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Virological Response and Hepatocarcinogenesis in Lamivudine-Resistant Hepatitis B Virus Genotype C Patients Treated with Lamivudine plus Adefovir Dipivoxil

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Key Words
Hepatitis B virus · Lamivudine · Adefovir dipivoxil · Hepatocellular carcinoma · Basic core promoter · Precore · Core region

Abstract
Aims: The long-term efficacy of adefovir dipivoxil in combination with lamivudine to chronic hepatitis B virus (HBV) infection is still unclear. ***Methods:*** Virological response and hepatocarcinogenesis during lamivudine + adefovir were investigated in 183 lamivudine-resistant Japanese patients with chronic genotype C-dominant HBV infection. As the predictors of virological response, an assessment of clinical parameters and a nucleotide (nt) sequence analysis of the negative regulatory element to core gene (nt 1611–2450) were performed at the start of adefovir. ***Results:*** The cumulative HBV-DNA non-detectable and ALT normalization rates were 93.6 and 97.6% at the end of 3 years, respectively. Multivariate analysis identified total bilirubin, AST, and nt substitutions (nt 1762, 1768, 1846, 1896, 2134, 2288, 2441) as determinants of early non-detectable HBV-DNA. The yearly incidence of hepatocellular carcinoma (HCC) during the first 3 years was 2.7%. At the diagnosis of HCC, ALT normalization, HBV-DNA non-detectable, and HBeAg-seronegative conversion rates were 75.0, 83.3, and 57.1%, respectively. Furthermore, the cumulative HBV-DNA non-detectable and ALT normalization rates were not significantly different according to the development of HCC or not. ***Conclusions:*** Lamivudine-resistant patients treated with lamivudine + adefovir could achieve the excellent virological response and biochemical response, but the low hepatitis activity was not enough to suppress hepatocarcinogenesis.

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 (HCC) [1, 2]. To date, interferon and five nucleoside and nucleotide analogs (lamivudine, adefovir dipivoxil, entecavir, telbivudine, and tenofovir) have been approved for the treatment of chronic HBV infection. Nucleoside and nucleotide analogues suppress HBV replication in most patients and

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improve transaminase levels and liver histology [3–7]. Especially lamivudine monotherapy to naive patients for nucleoside analogues suppresses hepatocarcinogenesis [8, 9], but prolonged therapy results in the emergence of drug-resistant mutants.

Most lamivudine-resistant strains show amino acid substitutions in the YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain of HBV polymerase [10, 11]. Both experimental and clinical studies have shown recently that adefovir and entecavir could suppress not only wild-type but also lamivudine-resistant strains and were confirmed as salvage therapy for lamivudine-refractory patients [12, 13]. Recently, Hosaka et al. [14] reported the efficacy of adefovir + lamivudine combination therapy in patients with lamivudine-resistant chronic HBV infection. However, the number of patients was limited and follow-up time was a short duration. Thus, the long-term efficacy in respect to viral response and suppression of hepatocarcinogenesis with lamivudine + adefovir is still unclear.

Virological predictors of viral response during the treatment of lamivudine + adefovir are insufficiently investigated. Negative regulatory element (NRE; nt 1611–1634), core upstream regulatory sequences (CURS; nt 1643–1742), basic core promoter (BCP; nt 1742–1849) are located mainly in the HBV X gene and play an important role in replication and hepatitis B core antigen/HBeAg formation [15–20]. Furthermore, in respect to the viral response to interferon, Erhardt et al. [21] reported that good response in HBeAg-positive patients was associated with a high number of mutations in the BCP and nt 1753–1766 as well as mutations at nt 1764, and that good response in HBeAg-negative patients correlated with a low number of mutations in the BCP and nt 1753–1766 and wild-type sequence at nt 1764. However, the significance of substitutions in NRE, CURS, BCP, precore, and core gene for viral response during the treatment of lamivudine + adefovir is still unknown.

The present study based on the long follow-up time included 183 lamivudine-resistant consecutive patients with chronic genotype C-dominant HBV infection treated with lamivudine + adefovir. The aims of the study were the following: (1) to evaluate the cumulative HBV-DNA non-detectable, alanine aminotransferase (ALT) normalization, and hepatocarcinogenesis rates during the treatment of lamivudine + adefovir, and (2) to analyze the predictive factors, including clinical parameters and a sequence analysis of the complete NRE, CURS, BCP, precore, and core gene, associated with early non-detectable HBV-DNA during the treatment of lamivudine + adefovir.

Table 1. Patient characteristics at the start of treatment with lamivudine + adefovir dipivoxil

Number	183
Male/female	150/33
Age, years[a]	47 (26–75)
Prior lamivudine therapy duration, years[a]	2.9 (0.6–10.8)
Lamivudine + adefovir treatment duration years[a]	2.2 (0.5–4.5)
HBeAg, number positive	109 (59.6%)
HBV-DNA, log copies/ml[a]	7.3 (3.3 to >7.6)
HBV genotype, number of A/B/C/D	7/7/168/1
Presence of cirrhosis	56 (30.6%)
Total bilirubin, mg/dl[a]	0.8 (0.2–6.0)
Aspartate aminotransferase, IU/l[a]	92 (18–1,413)
Alanine aminotransferase, IU/l[a]	130 (18–1,563)
γ-Glutamyl transpeptidase, IU/l[a]	58 (12–446)
Albumin, g/dl[a]	4.1 (2.3–4.7)
α-Fetoprotein, μg/l[a]	6 (2–282)
Creatinine, mg/dl[a]	0.8 (0.4–1.3)
Platelets, $\times 10^4/mm^3$ [a]	15.0 (3.1–38.8)
Mutant type of YMDD motif (YIDD/YVDD/YIDD+YVDD)	85/42/56

[a] Data are expressed as median (range).

Patients and Methods

Study Population

A total of 183 consecutive adult Japanese patients with chronic HBV infection were treated with adefovir at Toranomon Hospital, Tokyo, Japan, in addition to ongoing lamivudine treatment, for more than 24 weeks since 2002. Serum HBV-DNA and ALT levels re-increased despite the continuation of lamivudine, indicating breakthrough hepatitis, in all patients who then received adefovir along with the lamivudine. Enrolment in this study and the start of adefovir treatment were determined by the following criteria: (1) Increase in serum HBV DNA levels of ≥1 log copies/ml during lamivudine treatment on at least two consecutive occasions, compared with the nadir of initial antiviral efficacy. (2) Detection of mutations of the YMDD motif before the start of adefovir treatment by the PCR-based method described later and/or direct sequence analysis. (3) No history of treatment with other nucleoside analogues such as famciclovir and entecavir. The exclusion criteria were as follows: (1) patients with HCC; (2) serum creatinine levels ≥1.5 mg/dl; (3) patients coinfected with hepatitis C, hepatitis delta virus, or HIV, and (4) history of other liver diseases, such as autoimmune hepatitis, alcoholic liver disease, or metabolic liver disease.

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

Table 1 summarizes the profiles of the patients. They included 150 men and 33 women. The median duration of treatment

with lamivudine + adefovir was 2.2 years (range 0.5–4.5). Patients received a 10-mg once-daily dose of oral adefovir, in addition to ongoing lamivudine treatment (100 mg/day). Blood samples were obtained at least once every month before, during, and after treatment with lamivudine + adefovir, and analyzed for virological markers, biochemical markers associated with liver function and renal function, and complete blood cell counts every visit. The diagnosis of cirrhosis was based on liver biopsy histology and/or on clinical criteria, including imaging studies and signs of portal hypertension. As the indicators of low hepatitis activity, non-detectable HBV-DNA level by PCR assay and normalization of ALT level were evaluated. Adverse reactions were monitored clinically by careful interview and medical examination at least once every month. Patient compliance with treatment was evaluated by questionnaire. Follow-up time represented the time from the start of the treatment with lamivudine + adefovir until the last visit.

Laboratory Tests

HBsAg, HBeAg and antibody against HBeAg (anti-HBe) were determined by commercially available radioimmunoassay systems (Abbott Japan, Tokyo, Japan). HBV DNA serum level was determined by using the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan). The measurement range of the assay is $10^{2.6}$–$10^{7.6}$ copies/ml (2.6–7.6 log copies/ml). The HBV genotype was determined by enzyme-linked immunosorbent assay (ELISA) (HBV Genotype EIA, Institute of Immunology, Tokyo, Japan) based on the method of Usuda et al. [22]. Substitution at rtM204 of the YMDD motif was identified at baseline by using the Enzyme-Linked Mini-Sequence Assay with a commercial assay kit (PCR-ELMA; Genome Science, Tokyo, Japan).

Nucleotide Sequencing of Negative Regulatory Element, Core Upstream Regulatory Sequences, Basic Core Promoter, Precore, and Core Gene

The sequences of nt 1611–2450, including the complete NRE (nt 1611–1634), CURS (nt 1643–1742), BCP (nt 1742–1849), precore (nt 1814–1901), and core gene (nt 1901–2450), were determined by the direct sequencing method using sera at the start of adefovir treatment. Nucleotide sequences of HBV were compared with the prototype sequences of the HBV genotype C (accession No. AB033550) [23]. In the present study, the PCR genotyping could be performed in 148 patients; the remaining 35 patients could not be analyzed due to the lack of adequate serum samples obtained at the start of adefovir treatment.

HBV DNA was extracted with a Smitest EX-R&D kit (Genome Science). Nucleic acids were amplified by PCR using the following primers: (a) *Sequences of nt 1588–2130:* the single-round PCR was performed with HBVPCCPseqF01 (sense, 5′-GCT TCA CCT CTG CAC GTC GCA TG-3′ [nt 1588–1610]) and HBVPCCPseqR03 (antisense, 5′-TCC AAA TTA CTT CCC ACC CAG GT-3′ [nt 2130–2108]) primers. (b) *Sequences of nt 2022–2529:* the single-round PCR was performed with HBVCOREseqF01 (sense, 5′-CCT TAG AGT CTC CGG AAC ATT G-3′ [nt 2022–2043]) and HBVCOREseqR02 (antisense, 5′-GCC ACT CAG GAT TAA AGA CAG G-3′ [nt 2529–2508]) primers. All samples were initially denatured at 95° for 2 min. 45 cycles of amplification were set as follows: denaturation for 30 s at 94°, annealing of primers for 30 s at 60°, and extension for 30 s at 68° with an additional 7 min for extension. The amplified PCR products were purified by the QIA Quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (PerkinElmer, Tokyo, Japan). To avoid false-positive results, the procedures recommended by Kwok and Higuchi [24] to prevent contamination were strictly applied to these PCR assays. No false-positive results were observed in this study.

Liver Histopathological Examination

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained 6 or more portal areas. Histopathological diagnosis was made by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al. [25].

Diagnosis of Hepatocellular Carcinoma

Patients were examined for HCC by abdominal ultrasonography every 3–6 months. If HCC was suspected based on ultrasonographic results, additional procedures, such as computed tomography, magnetic resonance imaging, abdominal angiography, and ultrasonography-guided tumor biopsy if necessary, were used to confirm the diagnosis.

Statistical Analysis

The cumulative rates of non-detectable HBV-DNA and hepatocarcinogenesis were calculated using the Kaplan-Meier method and differences between the curves were tested using the log-rank test. Statistical analyses of non-detectable HBV-DNA and hepatocarcinogenesis were calculated using the period from start of treatment with lamivudine + adefovir. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with non-detectable HBV-DNA. The odds ratios and 95% confidence intervals (95% CI) were also calculated. Potential predictive factors associated with early HBV-DNA negativity included the following variables: age, sex, histological stage, HBV genotype, HBeAg, viremia level, mutant type of YMDD motif, total bilirubin, aspartate aminotransferase (AST), ALT, albumin, γ-glutamyl transpeptidase (GGT), α-fetoprotein (AFP), creatinine, platelets, nt substitutions in CURS to core gene. Each variable was transformed into categorical data consisting of two simple ordinal numbers for uni- and multivariate analyses. Variables that achieved statistical significance ($p < 0.05$) or marginal significance ($p < 0.10$) on univariate Cox proportional hazards model were tested by multivariate Cox proportional hazards model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, Ill., USA). All p values <0.05 by the two-tailed test were considered significant.

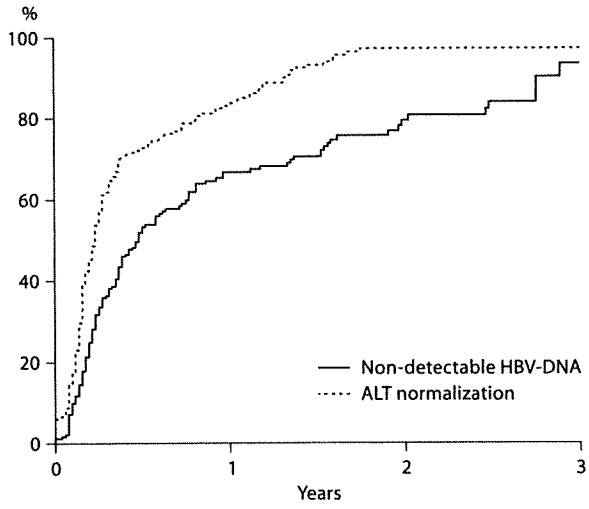

Fig. 1. Cumulative HBV-DNA non-detectable and ALT normalization rates. Patients treated lamivudine + adefovir dipivoxil could achieve the excellent virological response (non-detectable HBV-DNA) and biochemical response (ALT normalization) as an indicator of low hepatitis activity.

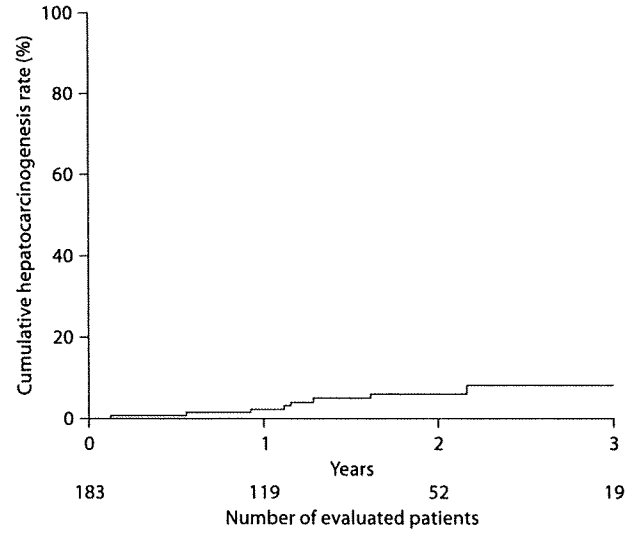

Fig. 2. Cumulative hepatocarcinogenesis rates during the treatment of lamivudine + adefovir dipivoxil. The yearly incidence of HCC during the first 3 years was 2.7%.

Results

Cumulative HBV-DNA Non-Detectable and ALT Normalization Rates

The cumulative HBV-DNA non-detectable rates were 48.4, 66.8, 79.4, and 93.6 at the end of 0.5, 1, 2, and 3 years, respectively. The cumulative ALT normalization rates were 72.4, 84.0, 97.6, and 97.6 at the end of 0.5, 1, 2, and 3 years, respectively. Thus, patients treated with lamivudine + adefovir could achieve the excellent virological response (non-detectable HBV-DNA) and biochemical response (ALT normalization) as an indicator of low hepatitis activity (fig. 1).

Predictive Factors Associated with Early Non-Detectable HBV-DNA by Uni- and Multivariate Analysis

The data for the whole population sample were analyzed to determine those factors that could predict early non-detectable HBV-DNA. Univariate analysis identified 21 parameters that tended to or significantly correlated with early non-detectable HBV-DNA. These included total bilirubin ($p = 0.027$), AST ($p = 0.004$), ALT ($p = 0.072$), HBV DNA ($p < 0.001$), HBeAg ($p < 0.001$), and nt substitutions [nt 1659 ($p = 0.073$), nt 1762 ($p = 0.040$), nt 1768 ($p = 0.084$), nt 1792 ($p = 0.077$), nt 1846 ($p < 0.001$), nt 1896 ($p < 0.001$), nt 1899 ($p = 0.031$), nt 1938 ($p = 0.019$), nt 2005 ($p = 0.058$), nt 2009 ($p < 0.001$), nt 2134 ($p = 0.074$), nt 2189 ($p = 0.017$), nt 2201 ($p = 0.031$), nt 2288 ($p = 0.038$), nt 2429 ($p = 0.042$), nt 2441 ($p < 0.001$)]. These factors were entered into multivariate analysis, which then identified 9 parameters that tended to or significantly influenced early non-detectable HBV-DNA independently; total bilirubin ($p = 0.002$), aspartate aminotransferase ($p = 0.077$), and nt substitutions [nt 1762 ($p = 0.092$), nt 1768 ($p = 0.001$), nt 1846 ($p = 0.034$), nt 1896 ($p = 0.001$), nt 2134 ($p = 0.034$), nt 2288 ($p = 0.016$), nt 2441 ($p = 0.019$)] (table 2).

Cumulative Hepatocarcinogenesis Rates and the Profiles of Patients Who Developed HCC

The cumulative hepatocarcinogenesis rates were 2.2, 5.9, and 8.1% at the end of 1, 2, and 3 years, respectively (fig. 2). The yearly incidence of HCC during the first 3 years was 2.7%. Table 3 summarizes the profiles of 12 patients who developed HCC during treatment with lamivudine + adefovir. They included 9 men and 3 women. The median age at the start of adefovir was 51 years (range 35–75). The median duration from the start of lamivudine to the diagnosis of HCC was 4.9 years (range 1.9–7.5), and the median duration from the start of adefovir

Table 2. Factors associated with early non-detectable HBV-DNA during the treatment with lamivudine + adefovir dipivoxil, identified by uni- and multivariate analysis

Factor	Category	Univariate Cox proportional hazards model		Multivariate Cox proportional hazards model	
		odds ratio (95% CI)	p	odds ratio (95% CI)	p
Total bilirubin, mg/dl	1: <1.0	1		1	
	2: ≥1.0	1.503 (1.047–2.159)	0.027	2.055 (1.289–3.279)	0.002
Aspartate aminotransferase, IU/l	1: <80	1		1	
	2: ≥80	1.695 (1.181–2.434)	0.004	1.506 (0.956–2.371)	0.077
Alanine aminotransferase, IU/l	1: <100	1		–	–
	2: ≥100	1.407 (0.970–2.041)	0.072	–	–
HBV DNA, log copies/ml	1: <7.0	1		–	–
	2: ≥7.0	0.488 (0.342–0.695)	<0.001	–	–
HBeAg	1: negative	1		–	–
	2: positive	0.428 (0.299–0.613)	<0.001	–	–
nt 1659	1: A	1		–	–
	2: not A	2.135 (0.931–4.895)	0.073	–	–
nt 1762	1: A	1		1	
	2: not A	1.988 (1.032–3.829)	0.040	1.987 (0.893–4.421)	0.092
nt 1768	1: T	1		1	
	2: not T	1.892 (0.917–3.903)	0.084	5.584 (2.096–14.88)	0.001
nt 1792	1: A	1		–	–
	2: not A	0.168 (0.023–1.211)	0.077	–	–
nt 1846	1: A	1		1	
	2: not A	2.080 (1.382–3.131)	<0.001	1.740 (1.043–2.902)	0.034
nt 1896	1: G	1		1	
	2: not G	2.207 (1.500–3.247)	<0.001	2.323 (1.430–3.775)	0.001
nt 1899	1: G	1		–	–
	2: not G	1.711 (1.049–2.789)	0.031	–	–
nt 1938	1: T	1		–	–
	2: not T	1.859 (1.107–3.124)	0.019	–	–
nt 2005	1: T	1		–	–
	2: not T	0.661 (0.431–1.014)	0.058	–	–
nt 2009	1: C	1		–	–
	2: not C	4.678 (2.191–9.986)	<0.001	–	–
nt 2134	1: C	1		1	
	2: not C	1.566 (0.957–2.561)	0.074	1.781 (1.044–3.038)	0.034
nt 2189	1: A	1		–	–
	2: not A	1.611 (1.087–2.385)	0.017	–	–
nt 2201	1: T	1		–	–
	2: not T	0.596 (0.373–0.953)	0.031	–	–
nt 2288	1: C	1		1	
	2: not C	1.518 (1.024–2.252)	0.038	1.733 (1.108–2.711)	0.016
nt 2429	1: C	1		–	–
	2: not C	2.573 (1.033–6.408)	0.042	–	–
nt 2441	1: T	1		1	
	2: not T	2.815 (1.656–4.783)	<0.001	2.001 (1.122–3.568)	0.019

Only variables that achieved statistical significance ($p < 0.05$) or marginal significance ($p < 0.10$) on uni- and multivariate Cox proportional hazards model are shown.

95% CI = 95% confidence interval.

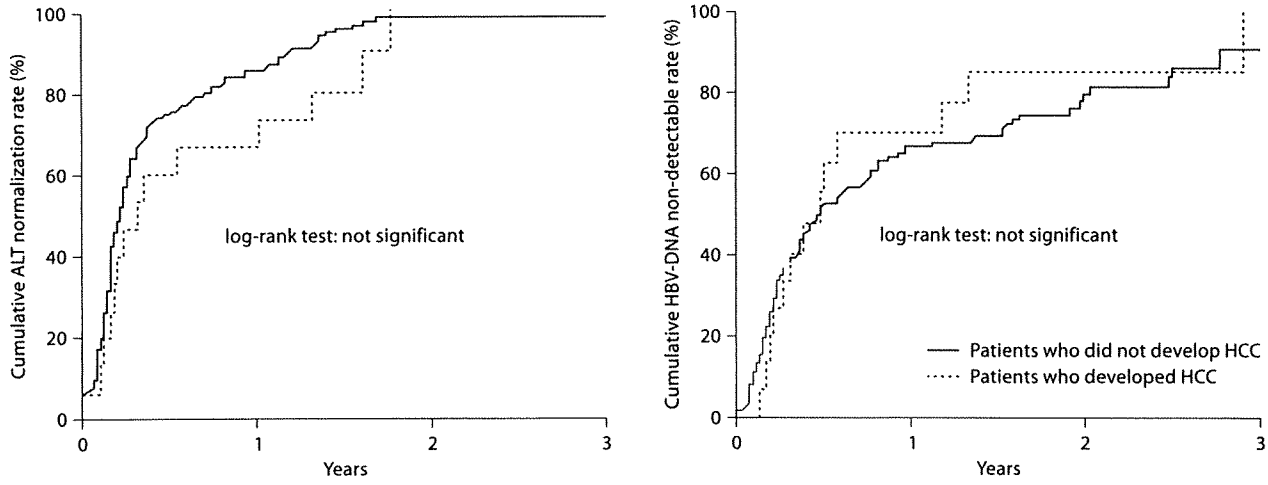

Fig. 3. Comparison of non-detectable HBV-DNA and ALT normalization in patients who developed HCC or not. The cumulative HBV-DNA non-detectable and ALT normalization rates were not significantly different according to the development of HCC or not. Low hepatitis activity during the treatment of lamivudine + adefovir dipivoxil was not enough to suppress hepatocarcinogenesis.

Table 3. Characteristics of 12 patients who developed HCC during treatment with lamivudine (LAM) + adefovir dipivoxil (ADV)

Case	Sex	Age years[a]	LAM to HCC years[b]	ADV to HCC years[c]	At the start of ADV			At the diagnosis of HCC		
					HBeAg	ALT IU/l	HBV-DNA log copies/ml	HBeAg	ALT IU/l	HBV-DNA log copies/ml
1	male	50	6.6	4.5	–	576	6.9	–	27	<2.6
2	male	40	4.9	3.8	–	124	6.3	–	24	<2.6
3	male	48	6.3	3.3	+	99	7.6	+	35	<2.6
4	female	58	7.4	3.3	+	214	4.4	–	13	<2.6
5	male	58	4.8	2.2	–	216	6.5	–	28	<2.6
6	male	35	7.5	1.6	+	164	7.5	–	35	<2.6
7	male	47	2.3	1.3	+	138	7.6	+	29	<2.6
8	male	50	2.5	1.1	+	272	7.6	–	72	<2.6
9	male	75	3.4	1.1	+	209	7.6	–	125	<2.6
10	female	51	1.9	0.9	–	130	5.3	–	73	<2.6
11	male	53	4.7	0.5	+	97	7.6	+	35	4.2
12	female	59	5.9	0.1	–	132	7.6	–	41	3.6

[a] Age at the start of adefovir dipivoxil.
[b] Duration from the start of lamivudine to the diagnosis of HCC.
[c] Duration from the start of adefovir dipivoxil to the diagnosis of HCC.

to the diagnosis of HCC was 1.5 years (range 0.1–4.5). At the diagnosis of HCC, 75.0% (9/12 patients) could achieve ALT normalization, and 83.3% (10/12) could achieve HBV-DNA non-detectable. 57.1% (4/7) of HBeAg-positive at the start of adefovir could achieve HBeAg-seronegative conversion at the diagnosis of HCC. Thus, they developed HCC in spite of the excellent virological response and biochemical response.

Comparison of Non-Detectable HBV-DNA and ALT Normalization in Patients Who Developed HCC or Not

The cumulative HBV-DNA non-detectable and ALT normalization rates were not significantly different according to the development of HCC or not (fig. 3). Thus, some of the patients during treatment with lamivudine + adefovir developed HCC in spite of the early non-detectable HBV-DNA and ALT normalization, and low hepatitis activity during treatment with lamivudine + adefovir was not enough to suppress hepatocarcinogenesis.

Discussion

This is the first report that investigates virological response and hepatocarcinogenesis during the treatment of lamivudine + adefovir in lamivudine-resistant patients with chronic genotype C-dominant HBV infection. Multivariate analysis identified total bilirubin, aspartate aminotransaminase, and nt substitutions (nt 1762, 1768, 1846, 1896, 2134, 2288, 2441) as determinants of early non-detectable HBV-DNA. Erhardt et al. [21] reported that the viral response to interferon was associated with a number of mutations in the BCP and nt 1753–1766 as well as mutation at nt 1764. As determinants of early non-detectable HBV-DNA, this study did not only identify nt substitutions in BCP (nt 1762, 1768), but also identified nt substitutions in precore (nt 1846, 1896), and core (nt 2134, 2288, 2441). This discrepancy between this results and previous findings may be explained by the difference of antiviral treatment, and design of this cohort study based on the only lamivudine-resistant patients during the treatment of lamivudine + adefovir. To our knowledge, the present study is the first to report that the precore, and core gene might influence viral response during lamivudine + adefovir. One limitation of the present study based on the small number of patients was that nt substitutions in areas other than the NRE, CURS, BCP, precore, and core gene of HBV genome, could not be examined. Further prospective studies based on the large numbers of patients, that examine the clinical impact of nt substitutions during lamivudine + adefovir (e.g., virological response and hepatocarcinogenesis) and the underlying mechanisms, should be conducted to confirm the above finding.

Lampertico et al. [26] recently reported the hepatocarcinogenesis during the treatment of lamivudine + adefovir in lamivudine-resistant patients with chronic genotype D-dominant HBV infection for a long-term follow-up period. To our knowledge, the present study is the first to report the hepatocarcinogenesis rates in patients with chronic genotype C-dominant HBV infection. Lamivudine-resistant patients treated with lamivudine + adefovir could achieve the excellent virological response and biochemical response, but the low hepatitis activity was not enough to suppress hepatocarcinogenesis. Kobayashi et al. [27] reported that the yearly incidence of HCC during the first 10 years was 3.3% in natural histories of patients with HBV genotype C-related-compensated cirrhosis without antiviral treatment, who have the higher risk for HCC development. This result showed that the yearly incidence of HCC during the first 3 years was 2.7% during the treatment of lamivudine + adefovir. Treatment of lamivudine + adefovir did not worsen natural histories of chronic HBV infection, but indicated the almost similar hepatocarcinogenesis rates in comparison to cirrhosis patients without antiviral therapy (namely, high-risk group for HCC development). Thus, lamivudine monotherapy to naive patients for nucleoside analogues without lamivudine-resistant HBV infection suppresses hepatocarcinogenesis [8, 9], but lamivudine-resistant chronic HBV patients might be also one of the high-risk groups for hepatocarcinogenesis. This study indicated the high cumulative hepatocarcinogenesis rates of 46.4% at the end of 4 years, and this reason is probably related to the small number of patients, in whom more than 4 years had elapsed since the induction of adefovir (data not shown). Further studies of a large group of patients for the longer-term follow-up period are required to clarify the true cumulative hepatocarcinogenesis rates during the treatment of lamivudine + adefovir.

Low hepatitis activity by suppression of viral replication was not enough to suppress hepatocarcinogenesis during the treatment of lamivudine + adefovir to lamivudine-refractory patients, in contrast to the suppression of hepatocarcinogenesis by lamivudine monotherapy to naive patients without lamivudine-resistant HBV infection [8, 9]. HBV DNA is often integrated into host chromosome in liver tumor tissue, possibly causing chromosomal instability [28–31]. Previous studies reported that antiviral treatment (e.g., lamivudine, adefovir, entecavir, peg-interferon) also diminished the amount of intrahepatic covalently closed circular DNA (cccDNA) as an important intermediate in the life cycle of HBV [32, 33]. However, it is possible that any residual cccDNA in the hepatocytes may still have had integrative capacity at the HBV-DNA non-detectable state during lamivudine + adefovir, and that those may induce hepatocarcinogenesis. Further investigations should be performed whether

cccDNA could influence hepatocarcinogenesis. In this study, it is regrettable that the associations of nt substitutions with the development of HCC could not be presented. This reason is related to the small number of HCC patients, who might provide misleading results (e.g., possible type II error). Further studies should be also conducted to investigate nt substitutions, which might affect the development of HCC during lamivudine + adefovir.

The hepatocarcinogenesis in many patients of this study might have started before the suppression of HBV replication under adefovir, since carcinogenesis begins several months or even years before HCC diagnosis. HCC was diagnosed from 0.1 to 1.6 years in 7 of 12 patients, so the potential beneficial effect of viral suppression might be expected to be seen after the first 2 years of adefovir therapy and only in patients who achieve HBV DNA nondetectable. Further studies should be performed to evaluate the HCC risk in patients who have remained at least 1 year in remission under adefovir to evaluate beneficial effect of viral suppression.

Previous studies reported that interferon monotherpy and lamivudine monotherapy to naive patients for nucleoside analogues without lamivudine-resistant HBV infection suppressed HBV-related hepatocarcinogenesis [8, 9, 34, 35]. In conclusion, lamivudine-resistant patients treated with lamivudine + adefovir could achieve the excellent virological response and biochemical response, but the low hepatitis activity by suppression of viral replication was not enough to suppress hepatocarcinogenesis. Further understanding including viral predictors should facilitate the development of more effective therapeutic regimens to reduce risk of hepatocarcinogenesis.

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Amino Acid Substitutions in the Hepatitis C Virus Core Region of Genotype 1b Are the Important Predictor of Severe Insulin Resistance in Patients Without Cirrhosis and Diabetes Mellitus

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Previous studies provided a direct experimental evidence for the contribution of HCV core protein in the development of insulin resistance (IR), but the clinical impact of HCV core region on IR is still not clear. The present study evaluated the impact of Amino acid (aa) substitutions of HCV-1b core region on IR in 123 Japanese patients infected with HCV-1b without cirrhosis and diabetes mellitus, and investigated the treatment efficacy of 48-week pegylated interferon (PEG-IFN) plus ribavirin (RBV) according to HOMA-IR values. Patients with IR (HOMA-IR $\geq$2.5) and severe IR (HOMA-IR $\geq$3.5) were present in 51.2% and 27.6%, respectively. Multivariate analysis identified body mass index ($\geq$25 kg/m^2) and hepatocyte steatosis ($\geq$5%) as significant determinants of IR. Furthermore, multivariate analysis identified hepatocyte steatosis ($\geq$5%), aa substitutions of the core region (Gln70 (His70) and/or Met91), and age ($\geq$55 years) as significant determinants of severe IR. Especially, significantly lower proportions of patients with Gln70 (His70) and/or Met91 were noted among those without severe IR (59.6%) than those with severe IR (82.4%). The rates of sustained virological response in patients with IR (50.0%) were not significantly different from those without IR (52.9%). Furthermore, the rates of non-virological response in patients with IR (28.9%) were not significantly also different from those without IR (20.6%). In conclusion, the present study indicated that substitutions of HCV-1b core region were the important predictor of severe IR in patients without cirrhosis and diabetes mellitus, but HOMA-IR values might be not useful as predictors of 48-week PEG-IFN plus RBV therapy. ***J. Med. Virol. 81:1032–1039, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: HCV; core region; genotype; HOMA-IR; hepatocyte steatosis; cirrhosis; diabetes mellitus

INTRODUCTION

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [Dusheiko, 1998; Ikeda et al., 1998; Niederau et al., 1998; Kenny-Walsh, 1999; Akuta et al., 2001]. Furthermore, HCV infection also affects an increased risk of diabetes mellitus [Allison et al., 1994; Caronia et al., 1999; Mason et al., 1999; Mehta et al., 2000, 2003; Zein et al., 2000, 2005; Antonelli et al., 2005] or insulin resistance (IR) [Hickman et al., 2003; Hui et al., 2003; Lecube et al., 2004, 2006]. IR and glucose metabolism impairment are associated with liver necroinflammation [Hui et al., 2003], hepatocyte steatosis [Fartoux et al., 2005; Cammà et al., 2006; Conjeevaram et al., 2007], cirrhosis [Petrides et al., 1994], and HCC [El-Serag et al., 2001; Lai et al., 2006; Veldt et al., 2008]. Especially, in patients infected with HCV genotype 1 (HCV-1), significant fibrosis is associated with IR independent from hepatocyte steatosis [Moucari et al., 2008; Petta et al., 2008].

Previous studies reported that HCV core protein induced HCC and IR in transgenic mice, and provided

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a direct experimental evidence for the contribution of HCV core protein in the development of HCC and IR in human HCV infection [Moriya et al., 1998; Shintani et al., 2004]. Amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of genotype 1b (HCV-1b core region) were predictors of poor virological response to 48-week pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy [Akuta et al., 2005, 2006, 2007a,b,c; Donlin et al., 2007; Okanoue et al., 2008], and also risk factors for hepatocarcinogenesis [Akuta et al., 2007d, 2008]. Thus, previous reports supported the oncogenic potential of the HCV core region and clinically linked substitutions of aa 70 and/or 91 in HCV-1b core region to HCC [Akuta et al., 2007d, 2008], but the clinical impact of HCV-1b core region on IR is still not clear. IR develops type 2 diabetes mellitus as its major late feature, and is also associated with advanced fibrosis [Petrides et al., 1994; Petta et al., 2008]. Hence, the biological mechanisms underlying the association between HCV core region and IR are probably multifactorial, and study based on patients without diabetes mellitus and cirrhosis, that might affect IR, should be performed to investigate whether HCV core region might affect IR clinically.

Previous reports showed that IR might be predictors of poor virological response to PEG-IFN plus RBV combination therapy [D'Souza et al., 2005; Romero-Gómez et al., 2005]. Chu et al. [2008] reported that IR was a major determinant of sustained virological response (SVR) in HCV-1 patients receiving 24-week PEG-IFN plus RBV. However, to our knowledge, there is little evidence that IR affects treatment efficacy of HCV-1b patients receiving 48-week PEG-IFN plus RBV combination therapy.

The aims of the present study conducted in Japanese patients infected with HCV-1b without cirrhosis and diabetes mellitus, were the following. (1) To evaluate the HOMA-IR values of patients infected with HCV-1b. (2) To identify the impact of aa substitutions in the core region on IR in such patients, and determine the factors associated with IR, and (3) to investigate the treatment efficacy of 48-week PEG-IFN plus RBV combination therapy according to HOMA-IR values.

PATIENTS AND METHODS

Study Population

At Toranomon Hospital, Tokyo, Japan, 221 HCV-infected Japanese patients were consecutively recruited into the study protocol of the combination therapy with PEG-IFNα-2b plus RBV between December of 2001 and June of 2005. Among these, 123 patients were selected in the present retrospective study based on the following criteria. (1) Negativity for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positivity for anti-HCV (third-generation enzyme immunoassay, Chiron Corp, Emerville, CA), and positivity for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostics, Mannheim, Germany). (2) They were infected with HCV-1b alone. (3) HOMA-IR values and substitutions of aa 70 and 91 in the HCV core region were determined at the commencement of treatment. (4) They were free of cirrhosis and hepatocellular carcinoma, based on biopsy examination, laboratory tests, and imaging studies at baseline. (5) None had diabetes mellitus. (6) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg (mild to moderate alcohol intake). (7) All were free of coinfection with human immunodeficiency virus. (8) None had other forms of hepatitis, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (9) Each signed a consent form of the study protocol that had been approved by the human ethics review committee. Table I summarizes the profiles and laboratory data of the 123 patients at the commencement of treatment. They included 71 males and 52 females, aged 20–70 years (median, 55 years). The treatment efficacy was evaluated by HCV-RNA positive based on qualitative PCR analysis at the end of treatment (non-virological response; NVR), and by HCV-RNA negative based on qualitative PCR analysis at 24 weeks after the completion of therapy (SVR).

Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for alanine aminotransferase (ALT) and HCV-RNA levels. The serum samples were frozen at −80°C within 4 hr of collection and thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA levels were measured by quantitative PCR (AMPLICOR GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems, Inc.) at least once every month before, during, and after therapy. The dynamic range of the assay was 5.0×10^3 to 5.0×10^6 IU/ml. Samples collected during and after therapy that showed undetectable levels of HCV-RNA ($<5.0 \times 10^3$ IU/ml) were also checked by qualitative PCR (AMPLICOR HCV v2.0, Roche Molecular Systems, Inc.), which has a higher sensitivity than quantitative analysis, and the results were expressed as positive or negative. The lower limit of the assay was 50 IU/ml.

Histopathological Examination of Liver Biopsies

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan). The biopsy material was fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained six or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis and liver cirrhosis were diagnosed

TABLE I. Profile and Laboratory Data of 123 Patients Infected With HCV Genotype 1b

Demographic data	
Number of patients	123
Sex (M/F)	71/52
Age (years)*	55 (20–70)
History of blood transfusion	41 (33.3%)
Family history of liver disease	37 (30.1%)
Body mass index (kg/m^2)*	23.6 (17.6–32.0)
Laboratory data*	
Serum aspartate aminotransferase (IU/L)	59 (17–266)
Serum alanine aminotransferase (IU/L)	81 (25–504)
Serum albumin (g/dl)	3.8 (3.1–4.5)
Gamma-glutamyl transpeptidase (IU/L)	50 (15–393)
Leukocytes ($/mm^3$)	4,800 (2,300–8,800)
Hemoglobin (g/dl)	14.4 (10.6–17.6)
Platelet count ($\times 10^4/mm^3$)	16.8 (7.5–27.7)
Indocyanine green retention rate at 15 min (%)	15 (4–41)
Serum iron (μg/dl)	138 (18–290)
Serum ferritin (μg/L)	130 (<10–711)
Creatinine clearance (ml/min)	99 (46–146)
Level of viremia (KIU/ml)	1,900 (23–>5,000)
Alpha-fetoprotein (μg/L)	6 (2–161)
Total cholesterol (mg/dl)	166 (96–294)
High-density lipoprotein cholesterol (mg/dl)	46 (10–83)
Low-density lipoprotein cholesterol (mg/dl)	101 (53–207)
Triglycerides (mg/dl)	95 (33–362)
Uric acid (mg/dl)	5.5 (2.3–9.4)
Fasting plasma glucose (mg/dl)	94 (62–120)
Fasting insulin (μU/ml)	10.5 (0.4–55.5)
HOMA-IR	2.6 (0.1–12.5)
Treatment*	
PEG-IFNα-2b dose (μg/kg)	1.4 (0.7–1.9)
Ribavirin dose (mg/kg)	11.0 (3.7–14.2)
Histological findings	
Stage of fibrosis (F1/F2/F3/ND)	53/32/20/18
Hepatocyte steatosis (<5% (Absent)/≥5% (Present)/ND)	38/64/21
Amino acid substitutions in the HCV	
Core aa 70 (arginine/glutamine (histidine))	69/54
Core aa 91 (leucine/methionine)	71/52
ISDR of NS5A (wild-type/mutant-type/ND)	94/22/7

HOMA-IR, homeostasis model for assessment of insulin resistance; ND, not determined.
Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.

based on histological assessment according to the scoring system of Desmet et al. [1994]. Hepatocyte steatosis was assessed as the percentage of hepatocytes containing fat droplet, and subjects were considered to have steatosis in the presence of fat droplets in ≥5% of hepatocytes.

Diagnosis of Liver Cirrhosis, Insulin Resistance, and Diabetes Mellitus

Liver cirrhosis was diagnosed based on the presence of markedly irregular surface with nodular formation in the liver, evident on peritoneoscopy, histological assessment according to the scoring system of Desmet et al. [1994], or on computed tomography or ultrasonography. Ascites, edema, and esophageal varicosities, facilitated the diagnosis when present.

The diagnosis of type 2 diabetes was based on the revised criteria of the American Diabetes Association using a value of fasting plasma glucose of ≥126 mg/dl on at least two occasions [American Diabetes Association, 2000]. IR was assessed by the Homeostasis Model for Assessment of Insulin Resistance (HOMA-IR) method [Matthews et al., 1985], using the following equation: HOMA-IR = fasting plasma glucose (mg/dl) × fasting insulin (μU/ml)/405. HOMA-IR values of ≥2.5, and ≥3.5 were evaluated as IR, and severe IR, respectively.

Detection of Amino Acid Substitutions in Core Region and NS5A Region

With the use of HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 aa in the core protein of genotype 1b was determined and then compared with the consensus sequence constructed on 50 clinical samples to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91) [Akuta et al., 2005]. The sequence of 2209–2248 aa in the NS5A of genotype 1b (IFN-sensitivity determining region [ISDR]) reported by Enomoto et al. [1995, 1996] was also determined, and the numbers of aa substitutions in ISDR were defined as wild-type (≤1) or mutant-type (≥2).

In the present study, aa substitutions of the core region and NS5A-ISDR were analyzed by direct

sequencing [Enomoto et al., 1995, 1996; Akuta et al., 2005]. HCV RNA was extracted from serum samples at the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). Nucleic acids were amplified by PCR using the following primers: (a) Nucleotide sequences of the core region: The first-round PCR was performed with CC11 (sense, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′) primers, and the second-round PCR with CC9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (antisense) primers. (b) Nucleotide sequences of NS5A-ISDR: The first-round PCR was performed with ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′) primers, and the second-round PCR with ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′) primers. ([a]; hemi-nested PCR. [b]; nested PCR). All samples were initially denatured at 95°C for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C with an additional 7 min for extension. Then 1 μl of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan).

Statistical Analysis

Non-parametric tests were used to compare variables between groups, including the Mann–Whitney U test, chi-squared test, and Fisher's exact probability test. Univariate and multivariate logistic regression analyses were used to determine the independent predictive factors of IR. The odds ratios and 95% confidence intervals (95%CI) were also calculated. All P-values <0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors of IR included the following pretreatment variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, aspartate aminotransferase (AST), ALT, albumin, gamma-glutamyl transpeptidase (γGTP), leukocyte count, hemoglobin, platelet, count, indocyanine green retention rate at 15 min (ICG R15), serum iron, serum ferritin, creatinine clearance, level of viremia, alfa-fetoprotein, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, uric acid, fasting plasma glucose, fasting insulin, HOMA-IR values, stage of fibrosis, hepatocyte steatosis, PEG-IFN dose/body weight, RBV dose/body weight, and aa substitution in the core and ISDR of NS5A. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL).

RESULTS

HOMA-IR Values of Patients Infected With HCV-1b Without Cirrhosis and Diabetes Mellitus

As a whole, 16.3% (20 of 123 patients), 32.5% (40 of 123), 23.6% (29 of 123), and 27.6% (34 of 123) indicated HOMA-IR values of ≤1.4, 1.5–2.4, 2.5–3.4, and ≥3.5, respectively (Fig. 1). Thus, patients with IR (HOMA-IR ≥2.5) were present in 51.2% (63 of 123), and exceeded 50%. Furthermore, patients with severe IR (HOMA-IR ≥3.5) were present in 27.6%. These results show that patients, infected with HCV-1b without cirrhosis and diabetes mellitus, might indicate IR frequently.

Factors Associated With Insulin Resistance in Univariate and Multivariate Analyses

The whole population sample of 123 patients were analyzed to determine factors that could be associated with IR. IR (HOMA-IR ≥2.5) was detected in 63 of 123 (51.2%) patients. Univariate analysis identified six parameters that tended to or significantly influenced IR. These included age (≥55 years, $P = 0.072$), body mass index (≥25 kg/m^2, $P < 0.001$), serum ferritin (≥200 μg/L, $P = 0.071$), family history of liver disease (Absent, $P = 0.077$), hepatocyte steatosis (Present (≥5%), $P = 0.002$), and aa substitutions of the core region (Gln70 (His70) and/or Met91, $P = 0.092$). Multivariate analysis identified two parameters that independently influenced IR, including body mass index (≥25 kg/m^2, $P = 0.001$) and hepatocyte steatosis (Present (≥5%), $P = 0.028$).

Severe IR (HOMA-IR ≥3.5) was detected in 34 of 123 (27.6%) patients. Univariate analysis identified five

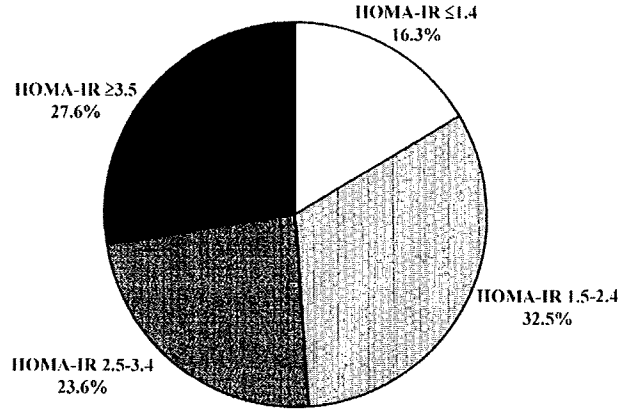

Fig. 1. HOMA-IR values of patients infected with HCV genotype 1b without cirrhosis and diabetes mellitus. As a whole, 16.3%, 32.5%, 23.6%, and 27.6% indicated HOMA-IR values of ≤1.4, 1.5–2.4, 2.5–3.4, and ≥3.5, respectively. These results show that patients, infected with HCV genotype 1b without cirrhosis and diabetes mellitus, might indicate IR frequently.

TABLE II. Factors Associated With Severe IR (HOMA-IR ≥3.5) in Patients Infected With HCV Genotype 1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	*P*
Hepatocyte steatosis	1: Absent (<5%)	1	
	2: Present (≥5%)	4.170 (1.235–14.08)	0.021
Substitution of aa 70 and 91	1: Arg70 and Leu91	1	
	2: Gln70 (His70) and/or Met91	3.654 (1.215–10.99)	0.021
Age (years)	1: <55	1	
	2: ≥55	3.015 (1.071–8.488)	0.037

parameters that tended to or significantly influenced severe IR. These included age (≥55 years, $P=0.015$), body mass index (≥25 kg/m^2, $P=0.025$), hepatocyte steatosis (Present (≥5%), $P=0.003$), triglycerides (≥100 mg/dl, $P=0.060$), and aa substitutions of the core region (Gln70 (His70) and/or Met91, $P=0.020$). Multivariate analysis identified three parameters that independently influenced severe IR, including hepatocyte steatosis (Present (≥5%), $P=0.021$), aa substitutions of the core region (Gln70 (His70) and/or Met91, $P=0.021$), and age (≥55 years, $P=0.037$) (Table II).

aa Substitutions of Core Region and HOMA-IR Values

The entire population sample was also analyzed to determine the relationship between aa substitutions of the core region and HOMA-IR values. HOMA-IR values of 81 patients with Gln70 (His70) and/or Met91 (median; 2.9) indicated the higher levels than those of 42 patients with Arg70 and Leu91 (median; 2.3), significantly ($P=0.022$) (Fig. 2).

Furthermore, the proportions of patients with Gln70 (His70) and/or Met91 among those with HOMA-IR values of ≤1.4, 1.5–2.4, 2.5–3.4, 3.5–3.9, and ≥4.0 were 60.0% (12 of 20 patients), 57.5% (23 of 40), 62.1% (18 of 29), 83.3% (5 of 6), and 82.1% (23 of 28), respectively (Fig. 3). Thus, the higher the proportion of patients with Gln70 (His70) and/or Met91, the higher HOMA-IR values, and significantly lower proportions of patients with Gln70 (His70) and/or Met91 were noted among those without severe IR (59.6% (53 of 89)) than those with severe IR (82.4% (28 of 34)) ($P=0.020$).

Treatment Efficacy of PEG-IFN Plus RBV Combination Therapy According to HOMA-IR Values

Of the 123 patients, 72 could be evaluated as 48-week regimen of PEG-IFN plus RBV combination therapy. Seventy-two patients received PEG-IFNα-2b combination therapy at a median dose of 1.5 μg/kg (range, 0.8–1.8 μg/kg) subcutaneously each week plus oral RBV at a median dose of 11.3 mg/kg (range, 9.7–14.2 mg/kg) daily for 48 weeks. 51.4% (37 of 72 patients) could achieve SVR, and 25.0% (18 of 72) had NVR.

In each groups with IR or without IR, SVR was achieved by 19 of 38 patients (50.0%) and 18 of 34 (52.9%), respectively. The proportions of SVR in group with IR was not significantly different from those in group without IR. Furthermore, in each groups with IR or without IR, NVR was identified in 11 of 38 patients (28.9%) and 7 of 34 (20.6%), respectively. The proportions of NVR in group with IR was not significantly different from those in group without IR.

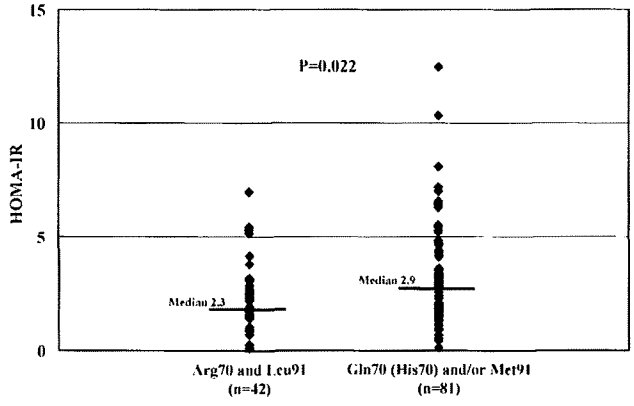

Fig. 2. aa substitutions of HCV core region and HOMA-IR values. HOMA-IR values of 81 patients with Gln70 (His70) and/or Met91 indicated the higher levels than those of 42 patients with Arg70 and Leu91, significantly ($P=0.022$).

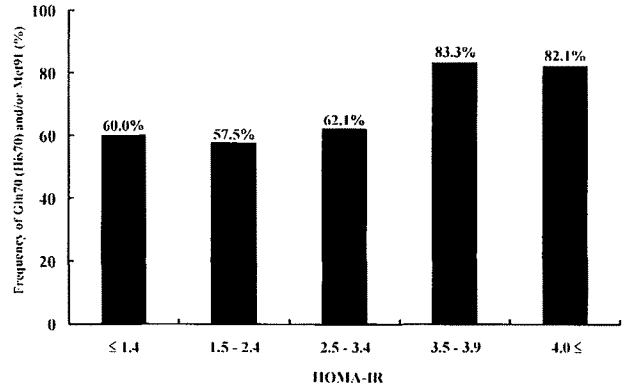

Fig. 3. The proportions of patients with Gln70 (His70) and/or Met91 and HOMA-IR values. Higher frequencies of Gln70 (His70) and/or Met91 correlated with higher HOMA-IR values. Significantly lower proportions of patients with Gln70 (His70) and/or Met91 were noted among those without severe IR (HOMA-IR <3.5) (59.6%) than those with severe IR (HOMA-IR ≥3.5) (82.4%) ($P=0.020$).

In each groups with severe IR or without severe IR, SVR was achieved by 11 of 20 patients (55.0%) and 26 of 52 (50.0%), respectively. The proportions of SVR in group with severe IR was not significantly different from those in group without severe IR. Furthermore, in each groups with severe IR or without severe IR, NVR was identified in 6 of 20 patients (30.0%) and 12 of 52 (23.1%), respectively. The proportions of NVR in group with severe IR was not significantly different from those in group without severe IR.

In this study, HOMA-IR values were not useful as pretreatment predictors of 48-week PEG-IFN plus RBV combination therapy in HCV-1b patients without cirrhosis and diabetes mellitus.

DISCUSSION

Shintani et al. [2004] reported that HCV core protein induced IR in transgenic mice, and provided a direct experimental evidence for the contribution of HCV core protein in the development of IR in human HCV infection. The results of the present study showed that higher frequencies of Gln70 (His70) and/or Met91 in HCV-1b core region might correlated with higher HOMA-IR values. Thus, the present results supported the potential of core region in the development of IR, and clinically linked substitutions of aa 70 and/or 91 in HCV-1b core region to IR. Especially, these findings without diabetes mellitus and cirrhosis suggest that the real connection between IR and HCV-1b infection is initiated at early stages of liver disease. The limitations of the present study were that it could not investigate an improvement of IR in patients who developed the viral eradication after antiviral treatment [Kawaguchi et al., 2007; Arase et al., 2008], as a direct evidence for the contribution of aa substitutions in HCV-1b core region. Further studies that examine the structural and functional impact of aa substitutions should be conducted to confirm the above finding.

To our knowledge, the present study is first report to identify the factors associated with IR of patients without diabetes mellitus and cirrhosis infected with HCV-1b. Especially, multivariate analysis identified age ($\geq$55 years), body mass index ($\geq$25 kg/m^2), hepatocyte steatosis (Present ($\geq$5%)), and aa substitutions of the core region (Gln70 (His70) and/or Met91) as significant determinants of IR (HOMA-IR $\geq$2.5) and/or severe IR (HOMA-IR $\geq$3.5). However, this study identified aa substitutions of the core region as significant determinants of severe IR, and did not identify as determinants of IR. The discrepant results may be due to one or more factors. The first reason for this is probably the small number of patients in the present study (e.g., possible type II error). Univariate analysis really identified aa substitutions of the core region that tended to influence IR. Furthermore, even if HOMA-IR values were also divided into two groups of $\geq$3.0 and $\leq$2.9, multivariate analysis identified aa substitutions of the core region as significant determinants of $\geq$3.0 (data not shown). Hence, further studies based on the large number of patients should be performed in the future. The second reason is probably the difference of objects, based on HCV-1b patients without diabetes mellitus and cirrhosis. Previous report indicated that HCV-related diabetes mellitus might occur in association with IR, hepatocyte steatosis, and high levels of both tumor-necrosis factor and CXCL10 [Antonelli et al., 2009], so patients with severe IR do not always have diabetes mellitus. However, IR develops type 2 diabetes mellitus as its major late feature, and is also associated with advanced fibrosis [Petrides et al., 1994; Petta et al., 2008]. Hence, the biological mechanisms underlying the association between HCV core region and IR are probably multifactorial, and the present study based on patients without diabetes mellitus and cirrhosis as confounding factors, that might affect IR, are very important for estimating the true relationship between HCV core region and IR. The present study is first report to identified aa substitutions of the core region (Gln70 (His70) and/or Met91) as significant determinants of severe IR, in HCV-1b patients without cirrhosis and diabetes mellitus.

Moriya et al. [1998] reported that HCV core protein induced HCC in transgenic mice, and provided a direct experimental evidence for the contribution of HCV core protein in the development of HCC in human HCV infection. Previous reports supported the oncogenic potential of the HCV core region and clinically linked substitutions of aa 70 and/or 91 in HCV-1b core region to HCC [Akuta et al., 2007d, 2008]. IR and glucose metabolism impairment are associated with HCC [El-Serag et al., 2001; Lai et al., 2006; Veldt et al., 2008]. The present study suggested the presence of IR-dependent pathway as a mechanism of HCV-1b core region-associated hepatocarcinogenesis, and the importance of eradication of the virus with Gln70 (His70) and/or Met91 in reducing the development of HCC through this pathway.

Treatment efficacy of 48-week PEG-IFN plus RBV combination therapy according to HOMA-IR values is controversial. Chu et al. [2008] reported that IR was a major determinant of SVR in HCV-1 patients receiving PEG-IFN plus RBV, but treatment duration was 24 weeks. Georgescu et al. [2008] reported that high HOMA-IR values could not affect treatment efficacy of 48-week PEG-IFN plus RBV therapy in HCV-1 patients, after excluding the patients of metabolic syndrome criteria. The present study based on HCV-1b patients without cirrhosis and diabetes mellitus also showed that HOMA-IR values might be not useful as predictors of 48-week PEG-IFN plus RBV therapy. This reason is probably related to exclude patients of diabetes mellitus as one of metabolic syndrome criteria, and the results might support the previous report of Georgescu et al. [2008]. To our knowledge, the present study is first report to investigate the relation between HOMA-IR values and treatment efficacy of HCV-1b patients, especially without cirrhosis and diabetes mellitus, receiving 48-week PEG-IFN plus RBV combination

therapy. Further studies based on the large number of patients should be performed in the future.

In conclusion, the results of the present study indicated that substitutions of HCV-1b core region were the important predictor of severe IR in patients without cirrhosis and diabetes mellitus. This finding highlights the importance of eradication of the virus with Gln70 (His70) and/or Met91 in reducing the development of severe IR. The limitations of the present study were that it did not investigate other genotypes apart from HCV-1b, the geographic diversities of HCV-1b core region (distribution of Arg70 or Gln70 (His70), and Leu91 or Met91), and the study of other races apart from Asians in Japan. Further prospective studies, matched for HCV genotype, aa substitutions of the core region, and race, of a large group of patients are required to determine the meaning of higher HOMA-IR values in HCV infection.

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Sustained Virological Response Reduces Incidence of Onset of Type 2 Diabetes in Chronic Hepatitis C

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Diabetes is present in patients with chronic hepatitis C virus infection. The aim of this retrospective cohort study was to assess the cumulative development incidence and predictive factors for type 2 diabetes after the termination of interferon therapy in Japanese patients positive for hepatitis C virus (HCV). A total of 2,842 HCV-positive patients treated with interferon (IFN) monotherapy or combination therapy with IFN and ribavirin were enrolled. The mean observation period was 6.4 years. An overnight (12-hour) fasting blood sample or a casual blood sample was taken for routine analyses during follow-up. The primary goal was the onset of type 2 diabetes. Evaluation was performed by using the Kaplan-Meier method and Cox proportional hazard analysis. Of 2,842 HCV patients, 143 patients developed type 2 diabetes. The cumulative development rate of type 2 diabetes was 3.6% at 5 years, 8.0% at 10 years, and 17.0% at 15 years. Multivariate Cox proportional hazard analysis revealed that type 2 diabetes development after the termination of IFN therapy occurred when histological staging was advanced (hazard ratio 3.30; 95% confidence interval [CI] 2.06-5.28; $P < 0.001$), sustained virological response was not achieved (hazard ratio 2.73; 95% CI 1.77-4.20; $P < 0.001$), the patient had pre-diabetes (hazard ratio 2.19; 95% CI 1.43-3.37; $P < 0.001$), and age was ≥50 years (hazard ratio 2.10; 95% CI 1.38-3.18; $P < 0.001$). *Conclusion:* Our results indicate sustained virological response causes a two-thirds reduction in the risk of type 2 diabetes development in HCV-positive patients treated with IFN. (HEPATOLOGY 2009;49:739-744.)

Hepatitis C virus (HCV) is one of the more common causes of chronic liver disease in world. Chronic hepatitis C is an insidiously progressive form of liver disease that relentlessly but silently progresses to cirrhosis in 20% to 50% of cases over a period of 10 to 30 years.[1-3] In addition, HCV is a major risk for hepatocellular carcinoma (HCC).[4-8] Moreover, chronic HCV infection has been associated with a variety of extrahepatic complications such as essential mixed cryoglobulinemia, porphyria cutanea tarda, membranoproliferative glomerulonephritis, autoimmune thyroiditis, sialadenitis, and cardiomyopathy.[9-13] Lately, data supporting a link between type 2 diabetes mellitus (T2DM) and chronic hepatitis C infection have been reported.[14,15]

Although there is growing evidence to support the concept that HCV infection is a risk factor for developing T2DM, there have been a few interventional studies confirming this issue. This issue needs to be confirmed with a long-term follow-up of patients with high risk of developing diabetes. Thus, prospective studies including metabolic evaluations are clearly needed to clarify these issues.

With this background in mind, the cohort study was initiated to investigate the cumulative incidence and risk factors of T2DM after prolonged follow-up in HCV-infected patients treated with interferon (IFN) monotherapy or combination therapy with IFN and ribavirin. The strengths of the current study are the large numbers of patients included and the long-term follow-up of patients.

Patients and Methods

Patients. There were 5,890 patients diagnosed with chronic HCV infection and treated with IFN mono-

Abbreviations: CI, confidence interval; FPG, fasting plasma glucose; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; SVR, sustained virological response; T2DM, type 2 diabetes mellitus.

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therapy or combination IFN + ribavirin therapy between September 1990 and March 2007 in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan. Of these, 2,842 patients satisfied the following criteria: (1) no evidence of diabetes mellitus for 3 months after the termination of IFN (plasma glucose concentration <126 mg/dL [6.9 mmol/L] in the fasting state, <200 mg/dL [11.0 mmol/L] in casual state and/or 2 hours after a 75-g oral glucose load); (2) features of chronic hepatitis or cirrhosis diagnosed via laparoscopy and/or liver biopsy before the initiation of IFN therapy; (3) positivity for serum HCV RNA before the initiation of IFN therapy; (4) period of ≤1 year of IFN therapy; (5) negativity for hepatitis B surface antigen (HBsAg), antinuclear antibodies, or antimitochondrial antibodies in serum, as determined via radioimmunoassay or spot hybridization; (6) no evidence of HCC nodules as shown on ultrasonography and/or computed tomography; and (7) no underlying systemic disease, such as systemic lupus erythmatosus or rheumatic arthritis.

Patients who were taking medications known to alter glucose tolerance or had illnesses that could seriously reduce their life expectancy or their ability to participate in the trial were excluded from the study. Patients were classified as having normal glucose or pre-diabetes based on fasting plasma glucose (FPG), casual plasma glucose, or 2-hour plasma glucose. The normal glucose group was regarded as having an FPG of <100 mg/dL, casual plasma glucose of <140 mg/dL, and/or 2-hour plasma glucose of <140 mg/dL. The pre-diabetes group was regarded as having an FPG of 100-125 mg/dL, casual plasma glucose of 140-200 mg/dL, and/or 2-hour plasma glucose of 140-200 mg/dL.[16]

Next, we assessed predictive factors for T2DM in chronic hepatitis C patients treated with IFN. The physicians in charge explained the purpose and method of this clinical trial to each patient and/or the patient's family. Informed consent was obtained from all living patients included in the present cohort study. The study was approved by the Institutional Review Board of our hospital.

Outcome Measures. The primary outcome was T2DM, diagnosed by the use of the 2003 criteria of the American Diabetes Association.[16] These criteria include (1) casual plasma glucose ≥200 mg/dL; (2) FPG ≥126 mg/dL; (3) 2-hour post-glucose (oral glucose tolerance test) ≥200 mg/dL.

Laboratory Investigation. Anti-HCV was detected using a second-generation enzyme-linked immunosorbent assay (ELISA II; Abbott Laboratories, North Chicago, IL). HCV-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, version 2.0; Roche, Tokyo, Japan). Hepatitis B surface antigen was tested via radioimmunoassay (Abbott Laboratories, Detroit, MI). The used serum samples were stored at −80°C at the first consultation. Diagnosis of HCV infection was based on detection of serum HCV antibody and positive RNA. Height and weight were recorded at baseline, and the body mass index was calculated as weight (in kg)/height (in m^2).

Evaluation of Liver Cirrhosis. Liver status of the 2,842 patients was mainly determined via peritoneoscopy and/or liver biopsy. Liver biopsy specimens were obtained using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin-eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The size of specimens for examination was more than six portal areas.[17]

Follow-up. The starting time of follow-up was 3 months after the termination of IFN therapy. After that, patients were followed up monthly to tri-monthly in our hospital. Physical examination and biochemical tests were conducted at each examination together with regular check-up. An overnight (12-hour) fasting blood sample or a casual blood sample was taken for routine analyses. These included aminotransferase activities, total cholesterol, platelet counts, and serum HCV RNA level. Three hundred twenty-four patients were lost to follow-up; because the appearance of T2DM and death was not identified in these patients, they were considered as censored data in the statistical analysis.[18] Moreover, patients retreated with antiviral agents were regarded as withdrawals at the time of starting the retreatment of antiviral agents.

Statistical Analysis. The cumulative appearance rate of T2DM was calculated from 3 months after the termination of IFN treatment to the appearance of T2DM using the Kaplan-Meier method. Differences in the development of T2DM were tested using the log rank test. Independent factors associated with the incidence rate of T2DM were analyzed by the Cox proportional hazard model. The following 11 variables were analyzed for potential covariates for incidence of T2DM at the time of termination of IFN therapy at our hospital: age, sex, state of liver disease (chronic hepatitis or liver cirrhosis), body mass index, glucose level, aspartate aminotransferase level, alanine aminotransferase level, type of IFN, total dose of IFN, efficacy of IFN therapy, hypertension, triglyceride level, and total cholesterol level. A P value of less than 0.05 was considered significant. Data analysis was performed using SPSS 11.5 for Windows (SPSS, Chicago, IL).

Results

Patient Characteristics. Table 1 shows the characteristics of the 2,842 HCV-positive patients treated with