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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.

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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 coinfected 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 kg 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 μg/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 univariate 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)	
	2: none	93% (37/40)	<0.001
γ-Glutamyl trans-peptidase, IU/l	1: ≥110	50% (11/22)	
	2: <110	76% (73/96)	0.020
Ribavirin dose, mg/kg	1: <11.0	62% (38/61)	
	2: ≥11.0	81% (46/57)	0.041
Leukocytes, /mm^3	1: <4,500	61% (28/46)	
	2: ≥4,500	78% (56/72)	0.061

[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	
	2: none	8.55 (2.34–31.26)	0.001
Ribavirin dose mg/kg	1: <11.0	1	
	2: ≥11.0	3.22 (1.28–8.09)	0.013

[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.

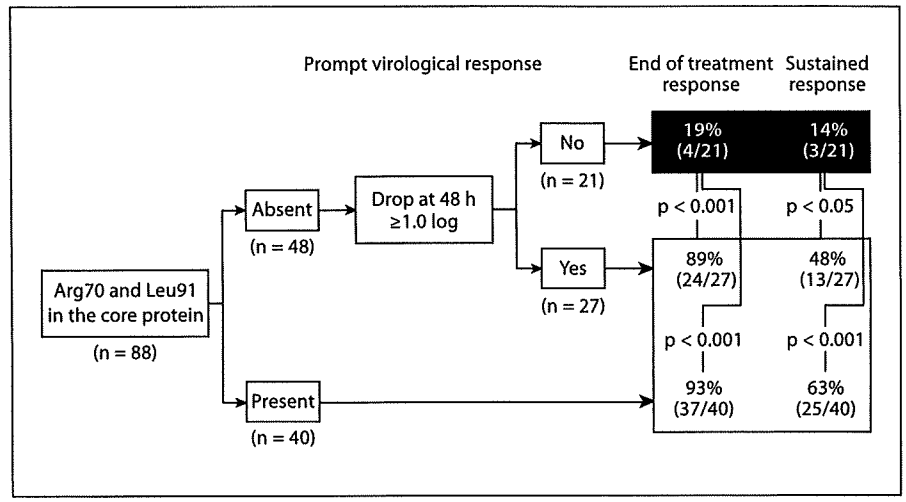

Fig. 1. ETR and SVR to combined Peg-IFN and ribavirin in patients with high-titer HCV-1b stratified by amino acid polymorphism and prompt virological response at 48 h for a decrease in HCV RNA by ≥1.0 log.

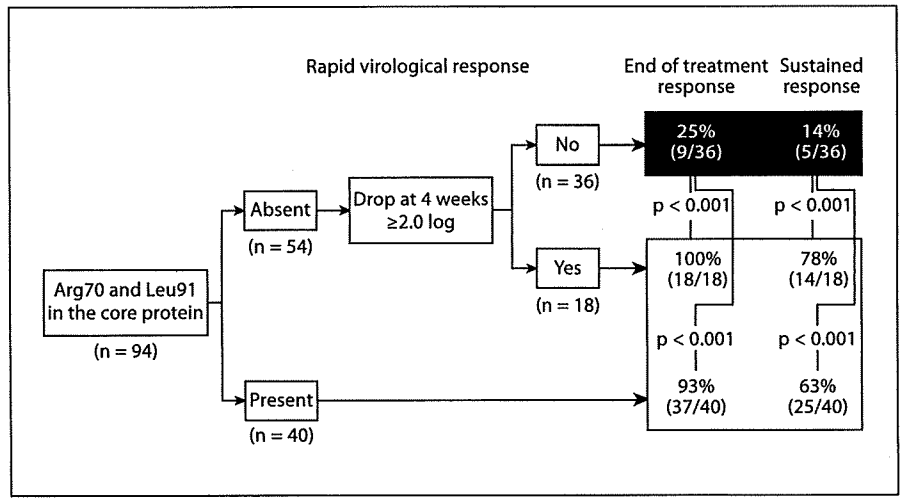

Fig. 2. ETR and SVR to combined Peg-IFN and ribavirin in patients with high-titer HCV-1b stratified by amino acid polymorphism and rapid virological response at 4 weeks for a decrease in HCV RNA by ≥2.0 logs.

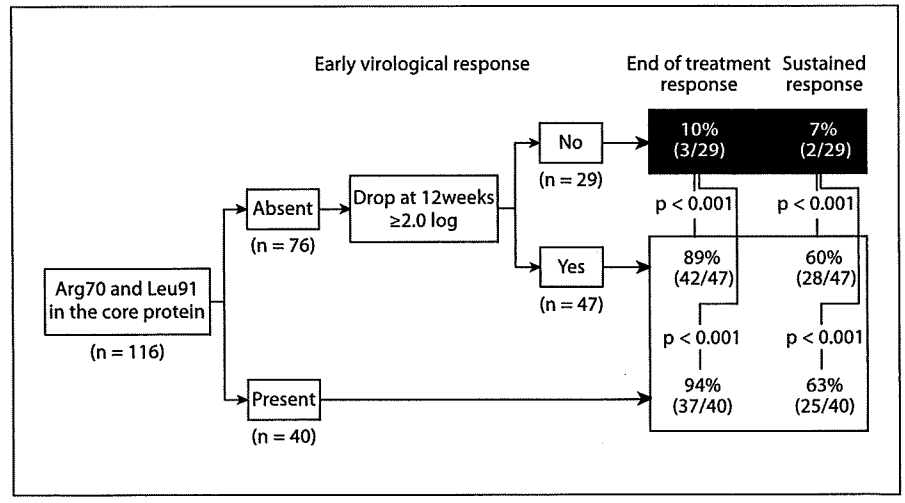

Fig. 3. ETR and SVR to combined Peg-IFN and ribavirin in patients with high-titer HCV-1b stratified by amino acid polymorphisms and early virological response at 12 weeks for a decrease in HCV RNA by ≥2.0 logs.

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 cascade. 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 antigenHBsAg,hepatitis B surface antigenHBV,hepatitis B virusORF,open reading framePCR,polymerase chain reactionRT,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 (Wallingfold, 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 H_2O. 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 *Eco*RI 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 pBluerscript 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 IC_{50}

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% CO_2. 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 (IC_{50}s) 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 15,000*g*. 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 ± 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).

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
	IFN	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
	LAM	71	24	3.3
		75	19	3.1

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

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.

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 IC_{50} 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.

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

				ETV	
	rt L180	rt M204	rt S202	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.
[a]NS, 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

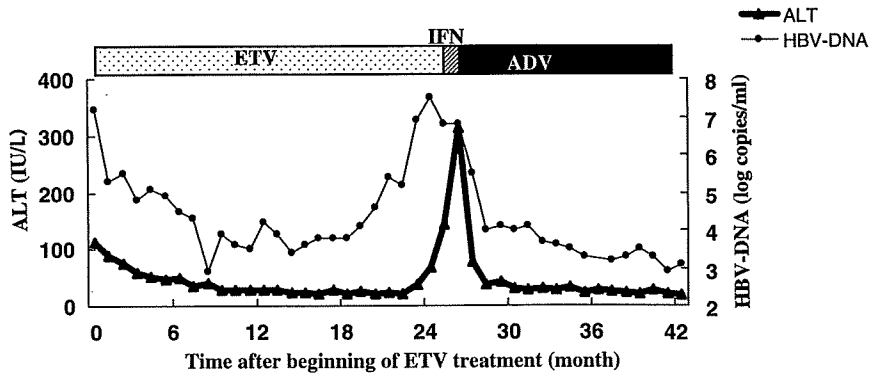

Fig. 2. Clinical course of a patient who developed breakthrough during entecavir therapy.

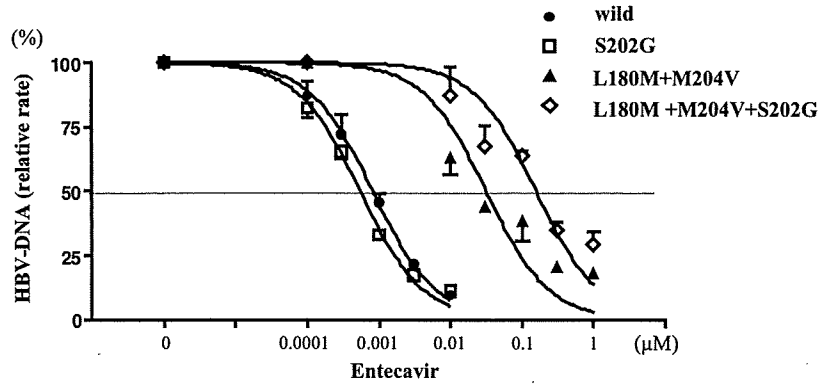

Fig. 3. In vitro analyses of susceptibilities of wild-type HBV and three mutants (rtS202G, rtL180M + M204V, rtL180M + M204V + S202G) to entecavir (ETV) after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4 genome lengths HBV and treated with the 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 IC_{50} 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.
[a]NS, 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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INTERNAL MEDICINE

CASE REPORT

Prolonged Hepatitis after Acute Infection with Genotype H Hepatitis B Virus

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Abstract

We present a case report of a Japanese patient who showed prolonged infection after acute hepatitis B with genotype H. The patient was a 60-year-old man who underwent an annual health care check every year for several years and was never pointed out to have any liver damage, and markers for hepatitis B and C were negative. He was found to be positive for hepatitis B surface antigen (HBsAg) at his health care check in December 2005. After one month, he had an elevated aminotransferase level with hepatitis B e antigen and a high level of serum HBV DNA. He was diagnosed as having acute hepatitis B. On HBV genotype, he had genotype H by the direct sequence method, and he was given a 100 mg of lamivudine daily. However, his acute hepatitis tended to go toward prolonged infection. After two months, he was treated with interferon daily for 28 days. He had negative HBsAg in August 2006. Genotype H, the newest type of hepatitis B, could be the type which shows a poor response to lamivudine. The present paper is the first report, describing the clinical course of acute hepatitis B with genotype H from onset to remission.

Key words: acute hepatitis B, HBV genotype H, prolonged infection

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Introduction

Hepatitis B virus (HBV) infection is related to many liver diseases, acute or fulminant hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. It is estimated that approximately 350 million are chronically infected. Annual mortality rate is 500 000-700 000 (1, 2). Many of the adult patients with acute hepatitis B are cured through the natural course (2). However, there are some individuals who are continuously with HBV and developed to cirrhosis or hepatocellular carcinoma.

Phylogenetic analysis has classified HBV into eight genotypes, designated A to H. The genotypes have different biological properties; these differences affect the clinical outcome and response to antiviral therapy (3). Genotype H has been newly found in Nicaragua and in the U.S.; it seems to be distributed in Central America (4). The prolonged prognosis and response to antiviral therapy in acute hepatitis B patients with genotype H is still obscure. We present here a patient who was suffered from acute hepatitis B with genotype H and had a prolonged clinical course after receiving intensive treatment for hepatitis B. This is the first report to describe the whole clinical course, including the period before onset, of a patient with acute hepatitis B due to HBV genotype H.

Case Report

The patient was a 60-year-old man. He underwent an annual health exam for several years. He had never been pointed out to have any liver damage, and he was negative for markers of hepatitis B or C. At the annual health care check in December 2005, he was found to be positive for serum hepatitis B surface antigen (HBsAg), along with aspartate aminotransferase (AST) 31 IU/l, alanine aminotransferase (ALT) 39 IU/l and was referred to the Toranomon Hospital. He did not have any complaints at that time. One

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Table 1. Laboratory Findings on Admission*

Parameter	Value	Parameter	Value
Hematology		K	4.2 mEq/l
White blood cells	5000/μl	Cl	107mEq/l
Hemoglobin	15.1g/dl	CRP	1.0mg/d
Platelets	25.6×10⁴/μl	Coagulation test	
Blood chemistry		Prothrombin test	89.2%
Total protein	7.2g/dl	Viral markers	
Albumin	3.9 g/dl	IgM anti-HAV(EIA)	0.2(-)
Total bilirubin	0.5mg/dl	IgM anti-HBV(CLIA)	21.9(+)
AST	150IU/l	HBsAg (RPHA)	2048(+)
ALT	434IU/l	HBeAg (CLIA)	1460(+)
LDH	221IU/l	Anti-HBe(CLIA)	0%(-)
ALP	293	HBV DNA(TMA)	8.2 LEG/ml
γ-GTP	127 IU/l	HBV genotype	H
Creatinine	0.8 mg/dl	Anti-HCV(CLEIA)	0.3(-)
Na	143mEq/l	Anti-HIV(CLEIA)	(-)

* AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, gamma glutamyl transpeptidase; CRP, C-reactive protein; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; anti-HBe, antibody to HBeAg.

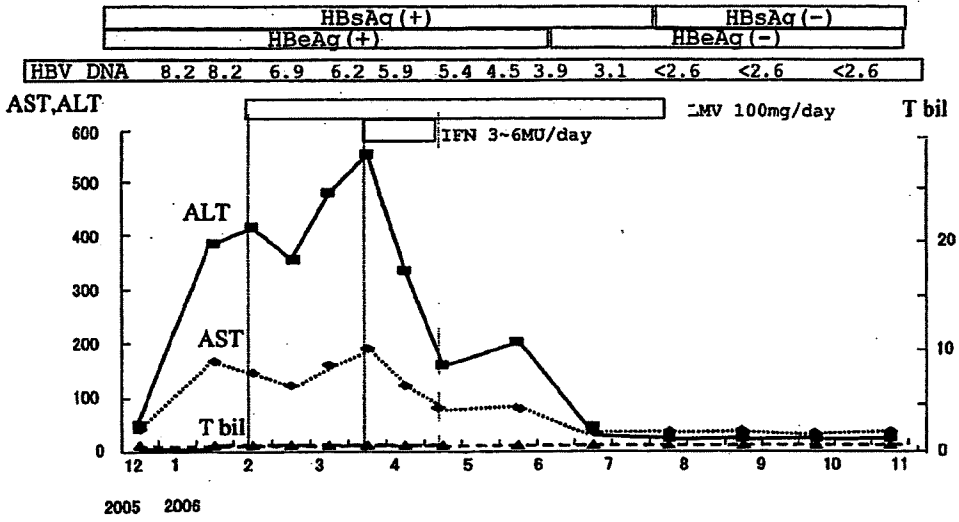

Figure 1. Clinical course of the patient with acute hepatitis B and genotype H.

month later, he just complained of slight fatigue and showed elevated AST and ALT.

He was admitted to our hospital for suspected acute hepatitis B in January, 2006. On admission he showed no jaundiced and was relatively healthy. He was positive for hepatitis B e antigen (HBeAg) and 8.2 LGE/ml of serum HBV-DNA as measured by transcription-mediated amplification and hybridization protect assay [Chugai Daiagnostics Science Co., Tokyo, Japan (5)]. Serum levels of AST and ALT were relatively low. Serological markers for HBsAg, HBeAg were strongly positive and serum level of HBV-DNA was high. IgM antibody to hepatitis B core antigen was high (21.9 S/CO) by the CLIA method (Abbott Japan Co., Ltd., Tokyo, Japan) as shown in Table 1. Therefore, he was diagnosed as having acute hepatitis B. No personal or family history of liver disease was recorded. Serological markers for antibodies to hepatitis C virus and antibodies to HIV type 1 and 2 were negative. However, he was a homosexual habit and went to a 'meeting' two to three times each month near his residence. In the meeting he had sexual contacts with unknown persons.

Lamivudine (LMV), a nucleoside analogue, was prescribed for him to reduce activity in the liver and HBV-DNA serum levels. He was given 100 mg of LMV daily. One month later from the initiation of lamivudine, his transaminase level began to increase, and natural interferon (IFN) beta (Toray Industiries, Inc., Tokyo, Japan) was started by intravenous injection from one more week later. Interferon was started at 6 MU daily. But neutropenia was seen in one week. The dose was then decreased to 3 MU. Unfortunately, three more weeks later, he had complained of depression which was suspected to be an interferon related side effect and IFN therapy was discontinued within one month. Over that time, HBV-DNA had gradually decreased (Fig. 1). Mu-

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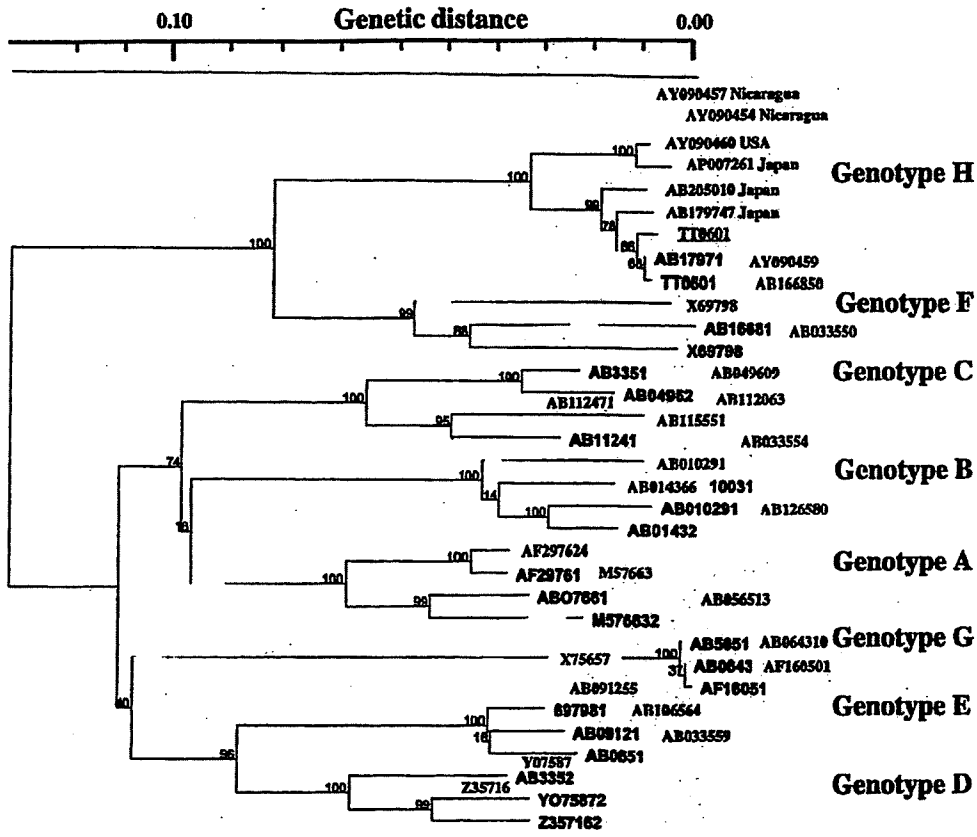

Figure 2. Phylogram generated by neighbor-joining analysis of genetic distance in the full-length sequence of HBV. Thirty strains (without TT0601; indicated by underline) were retrieved from the GenBank/EMBL/DDBJ database.

tation of the HBV DNA polymerase gene (rtM204I/V, L180M) was determined using polymerase chain reaction and restriction fragment length polymorphism as described previously (6). This patient did not show mutations at rt180 or 204 in the HBV DNA polymerase gene at the initiation of IFN therapy.

Full genome sequence analysis by PCR direct sequencing technique before treatment revealed that the patient was infected with genotype H virus (Fig. 2). The sequence was named HBV-TT0601. When compared with previously reported HBV isolates with full genome sequences, ST0404 showed high overall identity (99.2%) with a prototype of the Los Angeles strain (AY090460) and 97.5% identity with a Nicaragua strain (AY090457) of the genotype H group at the nucleotide level. Moreover, ST0404 showed higher overall identity (99.8%, 99.4% and 98.8%) with Japanese strains (AB179747, AB205010 and AP007261 respectively) (7-9).

Five months after the onset, needle liver biopsy under laparoscopy was performed. Portal Tracts had edematous enlargement with lymphocytic infilitration and increased collagen fiber. Moreover, the lobular area showed necroinflammatory activity. Inflammatory changes remained within the liver five months after the onset of acute hepatits B (Fig. 3). With the continuous treatment by LMV, eight months after onset from acute hepatitis, serum HBsAg converted to negative.

Discussion

Here, we report a 60-year-old man infected with genotype H HBV, who had a prolonged clinical course after onset of acute hepatitis B. The present case was suspected for infection from homosexual contact. The genotype H of this patient was reported three times in Japan previously (7-9).

This patient had several features. First, he showed a low level of serum aminotransferase and total bilirubin in spite of a high titer of serum HBV DNA level. In our previous report, we described that patients with a low serum level of aminotransferase and total bilirubin in acute hepatitis B have a high possibility of persistence (10). Low maximum ALT levels (<500 IU/l) and high baseline HBV-DNA levels (>8.7 LGE/ml) were going to persistent in patients with genotype A. Thus, we selected the intensive care for the present case of acute hepatitis B in order to prevent disease progression from acute to the chronic phase.

Second, acute hepatitis B with genotype H has the possibility of being prolonged or persistent in spite of intensive treatment. Generally, acute hepatitis B with HBV genotype A tends to be persistent (11). On the other hand, most patients with acute hepatitis B due to genotype B and C are

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Figure 3-1. Picture of liver biopsy. Panacinar necrosis of the portal tracts and parenchymal remnants leads to disruption of the lobular architecture. Portal tracts exhibited increased fibrosis.

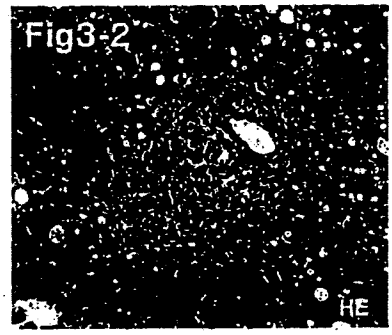

Figure 3-2. Picture of liver biopsy. Edematous enlargement with light lymphocytic infiltration of the portal tracts and parenchymal remnants was clear.

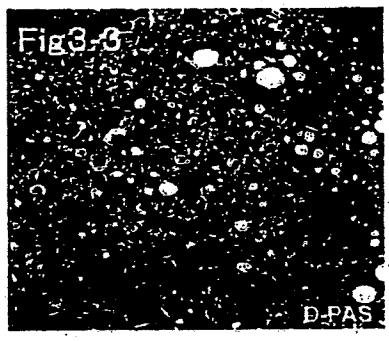

Figure 3-3. Picture of liver biopsy. Kupffer cells underwent hypertrophy and hyperplasia and were laden with lipofuscin pigment: red spot in D-PAS stain, indicating inflammatory persistence.

usually cured without antiviral drugs. The present patient showed a prolonged course after the onset of acute hepatitis by histological examination. HBV replicates by reverse transcription of an RNA intermediate, pregenomic RNA (pgRNA). For pgRNA to be encapsulated, its 5′ end is folded into a stem-loop structure, known as the encapsidation signal. PgRNA is transcribed from the distal Precore region and proximal C gene and consists of 60 nucleosides (positions 1847-1906, numbering from the EcoR1 site) (12-14). In general, the patients with HBV genotype A show adenosine at position 1858 in sequence. On the other hand, the patients with HBV genotype B or C show uracil at position 1858 in sequence. The present patient with genotype H had adenosine at position 1858 in sequence. We suggest that stability of pgRNA in HBV genotype A and H is associated with the clinical course after the onset of acute hepatitis B.

Thirdly, the present patient did not show a good response after lamivudine therapy. In most cases, acute hepatitis is cured with rest and observe. Therefore, antiviral treatment is rarely used for such cases. When antiviral drugs, such as lamivudine, are given the patients with acute hepatitis B in one or two months after onset, most patients show a decrease in the serum levels of ALT and HBV DNA level decrease (9). However, the present patient responded poorly to LMV treatment and had prolonged hepatitis. The serum level of ALT decreases slowly after the initiation of IFN therapy. IFN therapy may aid in decreasing aminotransferase.

Eight genotypes (A-H) of HBV have now been described. In brief, genotypes B and C are prevalent in Asia and the Far East, while genotype A is prevalent in northwestern Europe, North America and Africa. Genotype D is predominant in the Mediterranean area and India (15), while genotype E circulates in sub-Saharan Africa (16). Genotype F is found in Central and South America (17). Genotype G has been reported from France and North America (18). Genotype H has been described only recently, and the first report was from Central America (4). The strain in the present case showed high homology with those reported in Japan (7-9) and Los Angeles (4). However in the future, acute hepatitis B due to genotype H could be spread. Moreover, based on the difference of HBV-genotype, persistence rate is different (2, 10). Limitation of this case was other immunosuppressive factors. The patient was a homosexual. Homosexual men can be associated with poor responsibility for treatment of hepatitis (19).

In conclusion, the acute hepatitis B patients in Japan have shown various genotypes recently. We encountered a rare case of acute hepatitis B with genotype H which led to a prolonged state of acute hepatitis. LMV and IFN were effective for changing HBsAg to negative.

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