## INTRODUCTION

SINCE THE FISCAL year 2002, guidelines for the treatment of patients with viral hepatitis have been compiled annually by the Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis, under the auspice of the Ministry of Health Labor and Welfare of Japan, recruiting many specialists from all over the nation. They have been improved every year with many supplementary issues that have evolved, as our understanding of various aspects of viral hepatitis deepens and treatment options widen with time. For the fiscal year 2008, guidelines have been worked out for a comprehensive standardization of the treatment of chronic hepatitis and cirrhosis due to infection with hepatitis C virus (HCV) in Japan. It is hoped that these guidelines will be accepted widely and implemented for helping as many patients as possible who suffer from sequelae of persistent HCV infection.

Here, we relate excerpts of the 2008 guidelines for the treatment of patients with HCV-induced liver disease covering a wide range from those with normal aminotransferase levels to those with decompensated cirrhosis.

## GUIDELINES FOR THE PRIMARY TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS C

TABLE 1 SUMMARIZES the antiviral therapy of treatment-naive patients with chronic hepatitis C. In comparison with previous guidelines, the duration of combined treatment with pegylated interferon (Peg-IFN) and ribavirin is extended to 48–72 weeks for patients infected with HCV of genotype 1 in high viral loads (HVL: ≥5 log IU/mL by the Japanese criteria).[1,2] For patients infected with HCV of genotype 2 in HVL, Peg-IFN-α2b and ribavirin for 24 weeks are indicated. To patients with HCV-1 in low viral loads (LVL: <5 log IU/mL), either the standard IFN (not conjugated with polyethylene glycol) for 24 weeks, or the weekly monotherapy with Peg-IFN-α2a for 24–48 weeks, is given.[3] Patients with HCV-2 in LVL receive either the standard IFN for 8–24 weeks, or the weekly monotherapy with Peg-IFN-α2a for 24–48 weeks.

## GUIDELINES FOR THE RE-TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS C

FOR PATIENTS WHO receive re-treatment, first, it is imperatively prerequisite to: (i) identify factors for non-response to previous treatments; and (ii) decide whether to aim for clearance of HCV or to prevent the progression of hepatitis that can accelerate the development of hepatocellular carcinoma (HCC), and this can be monitored by alanine aminotransferase (ALT) and α-fetoprotein (AFP) levels toward normalizing or stabilizing their levels (Table 2).[4] Second, IFN combined with ribavirin is the mainstay of re-treatment of patients with chronic hepatitis C. Third, long-term IFN monotherapy is recommended to patients who are not indicated to IFN/ribavirin or who have failed to respond to the combination therapy. However, some patients do not tolerate IFN due to side-effects or their complicating morbidities. In addition, IFN monotherapy does not always improve ALT levels. Such patients need to receive liver supportive therapy including stronger neominophagen C (SNMC)[5] and ursodeoxycholic acid (UDCA),[6] as well as phlebotomy, either alone or in combination. Therapeutic target ALT levels are: (i) within ×1.5 the upper limit of normal (ULN) for patients in fibrosis stage 1 (F1); and (ii) less than 30 IU/L in those in fibrosis stages 2 or 3 (F2/F3), as far as possible.

Table 1 Guidelines for the primary treatment of patients with chronic hepatitis C

| Genotypes | Genotype 1 | Genotype 2 |
|---|---|---|
| Viral loads | | |
| High viral load<br>≥5.0 log IU/mL<br>≥300 fmol/L<br>≥1 Meq/mL | • Peg-IFN-α2b (Peg-Intron) + ribavirin (Rebetol) for 48–72 weeks<br>• Peg-IFN-α2a (Pegasys) + ribavirin (Copegus) for 48–72 weeks | • Peg-IFN-α2b (Peg-Intron) + ribavirin (Rebetol) for 24 weeks |
| Low viral load<br><5.0 log IU/mL<br><300 fmol/l<br><1 Meq/mL | • Standard IFN for 24 weeks<br>• Peg-IFN-α2a (Pegasys) for 24–48 weeks | • Standard IFN for 8–24 weeks<br>• Peg-IFN-α2a (Pegasys) for 24–48 weeks |

Peg IFN, pegylated interferon.

Table 2 Guidelines for re-treatment of chronic hepatitis C

Principles
Selection has to be made between termination of HCV infection and normalization/stabilization of ALT as well as AFP levels (toward preventing aggravation of liver disease and development of HCC), after evaluating factors for non-response in the primary IFN treatment.

1 "IFN plus ribavirin" is the mainstay of re-treatment of patients who have failed to respond to the primary IFN therapy.
2 Long-term IFN is recommended to patients in whom ribavirin is not indicated or who have failed to respond to IFN/ribavirin; self-injection at home is approved for IFN-α (not for Peg-IFN).
3 Patients who are not indicated to IFN or have failed to improve ALT and AFP levels, in response to IFN, receive liver supportive therapy (SNMC, UDCA) and phlebotomy, either alone or in combination.
4 For preventing aggravation of liver disease (and development of HCC), ALT levels need to be controlled within 1.5 × ULN in patients in stage 1 fibrosis (F1), and as far as possible, 30 IU/L or lower in those in fibrosis stages 2–3 (F2/F3).
5 In treatment combined with ribavirin, dose and mode need to be selected, taking into consideration factors contributing to the response, such as age, sex, progression of liver disease, mutations in the HCV genome (amino acid substitutions in the core protein [aa70/aa91] and ISDR) and HCV RNA titers determined by the real-time PCR.

AFP, α-fetoprotein; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; PCR, polymerase chain reaction; Peg-IFN, pegylated interferon; SNMC, stronger neo-minophagen C; UDCA, ursodeoxycholic acid; ULN, upper limit of normal.

## SUPPLEMENTS TO GUIDELINES FOR THE TREATMENT OF CHRONIC HEPATITIS C

IN THE FISCAL year 2009, the following items were supplemented to the treatment of chronic hepatitis C (Table 3).

1 The treatment of patients infected with HCV-1 in HVL with Peg-IFN/ribavirin for 72 weeks is modified by the early virological response (EVR) within 12 weeks after the start. Patients who have remained positive for HCV RNA detectable by the real-time polymerase chain reaction at 12 weeks after the start of treatment, but who turn negative for HCV RNA till 13–36 weeks on treatment.[7]
2 Patients with HCV-1 in HVL who fail to clear HCV RNA detectable by real-time PCR but in whom ALT levels normalize are continued on Peg-IFN/ribavirin until 48 weeks, so that normalized ALT levels endure longer after the completion of therapy.[7]
3 Patients who are not indicated to Peg-IFN/ribavirin, or who have failed to respond to previous treatments, receive long-term IFN monotherapy. During the first 2 weeks, IFN in the conventional dose is given daily or three times a week. Patients who do not clear HCV RNA during the maximal treatment period of 8 weeks receive half the conventional dose of IFN indefinitely.[8]

## GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS C IN NORMAL ALT LEVELS

AS IN PREVIOUS guidelines, patients with chronic hepatitis C having normal ALT levels are stratified into four groups by ALT levels and platelet counts (Table 4). Patients with chronic hepatitis C who have normal ALT levels are reported to gain the sustained virological response (SVR) to antiviral treatments comparably frequently as those having elevated ALT levels. Taking this into consideration, patients with ALT levels of 30 IU/L or less and platelet counts of $150 \times 10^3/mm^3$ or more are followed for ALT every

Table 3 Supplements to guidelines for chronic hepatitis C

1 Criteria for extending the duration of Peg-IFN/ribavirin (to 72 weeks) in patients infected with HCV-1b in HVL: patients who have remained positive for HCV RNA detectable by the real-time polymerase chain reaction at 12 weeks after the start of treatment, but who turn negative for HCV RNA till 13–36 weeks on treatment.
2 Patients with HCV-1b in HVL who fail to lose HCV RNA detectable by real-time PCR but in whom ALT levels normalize by 36 weeks, Peg-IFN/ribavirin is given till 48 weeks for maintaining normalized ALT levels long after the completion of treatment.
3 Long-term IFN monotherapy in patients who are not indicated to Peg-IFN/ribavirin, or have failed to respond to it, the usual dose of IFN daily or three times in week is given for the first 2 weeks, and when HCV RNA does not disappear within the maximal duration of 8 weeks, long-term treatment with half the usual dose of IFN is continued indefinitely.

ALT, alanine aminotransferase; HCV, hepatitis C virus; HVL, high viral loads; PCR, polymerase chain reaction; Peg-IFN, pegylated interferon.

Table 4 Guidelines for the treatment of patients with normal ALT levels toward preventing the development of HCC

| Platelets | $\geq 150 \times 10^3/mm^3$ | $<150 \times 10^3/mm^3$ |
|---|---|---|
| ALT | | |
| ≤30 IU/l | • Follow for ALT every 2–4 months.<br>• If ALT levels elevate, start antiviral treatments taking into consideration the possibility of SVR and risk for HCC. | • Liver biopsy, if possible, and consider antiviral treatments for patients in A2/F2.<br>• Follow for ALT every 2–4 months, and consider antiviral treatments when ALT levels elevate, for patients without biopsy. |
| 31–40 IU/L | • Consider antiviral treatments for patients younger than 65 years. | • Start treatments for chronic hepatitis C.<br>• Select treatments according to genotypes, viral load, age of patients, etc. |

ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; SVR, sustained virological response.

2–4 months. If ALT levels increase in them, antiviral treatments are considered based on the possibility of resolving HCV infection and the risk for developing HCC. In view of significant fibrosis present in patients with platelet counts of less than $150 \times 10^3/mm^3$, they are recommended to receive liver biopsy, if this is possible. Patients in fibrosis stage F2 or higher are evaluated for the indication to antiviral treatments. Patients with ALT levels between 31 and 40 IU/L are classified by platelet counts. Antiviral treatments are considered in those aged younger than 65 years who have platelet counts of $150 \times 10^3/mm^3$ or more, while guidelines for patients with chronic hepatitis are applied to those with platelet counts of less than $150 \times 10^3/mm^3$.[10]

## GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CIRRHOSIS DUE TO HCV

PATIENTS WITH COMPENSATED cirrhosis who are not infected with HCV-1 in HVL receive either IFN-β or IFN-α (Table 5). Since the fiscal year 2008, IFN-α has been approved for the treatment of patients infected with HCV-1 in HVL, with the aim of resolving infection and normalizing ALT as well as AFP levels by long-term therapy. Treatment duration was set at 1 year or longer, and because the longer the treatment duration the higher the SVR rate, 36 weeks has been recommended as the optimal treatment duration. Because the normalization of ALT/AST is important, even in patients who fail to clear HCV infection by these therapeutic regimens, treatment is better conducted for maintaining normal ALT/AST levels. Guidelines for maintaining liver function for preventing the development of HCC include liver supportive therapy with glycyrrhizin[5] and UDCA,[6] either alone or in combination. For treatment toward suppressing the development of HCC, branched chain amino acids (BCAA)[11] or phlebotomy are adopted. Also, nutrient supplements are applied for stabilizing liver function.

## SUPPLEMENTS TO GUIDELINES FOR THE TREATMENT OF CIRRHOSIS DUE TO HCV

THE FOLLOWING ITEMS have been appended to supplement guidelines for the treatment of type C cirrhosis (Table 6).

Table 5 Guidelines for treatment of type C cirrhosis

Principles
- Compensated: termination of HCV infection
- Decompensated: reversal to compensation and prevention of HCC

Methods
(1) Eradication of HCV and normalization of ALT/AST (for patients with compensated cirrhosis).
  a) HCV-1b in HVL (≥5 log IU/mL)
    IFN-α (Sumiferon)
  b) Others
    IFN-α (Sumiferon)
    IFN-β (Feron)
(2) Maintenance of liver function (improvement of ALT/AST and albumin) for preventing HCC.
  a) Liver supportive therapy
    Stronger neo-minophagen C (SNMC), ursodeoxycholic acid (UDCA), etc.
  b) Branched chain amino acids (BCAA [Livact])
  c) Phlebotomy
(3) Supplementation with nutrients (for stabilizing liver function in decompensated cirrhosis).

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HVL, high viral loads; IFN, interferon.

Table 6 Supplements to guidelines for type C cirrhosis

1 To start with, IFN for compensated cirrhosis is desired at 6 MIU daily for 2–8 weeks, as far as possible, and to continue for 48 weeks or longer, as for chronic hepatitis C.
2 In patients with compensated cirrhosis who fail to clear HCV RNA within 12 weeks on IFN, long-term therapy at 3 MIU should be considered for preventing HCC.
3 In patients with platelet counts $<50 \times 10^3/mm^3$, splenectomy or embolization of splenic artery is recommended before re-treatment, and after thorough evaluation has been made on the response to IFN to be expected.
4 For the prevention of HCC, not only IFN, but also liver supportive therapy (SNMC, UDCA, etc.), phlebotomy and branched chain amino acids, either alone or in combination, are recommended for improving ALT/AST and AFP levels.

AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; MIU, million international units; SNMC, stronger neo-minophagen C; UDCA, ursodeoxycholic acid.

1 For treatment of type C cirrhosis with IFN, the initial dose of 6 million international units (MIU) daily is continued as long as possible (2–8 weeks). Thereafter, long-term IFN for 48 weeks or longer is desired as in the treatment of chronic hepatitis C.
2 In the treatment of type C cirrhosis, patients who fail to achieve EVR with the clearance of HCV RNA from serum within 12 weeks should receive long-term IFN at a dose of 3 MIU.
3 For patients with type C cirrhosis who have platelet counts of less than $50 \times 10^3/mm^3$, splenectomy or embolization of the splenic artery is desirable before commencing IFN therapy, after the efficacy of IFN has been evaluated thoroughly.[12]
4 For preventing the development of HCC, improvement in ALT, AST and AFP levels are aimed. Toward this end, not only IFN, but also liver supportive therapy (SNMC and UDCA), phlebotomy and BCAA are used, either alone or in combination.

## DISCUSSION AND CONCLUSION

THE STUDY GROUP for the Standardization of Treatment of Viral Hepatitis including Cirrhosis, organized by the Ministry of Health, Labor and Welfare of Japan, has compiled a series of guidelines for the treatment of liver disease due to HCV ranging from chronic hepatitis to cirrhosis of various severities for the fiscal year 2008. The principal aim of these guidelines is to decrease the incidence of HCC due to HCV infection in Japan. In accord with this principle, supplements have been added to previous guidelines for the standardization of treatment of chronic hepatitis C. They are prepared on evidence-based data that have been accumulated by members and cooperators of the study group. It is necessary to improve these guidelines in the next fiscal year and thereafter, in accordance with many pieces of new evidence that are expected to emerge through enduring efforts of members and cooperators of the study group.

In the treatment of chronic hepatitis C, the duration of antiviral treatments is extended to 72 weeks, which has been approved as of the fiscal year 2008, and criteria for the eligibility of extended treatment duration are clearly defined. Long-term antiviral treatments, extended up to 72 weeks, are hoped to increase the SVR even further. In addition, comprehensive guidelines for the treatment of cirrhosis have been improved with substantial additions, and their criteria for the indication made explicit.

The Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis has drafted, and also displayed online (www.jsh.or.jp/medical/index.html [in Japanese]), guidelines for a spectrum of liver diseases due to HCV, from chronic hepatitis to cirrhosis of various severities. In view of the common goal of decreasing the incidence of HCC due to HCV infection, supplementation and adjustment are appended to previous guidelines, and new guidelines have been constructed for the treatment of cirrhosis due to HCV infection. As a general rule, antiviral treatments constitute the main body of guidelines for the treatment of chronic hepatitis C. Furthermore, the fundamental concept of these guidelines would need to be kept in mind always. It is our sincere hope that, for the treatment of each patient, readers will base their clinical practice on these guidelines, and refer to appropriate individual guidelines, when they make a decision on the treatment strategy, on a case-by-case basis. With respect to guidelines for the treatment of patients with cirrhosis, above all, expected achievable outcomes have to be taken into account in treatment choice.

It is our sincere desire that treatment of patients with chronic hepatitis and cirrhosis due to HCV will proceed following these guidelines. Efforts along these lines will rectify a wide gap in medical treatment served to the nation and raise substantial and efficient interest in the medical economy on the national basis. In practicing treatment according to these guidelines, it will be nec-

essary to evaluate their therapeutic efficacy, and revise or add necessary supplements to them as required in the future.

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# A Matched Case-Controlled Study of 48 and 72 Weeks of Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV Genotype 1b in Japan: Amino Acid Substitutions in HCV Core Region as Predictor of Sustained Virological Response

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Substitution of amino acid (aa) 70 and 91 in the core region of HCV genotype 1b is a useful pretreatment predictor of efficacy of 48-week peginterferon (PEG-IFN) plus ribavirin (RBV) therapy. Here, we determined the efficacy of 72-week PEG-IFN/RBV and the predictive factors to such therapy in a case–control study matched for sex, age, and periods from the start of treatment to initial point of HCV RNA-negative. We compared the treatment efficacy of 72-week regimen in 65 patients with that of 48-week in 130 patients, who were infected with HCV genotype 1b and treated with PEG-IFN/RBV. They consisted mainly of late virological responders (LVR) (HCV RNA-positive at 12 weeks and negative at 24 weeks after start of treatment). Sustained virological response (SVR) was achieved by 61.5% and 32.3% of patients of the 72- and 48-week groups, respectively, while non-virological response was noted in 9.2% and 29.2% of the respective groups. Multivariate analysis identified substitution of aa 70 and 91 (Arg70 and/or Leu91) and duration of treatment (72-week) as independent parameters that significantly influenced SVR. For Arg70 and/or Leu91 of core region, SVR rate was significantly higher in 72- (68.0%) than 48-week group (37.8%). For wild-type of ISDR, SVR rate was significantly higher in 72- (61.2%) than in 48-week group (29.3%). We conclude that 72-week PEG-IFN/RBV improves SVR rate for LVR, especially those with Arg70 and/or Leu91 of core region or wild-type of ISDR. Substitution of aa 70 and 91 is also a useful pretreatment predictor of response to 72-week PEG-IFN/RBV. ***J. Med. Virol. 81:452–458, 2009.*** © 2009 Wiley-Liss, Inc.

**KEY WORDS:** HCV; core region; NS5A-ISDR; peginterferon; ribavirin; 72-week; case–control study; LVR



## 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]. In patients with HCV-chronic hepatitis, treatment with interferon (IFN) can induce viral clearance and marked biochemical and histological improvement [Davis et al., 1989; Di Bisceglie et al., 1989]. Especially, peginterferon (PEG-IFN) plus ribavirin (RBV) combination therapy for 48 weeks can achieve a high sustained virological response (SVR) [Manns et al., 2001; Fried et al., 2002].

Although treatment of genotype 1-infected patients typically extends over 48 weeks, there has been interest in prolongation of therapy, particularly in late

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virological responders (LVR) (HCV RNA-positive at 12 weeks and negative at 24 weeks after the start of treatment), because high relapse rates in LVR may indicate that treatment was not administered for a sufficient duration [Ferenci et al., 2005]. Previous studies from Europe and United States have demonstrated that LVR improves SVR rates when treatment is extended to 72 weeks, compared with standard duration of therapy, largely as a result of reducing posttreatment relapse rates [Buti et al., 2003; Berg et al., 2006; Sánchez-Tapias et al., 2006; Pearlman et al., 2007]. Thus, prolongation of therapy in LVR may improve the virological response rate. However, it is not clear at present whether prolongation of treatment improves the SVR rate of treatment-resistant Japanese patients infected with HCV/genotype 1b [Akuta et al., 2007a,b,c].

Previous studies indicated that amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of genotype 1b were predictors of poor virological response to 48-week PEG-IFN plus RBV therapy [Akuta et al., 2005, 2006, 2007a,b,c; Donlin et al., 2007], and also risk factors for hepatocarcinogenesis [Akuta et al., 2007d, 2008a]. However, it is not clear at this stage whether aa substitutions in the core region can be used before therapy to predict the outcome of 72-week regimen.

The aims of the present study in HCV genotype 1b-infected Japanese adult patients, who received PEG-IFN plus RBV, were the following: (1) To conduct a case–control study matched for sex, age, and periods from the start of treatment to the initial point of HCV RNA-negative, to compare the treatment efficacy of 72-week regimen and 48-week regimen. (2) To identify the pretreatment factors that could predict treatment efficacy of the 72-week regimen, including pretreatment aa substitutions in the core region.

## PATIENTS AND METHODS

### Study Population

A total of 559 HCV genotype1b-infected Japanese adult patients were consecutively recruited into the study protocol of combination therapy with PEG-IFNα-2b plus RBV between 2001 and 2008 at Toranomon Hospital, Tokyo, Japan. They received PEG-IFNα-2b at a median dose of 1.4 μg/kg (range, 0.7–2.1 μg/kg) subcutaneously each week plus oral RBV at a median dose of 11.1 mg/kg (range, 3.4–16.0 mg/kg) daily. Among these, 383 patients, who could complete a total of 48 or 72 weeks of combination therapy, were enrolled in this retrospective study. The latter group consisted of 65 patients who extended combination therapy to 72-week (72-week group), and 318 patients who stopped combination therapy at the 48 weeks (48-week group). The decision to extend the combination therapy to 72 weeks was made by the patient. To compare the efficacy of the 72- and 48-week courses, all 65 patients of the 72-week group entered this study along with 130 patients of 48-week. The latter group was selected from among the 318 because they matched those patients of the 72-week group with respect to sex, age, and periods from the start of treatment to the initial point of HCV RNA-negative (matched case–control study). 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). Furthermore, LVR was defined as HCV RNA-positive at 12 weeks and negative at 24 weeks after the start of treatment, based on qualitative PCR analysis. All patients fulfilled 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, Manheim, Germany). (2) Infection with HCV genotype 1b only. (3) A high viral load ($\geq 100 \times 10^3$ IU/ml) by quantitative analysis of HCV RNA with PCR (AMPLICOR GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems Inc., Pleasanton, CA) within the preceding 2 months of enrolment. (4) No hepatocellular carcinoma. (5) Body weight > 40 kg. (6) Lack of coinfection with human immunodeficiency virus. (7) No previous treatment with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (8) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg. (9) None had other forms of liver diseases, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, or autoimmune liver disease. (10) None of the females was pregnant or a lactating mother. (11) All patients had completed a 24-week follow-up program after cessation of treatment, and SVR could be evaluated. (12) Each signed a consent form of the study protocol that had been approved by the human ethics review committee. The profile and laboratory data of 195 patients, who entered the matched case–control study, are summarized in Table I.

### 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 $5.0 \times 10^3$ to $5.0 \times 10^6$ 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.),

TABLE I. Patient Profile and Laboratory Data at Commencement of 48- and 72-Week Combination Therapy of Peginterferon Plus Ribavirin in Patients Infected With HCV Genotype 1b (Matched Case–Control Study)

| | 72-week group | 48-week group | |
|---|---|---|---|
| Matching data | | | |
| Number of patients | 65 | 130 | |
| Sex (M/F) | 28/37 | 57/73 | Matched |
| Age (years)* | 57 (22–70) | 56 (25–68) | Matched |
| Periods to the initial point of HCV RNA-negative (weeks)* | 17.4 (5.9–72.0) | 19.7 (6.0–48.0) | Matched |
| Demographic data | | | |
| History of blood transfusion | 18 (27.7%) | 42 (32.3%) | NS |
| Family history of liver disease | 21 (32.3%) | 31 (23.8%) | NS |
| Body mass index ($kg/m^2$)* | 22.6 (16.6–38.0) | 22.2 (17.0–32.4) | NS |
| Laboratory data* | | | |
| Serum aspartate aminotransferase (IU/L) | 49 (23–213) | 51 (21–217) | NS |
| Serum alanine aminotransferase (IU/L) | 64 (25–430) | 68 (20–391) | NS |
| Serum albumin (g/dl) | 3.9 (3.2–4.5) | 3.8 (3.2–4.6) | NS |
| Gamma-glutamyl transpeptidase (IU/L) | 40 (14–171) | 38 (15–581) | NS |
| Leukocytes ($/mm^3$) | 4,400 (2,300–8,800) | 4,600 (1,200–9,400) | NS |
| Hemoglobin (g/dl) | 14.0(11.3–17.8) | 13.9 (10.6–18.1) | NS |
| Platelet count ($\times 10^4/mm^3$) | 16.2 (8.2–30.7) | 15.8 (6.4–31.6) | NS |
| ICG R15 (%) | 13 (2–73) | 15 (2–45) | NS |
| Level of viremia (KIU/ml) | 2,650 (52->5,000) | 1,850 (49->5,000) | 0.013 |
| Alfa-fetoprotein ($\mu g/L$) | 6 (2–47) | 6(2–110) | NS |
| Total cholesterol (mg/dl) | 174(111–276) | 175 (104–274) | NS |
| High density lipoprotein cholesterol (mg/dl) | 45 (27–86) | 51 (24–78) | NS |
| Low density lipoprotein cholesterol (mg/dl) | 104 (49–204) | 107 (50–182) | NS |
| Triglycerides (mg/dl) | 91 (35–259) | 94 (35–315) | NS |
| Uric acid (mg/dl) | 5.3 (2.6–7.7) | 5.0 (2.3–8.7) | NS |
| Fasting blood sugar (mg/dl) | 95 (79–218) | 98 (76–157) | NS |
| Histological findings | | | |
| Stage of fibrosis (F1/F2/F3/ND) | 20/12/11/1/21 | 44/27/22/0/37 | NS |
| Hepatocyte steatosis (none to mild/moderate to severe/ND) | 40/2/23 | 78/8/44 | NS |
| Treatment | | | |
| PEG-IFN α-2b dose (μg/kg)* | 1.4 (0.8–2.1) | 1.4 (0.7–1.9) | NS |
| Ribavirin dose (mg/kg)* | 10.9 (6.6–16.0) | 10.8 (3.7–14.2) | NS |
| Amino acid substitutions in the HCV | | | |
| Core aa 70 (arginine/glutamine (histidine)/ND) | 37/23/5 | 11 47/6 | NS |
| Core aa 91 (leucine/methionine/ND) | 42/18/5 | 66/57/7 | 0.038 |
| ISDR of NS5A (wild-type/mutant-type/ND) | 49/5/11 | 99/17/14 | NS |

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values. ND: not determined.

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), 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 examinations contained six or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al. [1994]. Hepatocyte steatosis was graded as either none (absent), mild (less than 1/3 of hepatocytes involved), moderate (greater than 1/3 but less than 2/3 of hepatocytes involved), or severe (greater than 2/3 of hepatocytes involved) [D'Alessandro et al., 1991].

### 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 ($\leq 1$) or mutant-type ($\geq 2$).

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). 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 (Mann–Whitney *U*-test, chi-squared test and Fisher's exact probability test) were used to compare the characteristics of the groups. Univariate and multivariate logistic regression analyses were used to determine those factors that significantly contributed to SVR. The odds ratios and 95% confidence intervals (95% CI) were also calculated. All *P* values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. The potential pretreatment predictive factors associated with SVR included the following 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, platelets, indocyanine green retention rate at 15 min (ICG R15), level of viremia, alfa-fetoprotein, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, uric acid, fasting blood sugar, hepatocyte steatosis, stage of fibrosis, PEG-IFN dose/body weight, RBV dose/body weight, duration of treatment, and amino acid substitution in the core and ISDR of NS5A. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

## RESULTS

### Comparison of Treatment Efficacy Between 48-Week Group and 72-Week Group

Figure 1 shows comparison of the treatment efficacy between 48- and 72-week groups. SVR was achieved by 42 of 130 patients (32.3%) and 40 of 65 (61.5%) in the 48- and 72-week groups, respectively. The proportion of SVR was significantly higher in 72-week group than in the 48-week group ($P < 0.001$). Furthermore, NVR was identified in 38 of 130 patients (29.2%) and 6 of 65 (9.2%) in the 48- and 72-week groups, respectively. The proportion of NVR was significantly lower in the 72-week group than in 48-week group ($P = 0.002$).

### Predictive Factors Associated With SVR in Multivariate Analysis

Univariate analysis identified 13 parameters that influenced SVR either significantly or marginally: gender (female sex; $P = 0.002$), stage of fibrosis ($F_{1,2}$; $P = 0.008$), PEG-IFN dose/body weight (≥1.4 μg/kg; $P = 0.001$), RBV dose/body weight (≥11.0 mg/kg; $P = 0.029$), platelet count ($\geq 15.0 \times 10^4/mm^3$; $P = 0.002$), level of viremia (<1,000 KIU/ml; $P = 0.049$), γGTP (<50 IU/L; $P = 0.026$), ICG R15 (<15%; $P = 0.003$), triglycerides (<100 mg/dl; $P = 0.038$), high-density lipoprotein cholesterol (≥50 mg/dl; $P = 0.018$), α-fetoprotein (<20 μg/L; $P = 0.005$), substitution of aa 70 and 91 (Arg70 and/or Leu91; $P = 0.002$), and duration of treatment (72-week group; $P < 0.001$).

Multivariate analysis identified three independent parameters that either significantly influenced or tended to significantly influence SVR; substitution of aa 70 and 91 (Arg70 and/or Leu91; $P = 0.015$), duration of treatment (72-week group; $P = 0.014$), and high-density lipoprotein cholesterol (≥50 mg/dl; $P = 0.084$) (Table II).

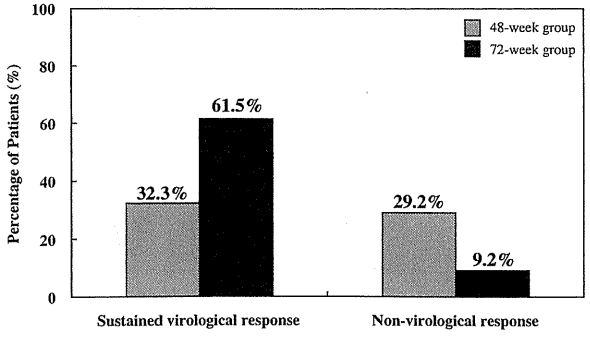


Fig. 1. Comparison of treatment efficacy between the 48-week group and 72-week group. The proportion of patients with sustained virological response in 72-week group was significantly higher than in 48-week group ($P < 0.001$). Furthermore, the proportion of patients with non-virological response in 72-week group was significantly lower than in 48-week group ($P = 0.002$).

TABLE II. Factors Associated With Sustained Virological Response to Combination Therapy of Peginterferon Plus Ribavirin in 195 Patients Infected With HCV Genotype1b, Identified by Multivariate Analysis

| Factor | Category | Odds ratio (95% CI) | *P* |
|---|---|---|---|
| Substitution of aa 70 and 91 | 1: Gln70 (His70) and Met91 | 1 | |
| | 2: Arg70 and/or Leu91 | 5.46 (1.39–21.3) | 0.015 |
| Duration of treatment (weeks) | 1: 48 | 1 | |
| | 2: 72 | 3.51 (1.28–9.62) | 0.014 |
| HDL-cholesterol (mg/dl) | 1: <50 | 1 | |
| | 2: ≥50 | 2.42 (0.89–6.58) | 0.084 |

*Only variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on multivariate logistic regression are shown.

### Treatment Efficacy According to Amino Acid Substitutions in Core Region

Figure 2 shows comparison of the treatment efficacy according to aa substitutions in the core region. In Gln70 (His70) and Met91, SVR was achieved by 4 of 26 patients (15.4%) and 3 of 10 (30.0%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was not significantly different than in 48-week group. In Arg70 and/or Leu91, SVR was achieved by 37 of 98 patients (37.8%) and 34 of 50 (68.0%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was significantly higher than in 48-week group ($P = 0.001$).

### Treatment Efficacy According to Amino Acid Substitutions in NS5A-ISDR

Figure 3 shows comparison of the treatment efficacy according to aa substitutions in NS5A-ISDR. In mutant-type, SVR was achieved by 9 of 17 patients (52.9%) and 3 of 5 (60.0%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was not significantly different from that in 48-week group. In wild-type, SVR was achieved by 29 of 99 patients (29.3%) and 30 of 49 (61.2%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was significantly higher than that in 48-week group ($P < 0.001$).

## DISCUSSION

This matched case-controlled study of PEG-IFN plus RBV for LVR infected with HCV genotype 1b, showed that treatment extension to 72 weeks seems to improve SVR rates in Japanese patients. To our knowledge, the present study is the first to report that 72-week regimen of PEG-IFN plus RBV might be also useful in Asians. Especially, the 72-week regimen significantly improved the SVR rates in LVR with Arg70 and/or Leu91 of core or wild-type of ISDR. The present study based on patients, who could complete a total of 48 or 72 weeks of combination therapy, did not show the frequencies of patients, who could not complete by side effects. Patients, who dropped out by side effects between 48 and 72 week for therapy prolonged to 72 weeks, were only 3 of 559 HCV genotype1b-infected Japanese adult patients (data not shown), so the frequencies of side effects with 72-week regimen might be nearly equal to those with 48-week regimen. Large-scale prospective study based on the intention to treat analysis should be conducted to confirm the above finding in future.

NS5A-ISDR, reported as predictor of treatment efficacy with IFN monotherapy by Enomoto et al. [1995, 1996], is also useful as predictor of 48-week PEG-IFN plus RBV combination therapy [Murayama et al., 2007; Shirakawa et al., in press; Yen et al., 2008]. Furthermore, the present study also indicated that

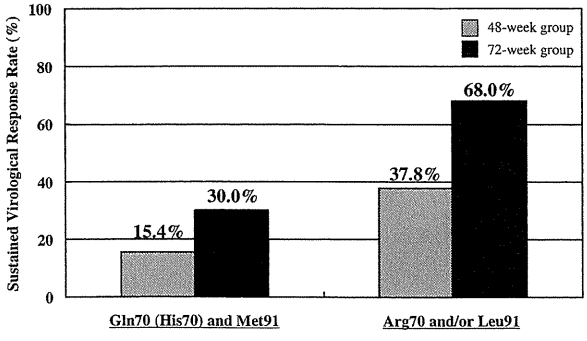


Fig. 2. Comparison of treatment efficacy according to amino acid substitutions in the core region. In Gln70 (His70) and Met91, the proportion of patients with sustained virological response in 72-week group was not significantly different from that in 48-week group. However, in Arg70 and/or Leu91, the proportion of patients with sustained virological response in 72-week group was significantly higher than in 48-week group ($P = 0.001$).

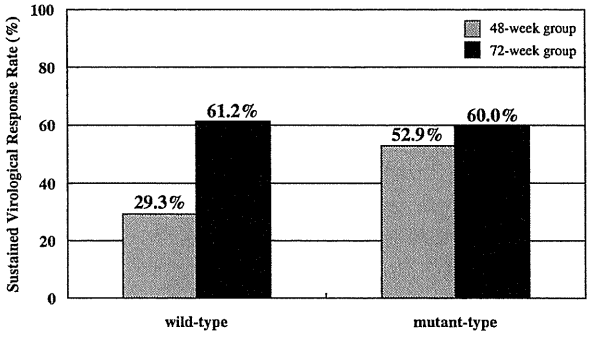


Fig. 3. Comparison of treatment efficacy according to amino acid substitutions in NS5A-ISDR. In mutant-type, the proportion of patients with sustained virological response in 72-week group was not significantly different from that in 48-week group. However, in wild-type, the proportion of patients with sustained virological response in 72-week group was significantly higher than in 48-week group ($P < 0.001$).

72-week regimen of PEG-IFN plus RBV significantly improved the SVR rate in LVR with wild-type of ISDR. Unfortunately, the 72-week regimen of PEG-IFN plus RBV did not improve the SVR rate in LVR with Gln70 (His70) and Met91 of the core region. Multivariate analysis also identified Gln70 (His70) and Met91 of the core region as independent parameter that significantly influenced non-SVR. PEG-IFN plus RBV carries potential serious side effects and is costly especially when used long enough to achieve higher SVR rates. For these reasons, we need to identify those patients who do not achieve SVR, to free them of unnecessary side effects and reduce costs, preferably before the start of the combination therapy. For patients unsuitable for PEG-IFN plus RBV, including LVR with Gln70 (His70) and Met91 of the core region, low-dose intermittent IFN monotherapy might be an efficacious therapeutic regimen, because it can lead to ALT normalization and thus reduce the risk of hepatocarcinogenesis [Akuta et al., 2008b].

One limitation of this study is that LVR could not be evaluated by the COBAS AmpliPrep/COBAS TaqMan HCV Test (the lower limit of this assay; 15 IU/ml), which has a higher sensitivity than AMPLICOR HCV v2.0 (the lower limit of this assay; 50 IU/ml) [Sizmann et al., 2007]. Rapid virological response (HCV RNA-negative at 4 weeks after the start of treatment) and early virological response (HCV RNA-positive at 4 weeks and negative at 12 weeks after the start of treatment) by AMPLICOR HCV v2.0 might be diagnosed as LVR by the COBAS AmpliPrep/COBAS TaqMan HCV Test. Further studies using highly sensitive real-time PCR assay should be performed to facilitate the development of more effective therapeutic regimens in future.

We previously reported that viral factors (e.g., aa substitutions in core region) and host factors (e.g., lipid metabolic factors, sex, and AFP) might be important predictors of treatment response to 48-week PEG-IFN plus RBV in Japanese patients infected with HCV genotype 1b, in addition to treatment-related factors (e.g., RBV dose) [Akuta et al., 2005, 2006, 2007a,b,c]. The present study also identified viral (aa substitutions in the core region), host (HDL-cholesterol), and treatment-related factors (duration of treatment) that can be useful as independent and significant pretreatment predictors of SVR. Thus, substitution of aa 70 and 91 is also useful as a pretreatment predictor of 72-week regimen. Further studies that examine the structural and functional impact of aa substitutions during combination therapy should be conducted to confirm the above finding.

Another limitation of our study was that we did not examine aa substitutions in areas other than the core region and NS5A-ISDR of HCV genome, such as the interferon/ribavirin resistance determining region (IRRDR), including V3 of NS5A region, although they should be investigated in future studies [El-Shamy et al., 2008; Muñoz de Rueda et al., 2008].

We conclude that treatment efficacy of 72-week PEG-IFN plus RBV seems to be based on a dynamic tripartite interaction of viral-, host-, and treatment-related factors. Further understanding of the complex interaction between these factors should facilitate the development of more effective therapeutic regimens.

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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.

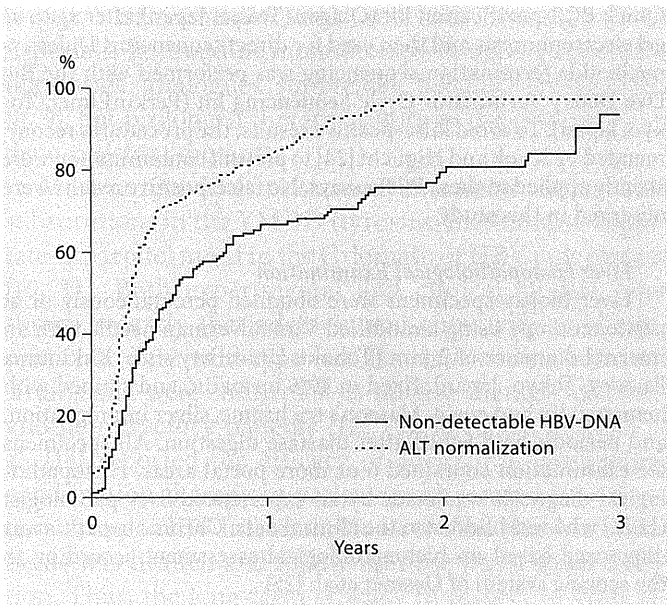


**Fig. 1.** Cumulative HBV-DNA non-detectable and ALT normalization rates. Patients treated lamivudine + adefovir dipivoxil could achieve the excellent virological response (non-detectable HBV-DNA) and biochemical response (ALT normalization) as an indicator of low hepatitis activity.

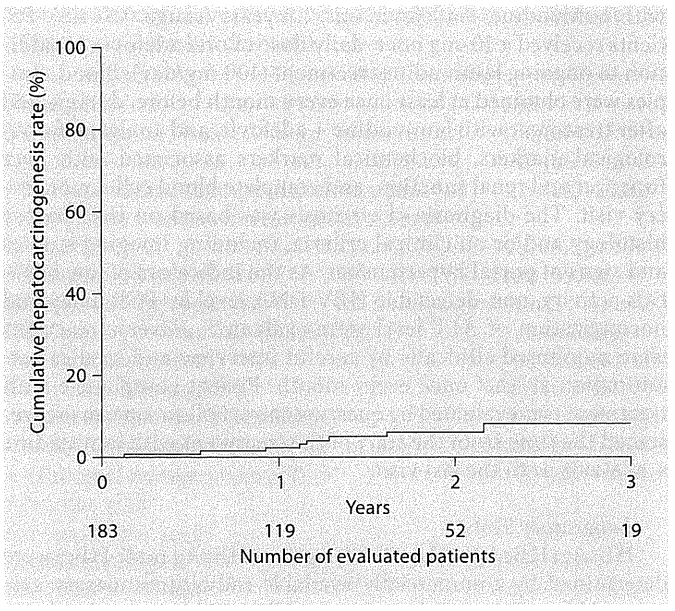


**Fig. 2.** Cumulative hepatocarcinogenesis rates during the treatment of lamivudine + adefovir dipivoxil. The yearly incidence of HCC during the first 3 years was 2.7%.

## 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.

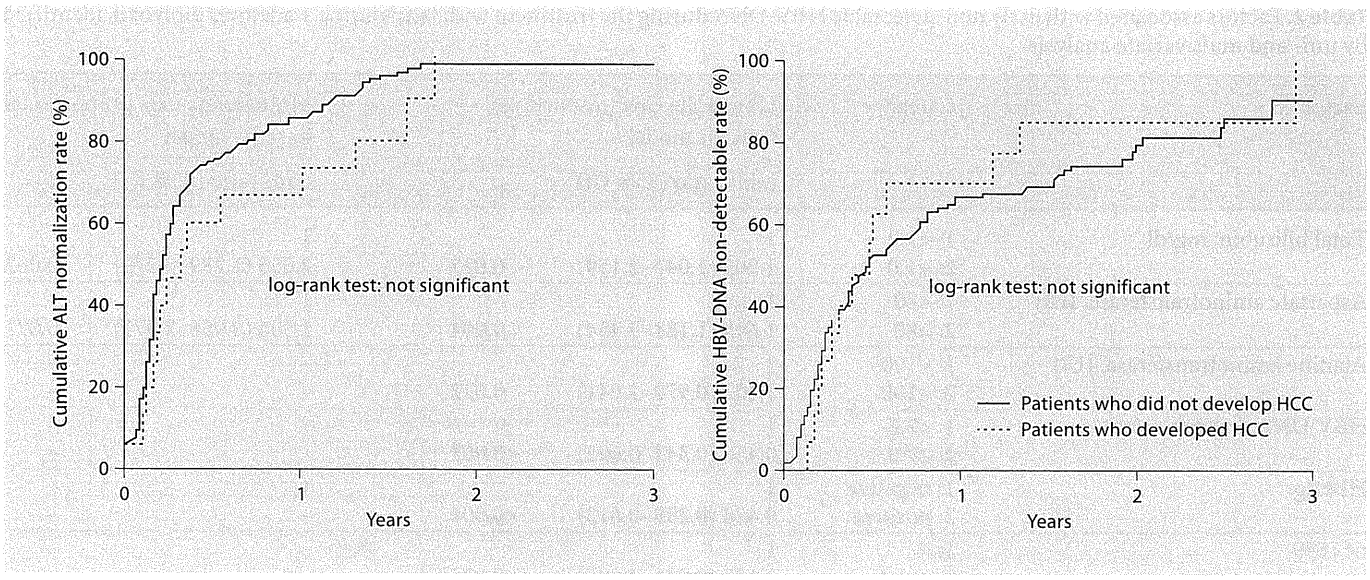


**Fig. 3.** Comparison of non-detectable HBV-DNA and ALT normalization in patients who developed HCC or not. The cumulative HBV-DNA non-detectable and ALT normalization rates were not significantly different according to the development of HCC or not. Low hepatitis activity during the treatment of lamivudine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