body weight ≥ 40 kg and not pregnant or lactating; (5) with the total

alcohol intake < 500 g in the past; (6) without HCC, hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic hepatitis or autoimmune hepatitis; (7) naive to ribavirin and not received antivirals or immunosuppressants during the past 3 months, and (8) compliant to the treatment protocol for 24 weeks or longer and followed at least monthly.

All the 118 patients were followed at least 6 months after completion of combination therapy with Peg-IFN and ribavirin, for the purpose of identifying virological and biochemical factors predictive of SVR as soon as possible after it was started. The study design was approved by the Ethics Committee of Toranomon Hospital, and every patient gave an informed consent on the purpose of this study.

Serum Markers of Hepatitis Viruses

Anti-HCV was determined by the third-generation enzyme-linked immunosorbent assay (ELISA) by commercial kits (Ortho HCV Ab ELISA Test 3; Chiron Corp., Emeryville, Calif., USA). HCV RNA was determined quantitatively by polymerase chain reaction (PCR) (Cobas Amplicor HCV Monitor Version 2.0, Roche Diagnostics, Tokyo, Japan) in serum diluted 10-fold at the baseline as well as at least monthly during and after treatment; it has a dynamic range between 5 and 5,000 KIU/ml. Sera negative for HCV RNA (< 5 KIU/ml) by the quantitative assay were tested by qualitative PCR (Amplicor, Roche Molecular Systems, Calif., USA) with the detection limit of 100 copies/ml. HBsAg was determined by ELISA with commercial kits (F-HBsAg; Sysmex, Kobe, Japan).

Amino Acid Polymorphisms in the Core Protein

With use of HCV-J (accession No. D90208) as a reference [16], the sequence of 1–191 aa in the core protein of genotype 1b was determined, and it was compared with the consensus sequence constructed on 50 clinical samples [14] for detecting polymorphisms at aa 70 of arginine or glutamine/histidine and aa 91 of leucine or methionine. In the present study, the PCR methods with primers specific for polymorphisms at aa 70 or 91 were performed on 109 patients [17]; the remaining 9 patients were analyzed by direct sequencing [13, 14].

Combined Peg-IFN and Ribavirin Therapy

Patients received subcutaneous Peg-IFN- α_{2b} (PEG-Intron, Schering Corp, N.J., USA) weekly at the median dose of 1.5 μ g/kg, along with oral ribavirin daily at the median dose of 11.0 mg/kg for 48 weeks. The dose of ribavirin was adjusted by the body weight: 600 mg for the patients weighing ≤ 60 kg, 800 mg for those between > 60 kg and < 80 kg, and 1,000 mg for those ≥ 80 kg. It was tapered in the 37 patients in whom levels of hemoglobin decreased during the combination therapy.

Evaluation of Virological Responses

Therapeutic efficacy of combined Peg-IFN and ribavirin was evaluated by the disappearance of HCV RNA from serum detectable by qualitative PCR (< 100 copies/ml) at the end of treatment (ETR) and 6 months after the completion of therapy (SVR). Dynamics of on-treatment HCV was assessed by prompt virological response (PVR) defined by a decrease in HCV RNA by ≥ 1.0 log at 48 h, rapid virological response (RVR) by that of ≥ 2.0 log or disappearance at 4 weeks, and by that of > 2.0 log or disappearance at 12 weeks (EVR) after the combination therapy was initiated.

Table 1. Baseline characteristics of the 118 patients infected with HCV of genotype 1b who received combination therapy with Peg-IFN and ribavirin

| | |
|---|--|
| Demographic data | |
| Male | 76 (64%) |
| Age, years | 55 (30–70) |
| History of blood transfusion | 42 (36%) |
| Family history of liver disease | 36 (31%) |
| Body mass index, kg/m ² | 23.3 (17.6–31.2) |
| Laboratory data | |
| Leukocytes, /mm ³ | 4,800 (2,300–8,800) |
| Hemoglobin, g/dl | 14.6 (10.6–17.6) |
| Platelets, × 10 ⁴ /mm ³ | 17.2 (6.6–30.9) |
| Albumin, g/dl | 3.7 (3.0–4.5) |
| Alanine aminotransferase, IU/l | 83 (25–504) |
| Aspartate aminotransferase, IU/l | 59 (17–266) |
| γ-Glutamyl transpeptidase, IU/l | 62 (15–393) |
| Ferritin, μg/l | 154 (<10–927) |
| Iron, μg/dl | 147 (18–308) |
| Retention of indocyanine green at 15 min, % | 15 (4–49) |
| Creatinine clearance, ml/min | 99 (51–146) |
| Histological findings | |
| Fibrosis stage (F1/F2/F3/F4/ND) | 52 (44%)/28 (24%)/18 (15%)/1 (1%)/19 (16%) |
| Steatosis (none–mild/moderate–severe/ND) | 87 (74%)/11 (9%)/20 (17%) |
| HCV RNA, KIU/ml | 2,100 (100–>5,000) |
| Polymorphism of amino acids in the core protein | |
| Arg70 and Leu91 | 40 (34%) |
| Gln70 (His70) and/or Met91 | 78 (66%) |
| Treatment for 48 weeks | |
| Peg-IFN-α2b (≥1.25 μg/kg) | 90 (76%) |
| Ribavirin (≥11.0 mg/kg) | 57 (48%) |

Number of patients with percentage in parentheses or the median value with a range in parentheses are shown. ND = Not determined.

Statistical Analysis

Non-parametric variables were compared between groups by the χ^2 test, Fisher's exact probability test and Mann-Whitney U test. Uni- and multivariate analyses for factors influencing the response to combination therapy were performed by the χ^2 test and logistic regression, respectively.

Results

Factors Predictive of ETR to Combination Therapy

Table 1 lists demographic, biochemical and virological characteristics of the 118 patients at the baseline. They all were infected with HCV-1b in high titers with the median of 2,100 KIU/ml. More than half (66%) of them possessed Gln70 and/or Met91 in the core protein. A total of 22 factors in table 1 were evaluated for association with the response to combination treatment. Factors influencing ETR to combination therapy were evaluated by uni-

variate analysis (table 2). There were three factors with significant difference ($p < 0.05$) and one with marginal significance (p between 0.05 and 0.1). Among them, Gln70 and/or Met91 in the core protein influenced the response to combination therapy with the highest significance ($p < 0.001$), followed by γ -GTP and ribavirin dose. Multivariate analysis identified only two factors predictive of ETR (table 3). They were Arg70 and Leu91 (i.e., without Gln70 or Met91) in the core protein and ribavirin dose ≥ 11.0 mg/kg. They increased chances for ETR by 8.55- and 3.22-fold, respectively.

Predicting ETR and SVR by Core Polymorphisms in Combination with Early Dynamics of HCV

Based on a strong power of Arg70 and Leu91 in predicting ETR (table 3), it was evaluated how they increase the predictive value when they were combined with PVR, RVR and EVR. The results are schematically depicted in

Table 2. Factors associated with ETR to combined Peg-IFN and ribavirin for 48 weeks in 118 patients with HCV-1b by univariate analysis

| Factor | Category | ETR | p value ^a |
|----------------------------------|-------------------|-------------|----------------------|
| Gln70 and Met91 | 1: either or both | 60% (47/78) | <0.001 |
| | 2: none | 93% (37/40) | |
| γ-Glutamyl trans-peptidase, IU/l | 1: ≥110 | 50% (11/22) | 0.020 |
| | 2: <110 | 76% (73/96) | |
| Ribavirin dose, mg/kg | 1: <11.0 | 62% (38/61) | 0.041 |
| | 2: ≥11.0 | 81% (46/57) | |
| Leukocytes, /mm ³ | 1: <4,500 | 61% (28/46) | 0.061 |
| | 2: ≥4,500 | 78% (56/72) | |

^a Evaluated by χ^2 test.

Table 3. Factors associated with ETR to combined Peg-IFN and ribavirin for 48 weeks on 118 patients with HCV-1b in multivariate analysis

| Factor | Category | Odds ratio (95% CI) | p value ^a |
|----------------------|-------------------|---------------------|----------------------|
| Gln70 and Met91 | 1: either or both | 1 | 0.001 |
| | 2: none | 8.55 (2.34–31.26) | |
| Ribavirin dose mg/kg | 1: <11.0 | 1 | 0.013 |
| | 2: ≥11.0 | 3.22 (1.28–8.09) | |

^a Evaluated by logistic regression analysis.

figures 1–3, respectively. Together they demonstrate three points: (1) efficacy of combination therapy was high in the patients with Arg70 and Leu91 who accomplished ETR at 93% and SVR at 63%, irrespective of any virological response; (2) even in the patients having Gln70 and/or Met91, those achieving PVR (fig. 1), RVR (fig. 2) and EVR (fig. 3) gained high ETR (89–100%) and considerable SVR (48–78%), and (3) ETR (10–25%) and SVR (7–14%) were the worst in patients who possessed Gln70 and/or Met91 and failed to achieve PVR, RVR or EVR (differences significant in all comparisons).

Performance of Core Polymorphisms Combined with On-Treatment Virological Responses in Predicting Non-ETR and Non-SVR

Table 4 evaluates performances, in predicting the lack of ETR, of Gln70 and/or Met91 combined with on-treatment HCV RNA decreases at three time points: 48 h

(PVR), 4 weeks (RVR) and 12 weeks (EVR) after the start of therapy. They were largely comparable, except for the specificity that was lower for RVR than EVR (86 vs. 96%, $p = 0.049$). Likewise, the performance in predicting the lack of SVR by combined factors was evaluated in table 5. The sensitivity was lower for PVR than RVR (38 vs. 62%, $p = 0.026$).

Discussion

Some patients infected with HCV-1 do not respond to combined therapy with Peg-IFN and ribavirin. Lest they should suffer from unrewarding side effects and high costs through 48 weeks, it is necessary to predict the lack of SVR for timing the withdrawal of treatment in them (stopping rule). The most popular stopping rule is based on two large-scale multinational studies involving 348 and 296 patients, respectively [6, 7]. Accordingly, Davis [18] has proposed the prediction of SVR by a fall of HCV RNA by ≥ 2 logs or to undetectable levels 12 weeks after the start of therapy (EVR) as the optimal predictor of SVR. Since only 3 of the 187 (1.6%) patients without EVR established SVR, he recommends to discontinue combination therapy in the patients infected with HCV-1 who fail to gain EVR. By applying his stopping rule, treatment costs are reduced by <16% and side effects avoided in 19% of HCV-1 patients [18].

Previous works have not established the optimal time for predicting non-SVR in patients with HCV-1 who receive the 48-week Peg-IFN/ribavirin therapy. Patients who gain SVR have cleared HCV RNA from serum by 2 weeks after the treatment start [19]. HCV RNA decreased by 3 logs within the first 4 weeks of treatment in all patients who have accomplished SVR [20, 21]; decreases in HCV RNA were almost identical at 1 and 3 months, however [22]. It is reported that lowering HCV RNA levels during the first phase is essential for efficient elimination of HCV during the second phase [23]. Decreases in HCV RNA titers within the first 24–48 h after the start of IFN, therefore, would be dependable estimates on the antiviral efficacy [24–27]. Early HCV dynamics may differ between patients receiving Peg-IFN and standard IFN [28], because it takes 3 days before the maximum serum level is reached with Peg-IFN. In our previous study [29], however, decreases in HCV RNA levels were significantly greater in patients with than without SVR from 24 h to 12 weeks after the start of combined Peg-IFN and ribavirin; they all were infected with HCV-1b in high loads.

Fig. 1. ETR and SVR to combined Peg-IFN and ribavirin in patients with high-titer HCV-1b stratified by amino acid polymorphism and prompt virological response at 48 h for a decrease in HCV RNA by ≥ 1.0 log.

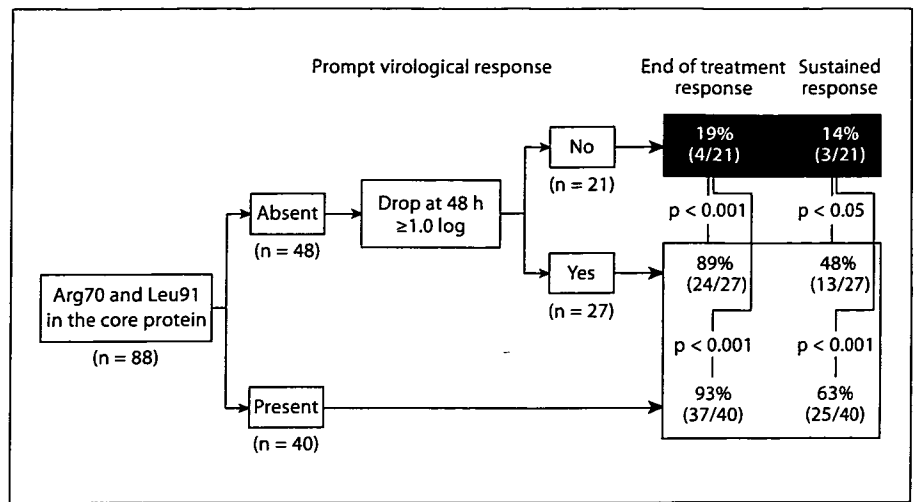


Fig. 2. ETR and SVR to combined Peg-IFN and ribavirin in patients with high-titer HCV-1b stratified by amino acid polymorphism and rapid virological response at 4 weeks for a decrease in HCV RNA by ≥ 2.0 logs.

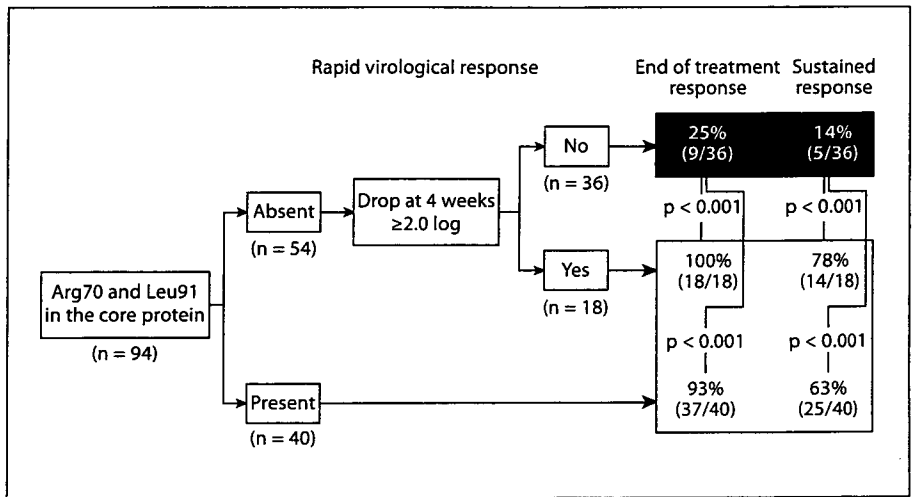


Fig. 3. ETR and SVR to combined Peg-IFN and ribavirin in patients with high-titer HCV-1b stratified by amino acid polymorphisms and early virological response at 12 weeks for a decrease in HCV RNA by ≥ 2.0 logs.

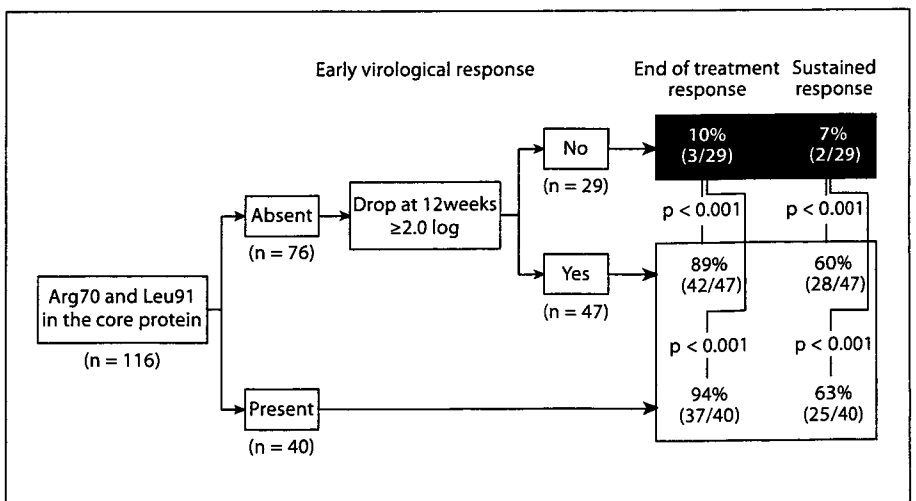


Table 4. Prediction of the lack of end-of-treatment response by polymorphism in the core protein combined with virological responses 48 h (PVR), 4 weeks (RVR) and 12 weeks (EVR) after the start of 48-week Peg-IFN and ribavirin in the patients with high-titer HCV-1b

| Performance | (1) Gln70 and/or Met91 plus non-response at 48 h (PVR [-]) (n = 88) | (2) Gln70 and/or Met91 plus non-response at 4 weeks (RVR [-]) (n = 94) | (3) Gln70 and/or Met91 plus non-response at 12 weeks (EVR [-]) (n = 116) | Differences (p value) ^a | | |
|------------------|---|--|--|------------------------------------|-------------|-------------|
| | | | | (1) vs. (2) | (2) vs. (3) | (1) vs. (3) |
| Sensitivity | 17/23 (74%) | 27/30 (90%) | 26/34 (76%) | NS | NS | NS |
| Specificity | 61/65 (94%) | 55/64 (86%) | 79/82 (96%) | NS | 0.049 | NS |
| Predictive value | | | | | | |
| Positive | 17/21 (81%) | 27/36 (75%) | 26/29 (90%) | NS | NS | NS |
| Negative | 61/67 (91%) | 55/58 (95%) | 79/87 (91%) | NS | NS | NS |

PVR = Prompt virological response; RVR = rapid virological response; EVR = early virological response; NS = not significant.

^a Evaluated by Fisher's exact probability test.

Table 5. Prediction of the lack of sustained virological response by polymorphism in the core protein combined with virological responses 48 h (PVR), 4 weeks (RVR) and 12 weeks (EVR) after the start of Peg-IFN and ribavirin in the patients with high-titer HCV-1b

| Performance | (1) Gln70 and/or Met91 plus non-response at 48 h (PVR [-]) (n = 88) | (2) Gln70 and/or Met91 plus non-response at 4 weeks (RVR [-]) (n = 94) | (3) Gln70 and/or Met91 plus non-response at 12 weeks (EVR [-]) (n = 116) | Differences (p value) ^a | | |
|------------------|---|--|--|------------------------------------|-------------|-------------|
| | | | | (1) vs. (2) | (2) vs. (3) | (1) vs. (3) |
| Sensitivity | 18/47 (38%) | 31/50 (62%) | 27/61 (44%) | 0.026 | NS | NS |
| Specificity | 38/41 (93%) | 39/44 (89%) | 53/55 (96%) | NS | NS | NS |
| Predictive value | | | | | | |
| Positive | 18/21 (86%) | 31/36 (86%) | 27/29 (93%) | NS | NS | NS |
| Negative | 38/67 (57%) | 39/58 (67%) | 53/87 (61%) | NS | NS | NS |

PVR = Prompt virological response; RVR = rapid virological response; EVR = early virological response; NS = not significant.

^a Evaluated by Fisher's exact probability test.

We have reported that polymorphisms of aa 70 and/or aa 91 in the core protein are associated with SVR in patients infected with HCV-1b in high loads (≥ 100 KIU/ml) and useful in predicting response to Peg-IFN and ribavirin prior to the institution of therapy [13, 14]. Both in uni- and multivariate analyses, Arg70 and Leu91 predicted ETR with the highest significance among 22 pretreatment variables in the present study (tables 2, 3); ribavirin dose ≥ 11 mg/kg was the only other factor predictive of ETR. A substantial weakness of Arg70 and Leu91 in predicting SVR, however, is their unsatisfactory sensitivity and specificity [13, 14]. Hence, Arg70 and Leu91 were combined with on-treatment virological responses in an attempt to achieve the maximum sensitivity and specificity in predicting SVR in the patients infected with high-titer HCV-1b.

As the results, PVR with a decrease in HCV RNA level ≥ 1.0 log by 48 h after the first dose of Peg-IFN and ribavirin, when it was combined with Arg70/Leu91, was highly specific in predicting SVR; it was comparable with RVR at 4 weeks or EVR at 12 weeks (93 vs. 89 or 96% (table 5)). Predicting SVR to Peg-IFN plus ribavirin in patients with high-titer HCV-1b, by combination with Arg70/Leu91 and PVR, would have clinical advantages. Patients with Arg70/Leu91 at the baseline can be continued aggressively on the 48-week combination therapy. Prediction of SVR would be particularly beneficial in the patients in old age or with hypertension [30], as well as those with insulin resistance [31, 32], who poorly respond to combination therapy. It would be particularly helpful in countries where HCC in the aged infected with HCV is coming to the fore [33, 34].

There are some theoretical bases for the association of polymorphisms in the HCV core protein with resistance to IFN. Both IFN- α and IFN- β bind to type-I IFN receptor, and one major pathway in type-I IFN signaling involves the signal transducer and activator of transcription (STAT) cascade [35]. Previous studies indicate possible association of HCV core region with the resistance to antiviral actions of IFN [36, 37]. Recently, de Lucas et al. [38] showed that the HCV core protein inhibits IFN- α -induced transcription of antiviral genes by reducing the binding of IFN-stimulated gene factor 3 to IFN-stimulated response element in HepG2 cells. Combined, polymorphisms of amino acids in the HCV core protein might be associated with resistance to the antiviral action of IFN therapy by influencing the Jak-STAT signaling cas-

cade. Further studies for the structural and functional impact of aa 70 and/or aa 91 in the core protein on combined IFN and ribavirin therapy are needed to verify this mechanism.

In conclusion, Arg70/Leu91 in combination with PVR at 48 h are predictive of SVR in the patients infected with high-titer HCV-1b. It would be useful for promptly lifting the burden of severe side effects and high costs from some of them who have virtually no chances of gaining benefit by continuing combination treatment, even before they receive the second dose of Peg-IFN. Insofar as Arg70/Leu91 and PVR act complementarily in predicting SVR, it would be useful to include these pre- and on-treatment predictors in the management of patients with HCV-1b in high loads.

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Successful Treatment of an Entecavir-Resistant Hepatitis B Virus Variant

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Emergence of a lamivudine (LAM)-resistant hepatitis B virus (HBV) with amino acid substitutions in the YMDD motif is a well-documented problem during long-term LAM therapy. Entecavir (ETV) is a new drug approved for treatment of HBV infection with or without LAM-resistant mutants. This report describes an ETV-resistant strain of HBV, which emerged after prolonged ETV therapy in a patient who did not respond to LAM therapy. Direct sequence analysis of the ETV-resistant strain showed appearance of amino acid substitution rtS202G in the reverse transcriptase (RT) domain, together with rtL180M + M204V substitution that had developed at the emergence of LAM-resistant mutant. In vitro analysis demonstrated that the rtL180M + M204V + S202G mutant strain displayed a 200-fold and a 5-fold reduction in susceptibility to ETV compared with the wild-type and the rtL180M + M204V mutant strain, respectively. Adefovir was effective against the ETV-resistant strain both in vitro and during the clinical course. In conclusion, this study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV and confirmed that the addition of rtS202G substitution to the rtL180M + M204V mutant strain is responsible for ETV resistance and we could treat the resistant mutant successfully. *J. Med. Virol.* 79:1811–1817, 2007. © 2007 Wiley-Liss, Inc.

KEY WORDS: HBV; rtS202G; lamivudine; adefovir; in vitro

INTRODUCTION

Hepatitis B virus (HBV) is a small enveloped DNA virus known to cause chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma [Bruix and Llovet, 2003; Ganem and Prince, 2004]. To date, interferon and three nucleoside and nucleotide analogs (lamivudine [LAM], adefovir dipivoxil [ADV], and entecavir [ETV]) have been approved for the treatment of chronic HBV infection. Nucleoside and nucleotide analogues suppress HBV replication in most patients and improve transaminase levels and liver histology [Nevens et al., 1997; Lai et al., 1998; Suzuki et al., 1999]. However, prolonged therapy results in the emergence of drug-resistant mutants.

LAM is associated with a higher rate of emergence of drug-resistant mutants than ADV or ETV, which is 24% and 70% after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels [Lai et al., 2003]. Most LAM-resistant

Abbreviations used: HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; ORF, open reading frame; PCR, polymerase chain reaction; RT, reverse transcriptase

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strains show amino acid substitutions in the YMDD (tyrosine–methionine–aspartate–aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed [Allen et al., 1998; Delaney et al., 2003].

Both in vitro and clinical studies have shown recently that ADV and ETV could suppress both wild-type and LAM-resistant strains and were confirmed as salvage therapy for LAM-refractory patients [Levine et al., 2003; Sherman et al., 2006; Rapti et al., 2007]. However, a few studies have already reported the emergence of resistant mutants to these drugs.

ADV-resistant mutations are infrequent and their appearance is delayed in treatment-naïve patients; mutation occurs at 0% after 1 year and 28% after 5 years and the selection of rtA181V/T or rtN236T mutant was associated with resistance to ADV [Maecellin and Asselah, 2005]. On the other hand, the emergence rate of ADV-resistant mutations in LAM-resistant patients was 18% after 48 weeks of ADV monotherapy [Lee et al., 2006]. A recent study reported patients treated with combination therapy of ADV with LAM did not develop resistance to ADV for 3 years [Rapti et al., 2007].

ETV is the most novel nucleotide analogue of the three drugs and displays greater in vitro potency than LAM or ADV against wild-type HBV. ETV-resistance is reported to be rare in treatment-naïve patients [Colonno et al., 2006]. However, ETV-resistant mutants appeared at 6–9% per year in LAM-refractory patients [Tenney et al., 2004, 2007; Sherman et al., 2006].

In the present study, an ETV-resistant strain of HBV was identified after prolonged ETV therapy in a patient who did not respond to LAM therapy. To our knowledge, this is the first report that breakthrough hepatitis was induced by emergence of an ETV-resistant strain and was successfully treated with ADV. This study checked the importance of amino acid substitutions in the HBV polymerase for resistance to ETV in vitro. Furthermore, the susceptibility of the mutant strain to ADV was analyzed.

MATERIALS AND METHODS

Antiviral Compounds

LAM [(–)-β-L-2', 3'-dideoxy-3'-thiacytidine] was provided by GlaxoSmithKline (Stevenage, Herts, UK). Adefovir {9-[2-(phosphonomethoxy)ethyl]-adenine} was provided by Gilead Sciences (Foster City, CA), and ETV {2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate} was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingford, CT).

Analysis of Virological Markers

Hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and antibody against HBeAg (anti-HBe) were determined by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using the Light Cycler

(Roche, Mannheim, Germany) by the polymerase chain reaction (PCR). The primers used for amplification were 5'-TTTGGGCATGGACATTGAC-3' and 5'-GGTGAA-CAATGTTCCGGAGAC-3'. The amplification condition included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Cloning of HBV-DNA and Plasmid Construction

HBV-DNA was extracted from 100 μl of serum samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μl H₂O. The full-length HBV-DNA was amplified using the above HBV-DNA samples by the method of Gunther et al. [1998]. Nucleotide sequence positions were numbered from the unique *EcoRI* site. The 1.4 genome lengths HBV-DNA amplified from the serum of a patient who showed ETV resistance was cloned into a plasmid vector pcDNA3 (Invitrogen, San Diego, CA). In brief, the PCR product amplified using serum from the patient was cleaved with *Bam*HI and *Apa*I (HBV positions 1,400–2,600) and cloned into pcDNA3, which was named pcDNA3-1. Similarly, the PCR product was cleaved with *Apa*I and *Bam*HI (HBV positions 2,600–3,215, 1–1,400) and cloned into pBluescript SK+ (Stratagene, La Jolla, CA), which was named pB-1. The *Kpn*I-*Bam*HI fragment from pB-1 and *Kpn*I-*Apa*I fragment from pcDNA3-1 were cloned into pcDNA3-1. To introduce the nucleotide substitutions into the rtL180M, M204V, and S202G, site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene). Four plasmids with/without amino acid substitutions were created and are listed in Table IV.

Cell Culture, Transfection, and Determination of IC₅₀

HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C under 5% CO₂. Cells were seeded to semi-confluence in 6-well tissue culture plates. Transient transfection of the plasmids into HepG2 cell lines was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC₅₀s) for each anti-viral drug, various concentrations of LAM, ADV, and ETV were added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The medium containing the drugs was changed at days 1, 3, and 4. All experiments were performed in triplicate. GraphPad prism (GraphPad Prism Software, Inc., San Diego, CA) was used to determine the best-fit values for individual dose–response equations.

Analysis of Replicative Intermediate of HBV by Quantitation

The cells were harvested at 5 days after transfection and lysed with 250 μl of lysis buffer (10 mM Tris-HCl [pH

7.4], 140 mM NaCl, and 0.5% (v/v) NP-40) followed by centrifugation for 2 min at 15,000g. The core-associated HBV genome was immunoprecipitated by mouse anti-core monoclonal antibody 2A21 (Institute of Immunology, Tokyo) and subjected to Southern blot analysis after SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR with cyber green using Light Cycler. The HBV-specific primers used for amplification were 5'-TTTGGGCATGGACATTGAC-3' and 5'-GGTGAACAATGTTCCGGAGAC-3'. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec, and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Statistical Analysis

Data are expressed as mean \pm SD. Group comparisons were performed using the Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Patient's Profile

An ETV-resistant strain of HBV was isolated from a 44-year-old Japanese woman with hepatitis B e antigen-positive chronic HBV infection (Fig. 1A). In this patient, LAM successfully reduced the HBV at the initial stage of

treatment. However, viral breakthrough was observed at 11 months after the beginning of LAM therapy and the HBV viral load reached up to 7.5 log copies/ml. After 17 months of LAM, interferon was added to LAM therapy for 6 months. However, after withdrawal of IFN, the viral load and ALT rebounded. Thus, the patient was switched to 0.5 mg of ETV. This resulted in reduction of HBV-DNA and normalization of ALT. After 12 months of ETV therapy, the viral load rebounded, and following 12 more months of ETV, breakthrough hepatitis was observed. After stopping ETV, because of the inadequate effect of IFN monotherapy for one month, the patient was switched to 10 mg of ADV. This treatment reduced both the viral load and ALT level to acceptable levels (Fig. 1).

Isolation of a Multiple Drug-Resistant Hepatitis Strain

Isolates from this patient were analyzed for substitutions in HBV reverse transcriptase (RT). Comparison of the nucleotide sequences by the direct sequence method obtained throughout the clinical course showed three amino acid substitutions in the RT domain of the polymerase (Table I). At the baseline of LAM, all three substitutions were of the wild-type by direct sequence analysis and clonal analysis (Table II). After breakthrough hepatitis induced by LAM, direct sequence analysis showed mixed type (YIDD and YVDD) mutant strain. The rtM204V mutant was detected in 65% of HBV clones and the rest were all the YIDD type. Importantly, at this point, there was no amino acid substitution at rt202. After 12 months of ETV therapy when the viral load was slightly increased, the rtL180M + M204V + S202G mutant was detected in 45% of the HBV clones, followed by decrease of the YIDD and YVDD mutants without substitution at rtS202G. Finally, after 24 months of ETV therapy, when the breakthrough hepatitis occurred, the rtL180M + M204V + S202G mutant was detected in 92% of the HBV clones and the rest were rtL180M + M204V mutants without substitution at rtS202G. Interestingly, the rtM204I + S202G strain never appeared during nucleotide therapy.

| treatment | month | ALT (IU/L) | HBV-DNA (log copies/ml) |
|-----------|-------|------------|-------------------------|
| | -3 | 246 | 7.2 |
| LAM | 0 | 46 | 5.2 |
| | 5 | 28 | 3.7 |
| | 11 | 33 | 4.1 |
| | 17 | 72 | 7.5 |
| | 18 | 1184 | 5.6 |
| | 20 | 39 | 3.9 |
| | 23 | 34 | 3.4 |
| | 27 | 117 | 7.1 |
| ETV | 31 | 112 | 7.2 |
| | 39 | 40 | 2.9 |
| | 43 | 28 | 4.2 |
| IFN | 56 | 140 | 6.8 |
| ADV | 57 | 313 | 6.8 |
| | 60 | 38 | 4 |
| | 71 | 24 | 3.3 |
| | 75 | 19 | 3.1 |

Fig. 1. Clinical course of a patient who developed entecavir resistant mutant.

Susceptibility of Mutants to Entecavir In Vitro

To analyze the role of the rtL180M, rtG202S, and rtM204V substitutions in ETV resistance, four patient-specific strains were transfected into HepG2 cells (Table III). ETV was added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The core-associated HBV genome was extracted from cells and quantified by real-time PCR. The double amino acid substitutions rtL180M + M204V, which is related to LAM resistance, displayed a 38-fold decrease in susceptibility to ETV compared with the wild-type. Moreover, triple amino acid substitutions rtL180M + M204V + S202G, isolated from the patient

TABLE I. Direct Sequence Analysis of Samples From Our Patient With Entecavir (ETV) Resistance

| | rt L180 | rt S202 | rt M204 |
|-----------------------------|---------|---------|---------|
| (1) At the beginning of LMV | — | — | — |
| (2) At the beginning of ETV | L/M | — | I/V |
| (3) One year after ETV | M | G/S | V |
| (4) Two years after ETV | M | G | V |

LMV, lamivudine.

who developed breakthrough hepatitis during ETV therapy, induced 198 times greater resistance than the wild-type. In agreement with the above data, the appearance of the rtS202G substitution in the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. On the other hand, only a single amino acid substitution rtS202G, which was artificial and did not truly exist, had little effect on the susceptibility to ETV (Table III, Fig. 3).

Susceptibility of Mutants to Lamivudine and Adefovir In Vitro

The susceptibility of the rtL180M + M204V and rtL180M + M204V + S202G mutants to LAM was also analyzed using transient transfection assay with HepG2 cells. Both strains displayed strong resistance to LAM (>1,000-fold). We also examined whether ADV was as effective against the rtL180M + M204V + S202G mutant strain as the wild-type. The IC₅₀ values of the mutant strain and wild-type for adefovir were almost the same, which displayed the same result in vivo (Fig. 2, Table IV).

DISCUSSION

The present study describes the identification of an ETV-resistant strain of HBV after prolonged ETV therapy in a patient who was resistant to LAM therapy. Using direct sequencing and clonal analysis, the results demonstrated that the addition of rtS202G mutation to the LAM-resistant mutant strain correlated with the ETV-resistance. To our knowledge, this is the first report of a patient who developed not only virologic breakthrough but also biochemical breakthrough, followed by successful treatment with ADV (Fig. 1).

Clonal analysis showed mixed type of LAM-resistant strains at the commencement of ETV treatment. All of

the rtM204V mutant strains were accompanied by rtL180M mutation, but none of the rtM204I mutant did. After 1 year of ETV therapy, the rtL180M + M204V + S202G mutant emerged in 45% of the HBV clones. Furthermore, almost all clones became the rtL180M + M204V + S202G variant 2 years after ETV therapy. These results suggest two important things. Firstly, the addition of the rtS202G mutant to the rtM204V mutant induced the ETV resistance. Secondly, the S202G was induced only in the mutant strains with rtM204V not in the rtM204I.

The in vitro study described in this article demonstrated that the rtL180M + M204V mutation reduced the susceptibility to ETV by 38-fold compared with wild-type (Table III). Furthermore, the addition of the rtS202G substitution to the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. Interestingly, the single S202G substitution did not induce ETV resistance in vitro. Thus, it appears that the rtS202G substitution never reduced the susceptibility to ETV in the absence of rtM204V substitution. The amino acid substitutions rtS202G have been reported to emerge with resistance against ETV [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. In all previous studies, the rtS202G mutation was accompanied by rtM204V substitution and our results are similar to those of the reported in vitro studies. It is known that other amino acid substitutions, rtT184 and rtM250 in the RT domain are associated with ETV resistance and they also need the substitution at rt204 to achieve such resistance. Tenney et al. [2004] reported that the rates of T184, S202, and M250 mutations in LAM-resistant patients before ETV treatment were 5.2%, 1.2%, and 1.8%, respectively. Moreover, these ETV-resistance-related residues emerged in 6% more patients by 1-year ETV therapy and 8% more patients by 2-year therapy.

TABLE II. Clonal Analysis of Samples From the Patient With Entecavir (ETV) Resistance

| | Relative rate (%) of clones (no. of clones/total) | | | |
|-----------------------------|---|-----------|---------------|-----------------------|
| | Wild | M204I | L180M + M204V | L180M + M204V + S202G |
| (1) At the beginning of LMV | 100 (6/6) | 0 | 0 | 0 |
| (2) At the beginning of ETV | 0 | 35 (7/20) | 65 (13/20) | 0 |
| (3) 12 months after ETV | 0 | 14 (3/22) | 41 (9/22) | 45 (10/22) |
| (4) 24 months after ETV | 0 | 0 | 8 (1/13) | 92 (12/13) |

LMV, lamivudine.

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TABLE III. In Vitro Susceptibility of rtL180/rtM204/rtS202 Mutants to Entecavir

| | rt L180 | rt M204 | rt S202 | ETV | |
|-----------------------|---------|---------|---------|-----------------------|-------------------|
| | | | | IC ₅₀ (μM) | Resistance (fold) |
| Wild | — | — | — | 0.00081 | 1 |
| S202G | — | — | G | 0.00054 | 0.67 ^a |
| L180M + M204V | M | V | — | 0.031 | 38** |
| L180M + M204V + S202G | M | V | G | 0.16 | 198** |

Experiments were performed in triplicates.

^aNS, not significant.

***P* < 0.001 compared with the wild-type.

In the present study, clonal analysis showed the rtS202G substitution was induced only in the mutant strains with rtM204V but not in the rtM204I, as described recently [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. A recent study demonstrated similar results; all 16 patients with virologic rebounds with ETV resistance had the rtM204V substitution, either alone or in combination with rtM204I substitution [Tenney et al., 2007]. Ono et al. [2001] reported that the clinical frequency of LAM-resistant mutants was 18.6% for the rtM204I, 1.4% for the rtM204V, 11.4% for the rtL180M + M204I, and 64.3% for the rtL180M + M204V. In other words, most of the YVDD mutants were accompanied with rtL180M mutation. On the other hand, only about one-third of YIDD mutants were accompanied with rtL180M. Previous in vitro studies demonstrated that both the rtM204I and rtL180M + rtM204V substitutions had incomplete cross-resistance to ETV, and reported that the rtL180M + rtM204V mutant was more susceptible than the rtM204I mutant. The replication capacity of the rtL180M + rtM204V was four-times larger than the rtM204I mutant [Ono et al., 2001]. Thus, it was considered that the addition of rtS202G substitution to the rtL180M + rtM204V mutant could strengthen the replication ability, or could reduce susceptibility to ETV more strongly than the rtM204I mutant. Further studies are needed to confirm the above hypothesis.

There is no consensus regarding the management of patients with ETV resistance. There are few reports of successful treatment of ETV resistant viruses in vivo.

Villet et al. [2007] reported that ADV was clinically effective for virological breakthrough caused by ETV-resistant HBV variant. However, different from the previous report, the present study demonstrated the emergence of biochemical breakthrough after viral rebound caused by ETV resistance. Moreover, it was confirmed that ADV was effective in not only viral breakthrough but also biochemical breakthrough. Our in vitro study also indicated that the rtL180M + M204V + S202G mutant had no resistance against ADV. This result is compatible with the response in vivo. In this regard, recent studies demonstrated that ADV and tenofovir are effective for ETV-resistance in vitro and that ADV was definitely effective against other ETV-related amino acid substitutions S184 and M250 in vitro [Tenney et al., 2007; Villet et al., 2007]. However, the clinical effect has never been reported.

In conclusion, the present study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV. It was confirmed that the addition of rtS202G substitution to the rtM204V mutant strain is responsible for ETV resistance and the resistant mutant could be treated successfully. While ETV resistance is rare in treatment-naïve patients, the amino acid substitution associated with ETV resistance is similar to the substitution seen in patients with LAM-resistance. Thus, it is considered that the successful salvage therapy described in this study could be a potentially helpful for similar events during ETV therapy. The possibility of emergence of novel mutants resistant to

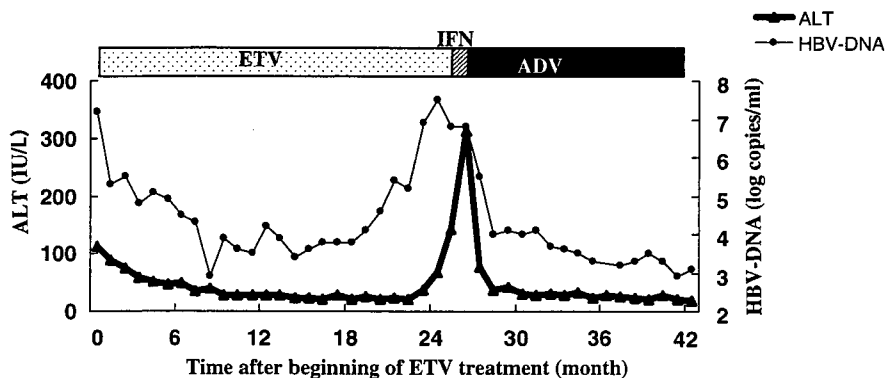


Fig. 2. Clinical course of a patient who developed breakthrough during entecavir therapy.

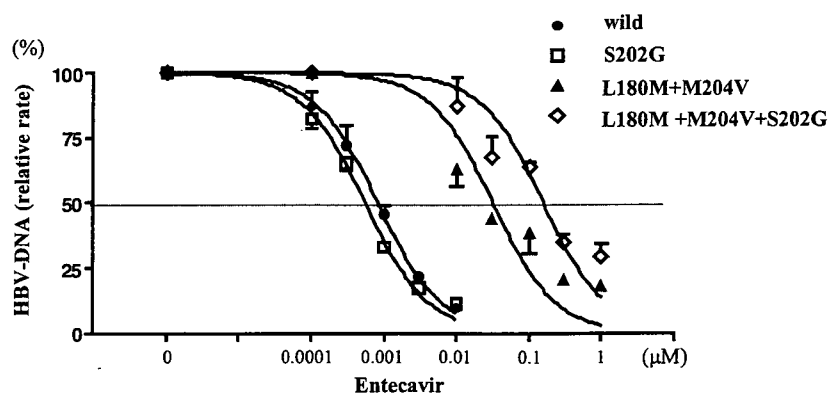


Fig. 3. In vitro analyses of susceptibilities of wild-type HBV and three mutants (rtS202G, rtL180M + M204V, rtL180M + M204V + S202G) to entecavir (ETV) after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4 genome lengths HBV and treated with the indicated amount of entecavir. Data are the dose-response curves of the four HBV strains against entecavir. The strains were used to estimate the entecavir IC₅₀ values for each HBV strains. Values are relative to no entecavir treatment controls for each strain. Experiments were performed in triplicates.

TABLE IV. In Vitro Susceptibility of rtS202/rtM204 Mutant to Lamivudine (LAM) and Adefovir (ADV)

| | LAM | | ADV | |
|-----------------------|-----------------------|-----------------|-----------------------|-------------------|
| | IC ₅₀ (μM) | Fold resistance | IC ₅₀ (μM) | Fold resistance |
| Wild | 0.1 | 1 | 0.39 | 1 |
| L180M + M204V | >100 | >1,000** | — | — |
| L180M + M204V + S202G | >100 | >1,000** | 0.32 | 0.82 ^a |

Experiments were performed in triplicates.

^aNS, not significant.

**P < 0.001 compared with the wild-type.

multiple anti-HBV drugs is real. Therefore, further studies are necessary to develop safes and more useful treatment strategies.

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