Serum HBV RNA is a Predictor of Early Emergence of the YMDD Mutant in Patients Treated with Lamivudine

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Lamivudine (LAM) is a nucleoside analogue widely used for the treatment of chronic hepatitis B virus (HBV) infection. Emergence of resistant strains with amino acid substitutions in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of reverse transcriptase is a serious problem in patients on LAM therapy. The amount of covalently closed circular DNA in the serum is reported to be higher in patients who develop YMDD mutants than in those without mutants. However, there is no useful serum marker that can predict early emergence of mutants during LAM therapy. Analysis of patients who were treated with entecavir (n = 7) and LAM (n = 36) showed some patients had high serum levels of HBV RNA. Median serum levels of HBV RNA were significantly higher in patients in whom the YMDD mutant had emerged within 1 year (n = 6, 1.688 log copies/ml) than in those in whom the YMDD mutant emerged more than 1 year after treatment (n = 12, 0.456 log copies/ml, $P = 0.0125$) or in whom the YMDD mutant never emerged (n = 18, 0.688 log copies/ml, $P = 0.039$). Our results suggest that HBV RNA is a valuable predictor of early occurrence of viral mutation during LAM therapy. (HEPATOLOGY 2007;45:1179-1186.)

The hepatitis B virus (HBV) is a member of the hepadnaviridae family. Worldwide, approximately 350 million people are estimated to be chronically infected with HBV.[1] Patients with chronic HBV infection develop chronic hepatitis, cirrhosis, and hepatocellular carcinoma, accounting for approximately 1 million deaths per year.[2] Recently, inhibitors of reverse transcriptase have been developed and widely used for patients with chronic HBV infection. Lamivudine (LAM), a cytosine nucleoside analogue, was first developed as an antiviral agent against HIV and later was used effectively against HBV because HBV also uses reverse transcriptase for replication.[3,4] Because LAM suppresses HBV replication, patients who are treated with LAM show a decreased level or disappearance of HBV DNA in serum and hepatitis B e antigen, normalization of serum alanine aminotransferase (ALT) level, and histological improvement.[5-12] However, discontinuation of therapy often leads to reactivation of HBV.[6,8,13,14] Therefore, long-term therapy is necessary for many patients with chronic HBV infection. During long-term LAM therapy, drug-resistant mutants with amino acid substitutions in the tyrosine-methionine-aspartate-aspartate (YMDD) motif emerge, resulting in expression of HBV DNA increasing again and in worsening of hepatitis.[6,10,15-18] Moreover, some patients develop a severe flare-up of hepatitis that could lead to fatal hepatic failure. Therefore, prediction of the emergence of YMDD mutants is an important issue.

In our hunt for useful serum markers to detect the early emergence of YMDD mutants, we noticed some patients who showed a discrepancy in the expression of HBV DNA measured by the transcription-mediated amplifica-

Abbreviations: cccDNA, covalently closed circular DNA; ETV, entecavir; HBV, hepatitis B virus; LAM, lamivudine; PCR, polymerase chain reaction; RT, reverse transcription; TMA-HPA, transcription-mediated amplification and hybridization protection assay; YMDD, tyrosine-methionine-aspartate-aspartate.

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Table 1. Clinical Characteristics of the 3 Groups

	Group A	Group B	Group C
Number	6	12	18
Age, median (range)	50 (37-67)	49 (31-66)	49 (27-68)
Sex (M:F)	3:3	9:3	13:5
Observation period (months)	34.5 (13-58)	38 (16-64)	34 (13-58)
Time before emergence of mutants (months)	8.5 (4-11)	19 (13-36)	
HBV DNA (LGE/ml)	7.8 ± 0.95	6.13 ± 0.84	6.64 ± 1.63
Hbe-antigen-positive	4 (66.7%)	6 (50%)	10 (55.6%)
Hbe-antibody-positive	1 (16.7%)	6 (50%)	9 (50%)
ALT (U/l)	136.1 ± 122.8	114.5 ± 104.1	129.8 ± 206.4

Group A: patients who showed early emergence of the mutants (within 1 year).
Group B: patients who developed resistance after 1 year of LAM therapy.
Group C: patients in whom mutants did not develop.

tion and hybridization protection assay (TMA-HPA) and that measured by the Amplicor HBV Monitor test. Because the former method detects both HBV DNA and HBV RNA, we thought that the difference in measurement by the 2 methods was a result of the presence of a large amount of HBV RNA.[19-21] We thus studied patients with chronic HBV infection who were being treated with LAM or entecavir (ETV) for the presence of HBV RNA. We also assumed that the presence of a large amount of HBV RNA would indicate that transcription and virus particle formation were still active in such patients. We thus assessed the value of this indicator in the prediction of the emergence of YMDD mutants during LAM therapy.

Patients and Methods

Patients. We studied 36 patients with chronic hepatitis B who were being treated with LAM from 2001 to 2006 at Hiroshima University Hospital, Kawakami Clinic, and Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital. We also analyzed 7 patients who were being treated with ETV from 2004 to 2006 at Hiroshima University Hospital. No patients showed clinical signs of cirrhosis or hepatocellular carcinoma. They were not treated with other antiviral agents, corticosteroids, or immunosuppressant drugs during LAM/ETV therapy. The LAM-treated patients were 25 men and 11 women whose median age was 52 years (range 27-68 years; Table 1). They were divided into 3 groups (groups A, B, and C) according to how long it took for YMDD mutants to appear. Group A (n = 6) was composed of patients who showed early emergence of the mutants (within 1 year); group B (n = 12) had patients who developed resistance after 1 year of LAM therapy; and group C (n = 18) was composed of patients who did not show resistance to LAM therapy. Each of the 36 patients received 100 mg of LAM daily for 4-58 months (median, 21.5 months). All patients continued LAM therapy throughout the course of the study. Patients in the ETV group were 6 men and 1 woman whose median age was 37 years (32-50 years). They received 0.01-0.5 mg of ETV daily for 21-28 months (median, 25 months), and all patients continued ETV therapy throughout the course of the study. Blood samples were obtained from patients of both groups just before commencement of antiviral therapy and every 4 weeks during therapy. Informed consent was obtained from each patient.

Quantification of HBV DNA. HBV DNA serum level was determined by using the TMA-HPA (Fujirebio Inc., Tokyo, Japan) and the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan). The measurement range of the former assay is $10^{3.7}$-$10^{8.7}$ genome equivalents (GE)/ml (3.7-8.7 LGE/ml),[22] whereas the range of the latter test was $10^{2.6}$-$10^{7.6}$ copies/ml (2.6-7.6 log copies/ml).[23] These quantitative assays of HBV DNA were performed at the Special Reference Laboratory (Tokyo, Japan).

Extraction of Nucleic Acid of HBV and Reverse Transcription. Nucleic acid was extracted from 100 μL of serum by the SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL of H_2O for DNA analysis or 8.8 μL of ribonuclease-free H_2O for RNA analysis. The latter solution was reverse-transcribed by using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan). In the next step, 25 pM of random primer was added to 8.8 μL of nucleic acid extract and heated at 65°C for 5 minutes. The samples were set on ice for 5 minutes. Then 4 μL of 5× reverse transcription (RT) buffer, 2 μL of 10 mM dNTPs, 2 μL of 0.1 M dithiothreitol (DTT), 8 units of ribonuclease inhibitor, and 100 units of M-MLV reverse transcriptase were added to each sample. The reaction mixture was incubated at 30°C for 10 minutes and 42°C for 60 minutes, followed by inactivation at 99°C for 5 minutes.

Quantitative Analysis of HBV DNA by Real-Time Polymerase Chain Reaction. One microliter of DNA solution or cDNA solution was amplified by real-time polymerase chain reaction (PCR) with an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the instructions provided by the manufacturer. Amplification was performed in a 25-μL reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 200 nM of forward primer (5′-TTTGGGGCATGGACATTGAC-3′, nucleotides 1893-1912), 200 nM of reverse primer (5′-GGTGAACAATGGTCCGGAGAC-3′, nucleotides 2029-2049), and 1 μL of DNA or cDNA solution. After incubation for 2 minutes at 50°C, the sample was heated for 10 minutes at 95°C for denaturing, followed by a PCR cycling program consisting of 40 2-step cycles of 15 seconds at 95°C and 60 seconds at 60°C. The lower detection limit of this assay was 10^3 copies/ml.

Confirmation of Presence of HBV RNA in Serum by RNase Digestion. To confirm the presence of HBV RNA, nucleic acid extracted from the serum samples by SMITEST (Genome Science Laboratories, Tokyo) was digested with 1 μg/μL of RNase A (Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 60 minutes, digested with proteinase K (New England Biolabs Inc., Ipswich, MA) at 37°C for 60 minutes, extracted with phenol/chloroform, precipitated with ethanol, and dissolved in water. Treated nucleic acid with or without RNase was analyzed by real-time PCR after reverse transcription with a random primer and reverse transcriptase, as already described.

Detection of YMDD Mutant. Mutations in the YMDD motif of reverse transcriptase of HBV were examined by PCR with peptide nucleic acid clamping, as described previously.[24]

Statistical Analysis. Differences between groups were examined for statistical significance using the Student t test, and correlations of parameters were examined by the Spearman's rank correlation. A difference with a P value less than 0.05 was considered statistically significant. All statistical analyses were performed with StatView version 5.0 (SAS Institute, Cary, NC).

Results

HBV DNA Levels Determined by TMA-HPA and Amplicor HBV Monitor Test During ETV Therapy. High expression of HBV RNA was initially observed by measuring HBV nucleic acid with the TMA-HPA and HBV DNA with the Amplicor HBV monitor test. As shown in Fig. 1, expression of HBV nucleic acid was higher than HBV DNA during the initial 6 months of ETV therapy. We assumed that the discrepancy in the measurements by these 2 methods was a result of the large amount of HBV RNA in the serum because the TMA-HPA measures both HBV DNA and HBV RNA, whereas the Amplicor HBV monitor test detects only HBV DNA. We measured the HBV nucleic acid levels in the 7 patients who received ETV therapy 3 and 6 months after the start of therapy. The HBV nucleic acid levels of all 7 patients determined by the TMA-HPA were 10-100 times higher than those determined by the Amplicor HBV Monitor test except for 2 patients who received a small amount (0.01 mg) of ETV (Fig. 2). The small dif-

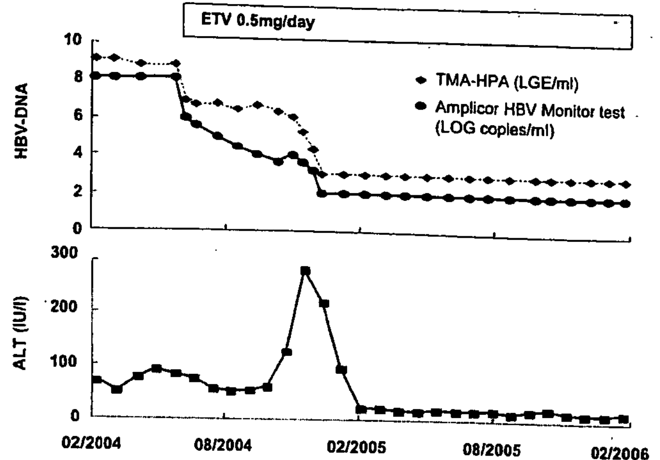

Fig. 1. Time courses of serum HBV DNA and ALT levels of patients treated with ETV. Expression of HBV nucleic acids determined by the TMA-HPA was higher than that determined by the Amplicor HBV Monitor test soon after beginning administration of ETV. The discrepancy was less marked when both measurements were low and when both were negative.

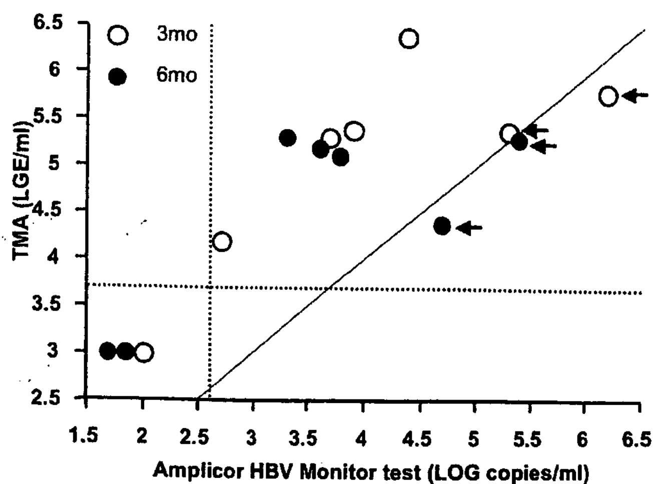

Fig. 2. Correlation of HBV nucleic acid levels determined by the TMA-HPA with HBV DNA levels determined by the Amplicor HBV Monitor test during ETV therapy. Serum samples obtained from the 2 patients who received low-dose ETV (0.01 mg) are indicated by arrows. The vertical and horizontal dotted lines indicate the lower detection limits of the Amplicor HBV Monitor test and the TMA-HPA, respectively.

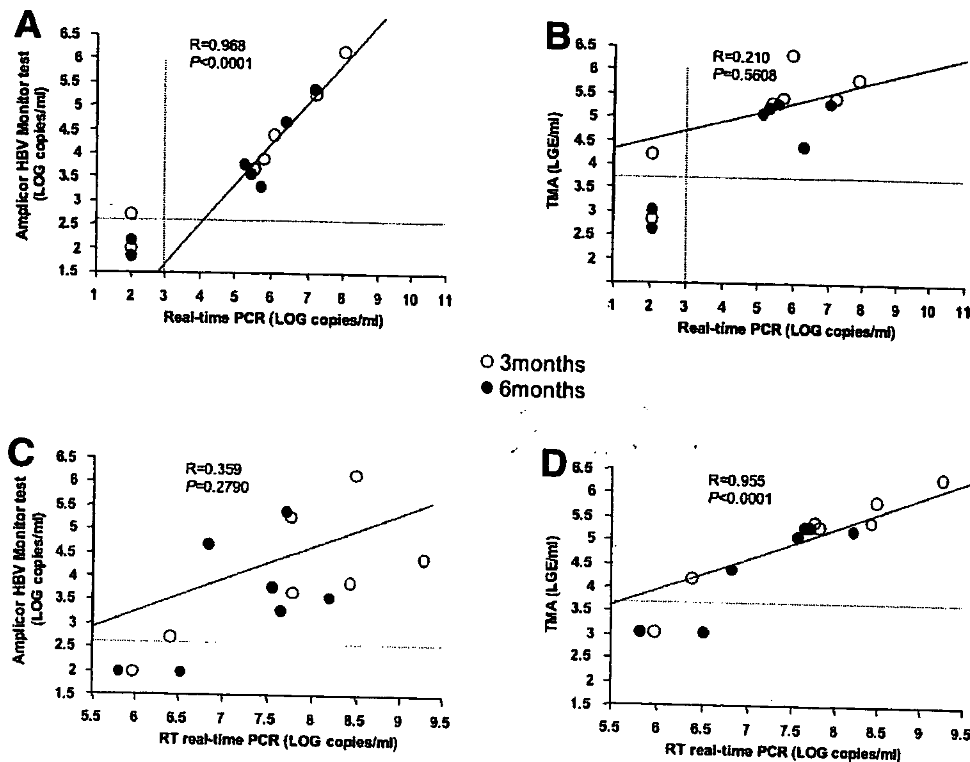

Fig. 3. Correlation between HBV nucleic acid and HBV DNA measurements after 3 and 6 months of ETV therapy. (A) Correlation between HBV DNA level determined by Amplicor HBV Monitor test and that determined by in-house real-time PCR. (B) Correlation of HBV nucleic acid level determined by the TMA-HPA and of HBV DNA determined by real-time PCR. (C) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with HBV nucleic acid level determined by real-time RT-PCR. (D) Correlation of HBV nucleic acid level determined by the TMA-HPA with that determined by real-time RT-PCR. The vertical and horizontal dotted lines represent the lower detection limits of the Amplicor HBV Monitor test or TMA-HPA and in-house real-time PCR, respectively.

ference in nucleic acid level of these patients is probably a result of the small effect of the small amount of ETV.

Comparisons of HBV Nucleic Acid and DNA Values Determined by 4 Measurement Methods—TMA-HPA, Amplicor Monitor Test, In-House Real-Time PCR Assay, and Real-Time RT-PCR—in Patients Treated with ETV. We measured HBV DNA by in-house real-time PCR and HBV nucleic acid by real-time RT-PCR using serum samples obtained from the patients after 3 and 6 months of ETV therapy and compared these values with those obtained by the TMA-HPA and the Amplicor monitor test. HBV DNA determined by real-time PCR correlated well with that obtained by the Amplicor HBV Monitor test (r = 0.968, $P < 0.0001$; Fig. 3A), but not with HBV nucleic acid determined by the TMA-HPA (r = 0.210, P = 0.5608; Fig. 3B). Expression of HBV DNA determined by the in-house real-time PCR assay was $10^{1.5}$-10^{2} higher than that determined by the Amplicor HBV Monitor test. We confirmed the accuracy of our assay using limiting dilution and detection with nested PCR assay. When we diluted the standard samples used in our in-house assay to 1 copy/μL, we detected them by nested PCR using 1 μL of such samples. Three of the 10 (30%) samples tested positive by nested PCR. We thus conclude that our assay accurately measure the amount of HBV DNA in serum.

To examine if measurement by the TMA-HPA reflected the total amount of HBV RNA and HBV DNA in serum samples, we performed real-time RT-PCR using serum samples obtained from patients after 3 and 6 months of ETV therapy. In contrast to the values determined by real-time PCR without RT, the measurement of HBV nucleic acid determined by RT-PCR did not correlate well with that obtained by the Amplicor HBV Monitor test (r = 0.359, P = 0.2790; Fig. 3C), but did correlate well with that obtained with the TMA-HPA (r = 0.955, $P < 0.0001$; Fig. 3D). These results show that the TMA-HPA measures both HBV DNA and HBV RNA in serum. To further confirm the presence of HBV RNA, we digested 3 nucleic acid samples arbitrarily picked from serum samples obtained from patients treated by lamivudine for 3 months, by RNase A. As shown in Fig. 4, RNase treatment reduced the amount of HBV DNA detected by real-time RT-PCR to about 1% of that originally detected.

HBV DNA Levels Determined by TMA-HPA and Amplicor HBV Monitor Test during LAM Therapy. We then investigated the levels of HBV DNA in serum samples obtained from 36 patients after 3 and 6 months of LAM therapy. In some patients, HBV DNA was already negative after 3 and 6 months of therapy (Fig. 5). Similar to the results obtained from patients treated with ETV, comparisons of values obtained from patients who showed measurable HBV DNA levels revealed that HBV nucleic acid levels determined by the TMA-HPA tended to be higher than those determined by the Amplicor HBV Monitor test (Fig. 4).

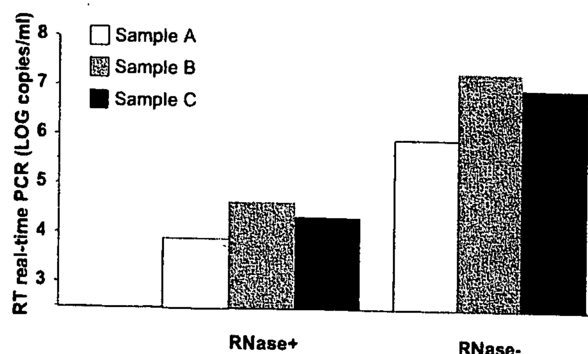

Fig. 4. Presence of HBV RNA confirmed by RNA treatment of 3 nucleic acid samples (samples A-C) obtained from patients after 3 months of LAM therapy. Extracted nucleic acid samples with or without RNase digestion were further digested by proteinase K and ethanol-precipitated after phenol/chloroform extraction. The amount of HBV DNA in each sample was then measured by real-time RT-PCR.

Comparisons of HBV Nucleic Acid Values and HBV DNA Determined by 4 Measurement Methods—TMA-HPA, Amplicor Monitor Test, In-House Real-Time PCR Assay, and Real-Time RT-PCR—in Patients Treated with LAM. We measured HBV nucleic acid and DNA levels by the same 4 methods and investigated the correlations between them after 3 and 6 months of LAM therapy (Fig. 6). HBV DNA levels determined by real-time PCR correlated better with those determined by the Amplicor HBV Monitor test ($r = 0.653$, $P = 0.0083$; Fig. 6A) than with those determined by the TMA-HPA ($r = 0.456$, $P = 0.1173$; Fig. 6B). Similarly, measurement of HBV nucleic acid by RT-PCR did not correlate well with that obtained by the Amplicor HBV Monitor test (Fig. 6C), but showed better correlation with that obtained by the TMA-HPA ($r = 0.452$, $P = 0.0907$, and $r = 0.675$, $P = 0.0114$, respectively; Fig. 6D). These results also showed that the TMA-HPA detects both HBV RNA and HBV DNA.

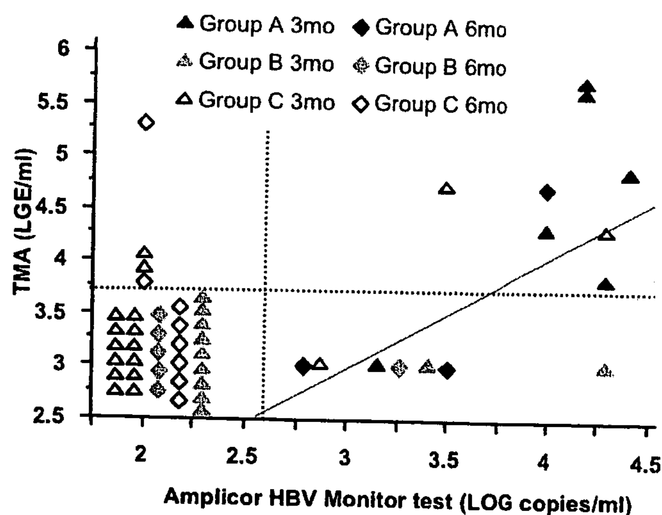

Fig. 5. Correlation of HBV nucleic acid levels determined by the TMA-HPA with HBV DNA levels determined by the Amplicor HBV Monitor test during LAM therapy. During ETV therapy the TMA-HPA showed higher expression of HBV DNA in patients regardless of the presence of the mutation than did the Amplicor HBV Monitor test. The vertical and horizontal dotted lines indicate the lower detection limits of the Amplicor HBV Monitor test and the TMA-HPA, respectively.

HBV RNA in Serum after 3 Months of LAM Therapy Is Higher in Patients Who Showed Early Emergence of YMDD Mutants. In LAM-treated patients, it was assumed that a high serum level of HBV RNA was a marker of the active transcription form of covalently closed circular DNA (cccDNA) and packaging of HBV RNA in the liver. We assumed that YMDD mutants easily emerged under such condition. We compared HBV RNA values (HBV nucleic acid determined by real-time RT-PCR minus HBV DNA determined by real-time PCR) in patients who showed early emergence of mutants (within 12 months) with those who showed late emergence of mutants (more than 12 months) and those who did not show emergence of mutants (Table 1). As shown in Fig. 7, HBV RNA levels were significantly higher in patients who showed early emergence of mutants than the other 2 groups after 3 months of LAM therapy. There was no significant difference in the amount of HBV RNA between group A (patients who showed emergence of mutants within 12 months) and the other 2 groups at the beginning of LAM therapy (data not shown).

Discussion

In this study, we addressed the discrepant measurements of HBV nucleic acid by the TMA-HPA and the Amplicor Monitor test. The presence of HBV RNA in serum samples of patients with HBV infection has been previously reported.[19-21] Because the TMA-HPA uses RNA transcription and amplification of transcripts by T7 RNA polymerase,[22] we assumed that the discrepancy was a result of the presence of HBV RNA in the serum of LAM- and ETV-treated patients. The presence of HBV RNA in a patient treated with LAM was reported previously.[21] In that report, the authors mainly analyzed truncated HBV RNA, which they assumed was transcribed from the integrated genome.[20, 21] They showed a large difference between HBV DNA and truncated HBV RNA, which did not decrease during LAM therapy. We also detected HBV DNA and HBV nucleic acid by real-time PCR and real-time RT-PCR. The values determined by these 2 methods showed less than a 1 log difference (data not shown); we assume that the effect of truncated HBV RNA in serum was only minimal in our study. As we demonstrated in this study, HBV nucleic acid measured by real-time RT-PCR correlated with that determined by the TMA-HPA. This finding suggests that the

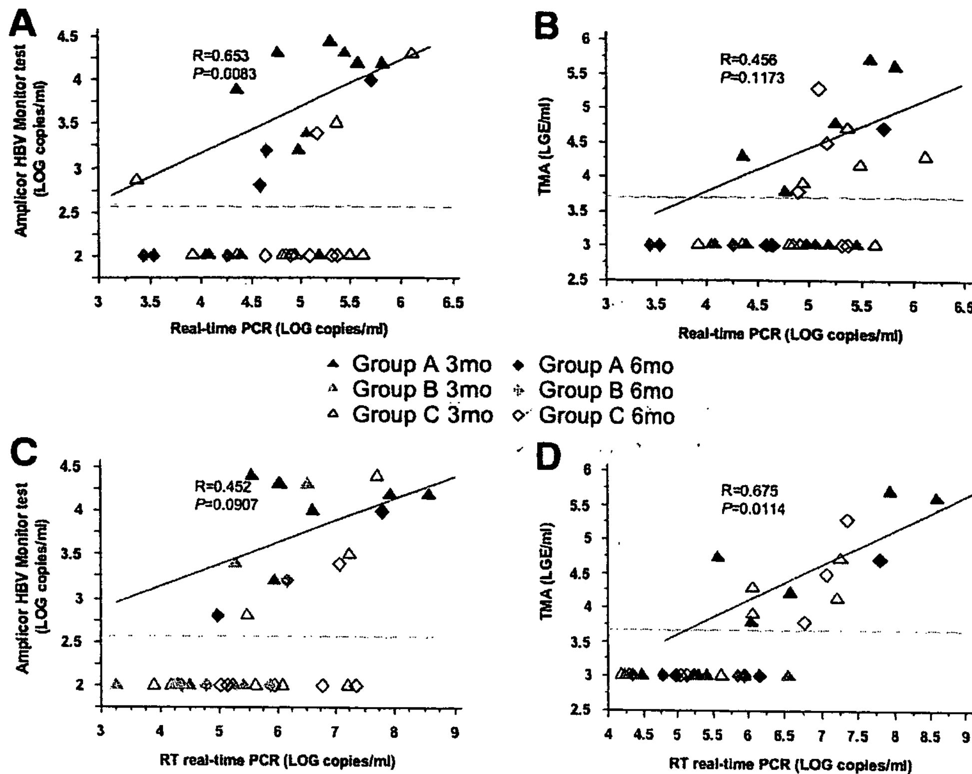

Fig. 6. Correlations between HBV nucleic acid and HBV DNA measurements after 3 and 6 months of LAM therapy. (A) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with that determined by in-house real-time PCR. (B) Correlation of HBV nucleic acid level determined by the TMA-HPA with HBV DNA by real-time PCR. (C) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with HBV nucleic acid level determined real-time RT-PCR. (D) Correlations of HBV nucleic acid level determined by the TMA-HPA with that determined by real-time RT-PCR. The vertical and horizontal dotted lines represent the lower detection limits of the Amplicor HBV Monitor test or TMA-HPA and in-house real-time PCR, respectively.

discrepancy in the values measured by the TMA-HPA and the Amplicor Monitor test is a result of the presence of HBV RNA in the serum.

We showed that a large amount of HBV RNA in the serum was produced during the early stage of ETV (Fig. 1) and LAM treatments (within 6 months). Because ETV and LAM work only on reverse transcription, it is difficult to conceive that the level of transcription from the cccDNA was altered by these drugs. Thus, the slow decrease in HBV RNA seems to reflect that a certain amount of cccDNA still existed in the liver and that the virus replication machinery was still actively operational. This is consistent with previous reports that showed that the amount of cccDNA in the liver tissues[25, 26] and in serum,[26] which correlated well with intrahepatic cccDNA,[27] reflected the effect of LAM and is a marker for cessation of therapy without viral level increasing again after stopping the therapy.

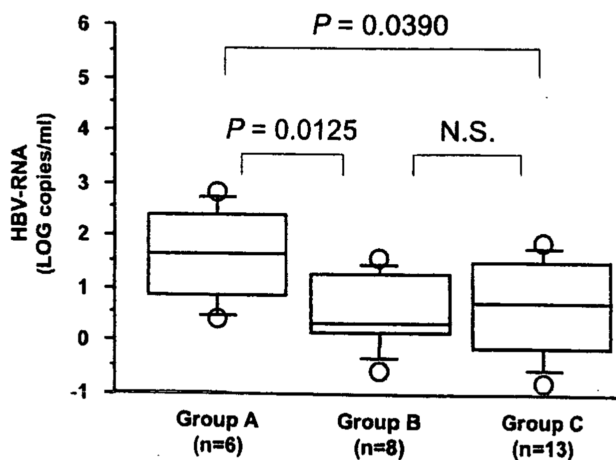

Fig. 7. Box plots of HBV RNA levels of patients in group A (patients who showed emergence of the mutants within 1 year, group B (those who developed resistance after 1 year of LAM therapy), and group C (patients who did not show resistance to LAM therapy). HBV RNA level represents the difference between HBV nucleic acid level determined by real-time RT-PCR minus HBV DNA level determined by in-house real-time PCR. Nine samples that tested negative for in-house real-time PCR were omitted from the analysis (4 samples of group B and 5 samples of group C).

Whether a large amount of HBV RNA originates from a large amount of cccDNA template in hepatocytes or from active transcription (or both) is actually unknown. However, it is assumed that the probability of developing mutants is high in patients who have large amounts of HBV RNA. We thus analyzed the amount of HBV RNA in patients treated with LAM and compared it in patients who showed early emergence of mutants and those who did not. As expected, the amount of HBV RNA in the serum was significantly higher in patients who showed early emergence of mutants than in those who showed late emergence and those who did not show emergence of mutants.

Using complex analysis, previous studies identified several factors predictive of emergence of YMDD mutants such as HBV genotype,[28] ALT level,[29, 30] HBV DNA level

before therapy,[28,30-32] degree of decline of HBV DNA level during therapy,[33,34] presence of hepatitis B e antigen,[17,29,31,32,35] presence of core promoter mutations,[36] deletion of pre-S region,[37] and HBV core–related antigen.[38] We also showed that a slow decrease in HBV nucleic acid measured by the TMA-HPA is a marker of early emergence of mutants. Our finding is important because this assay is routinely used in daily clinical practice. However, the results did not reach statistical significance, probably because of the small number of patients analyzed in our study and the low sensitivity of the assay (detection limit 3.7 log copies/ml). We assume that a sensitive measurement of HBV RNA is useful for predicting the emergence of mutants. Development of such an assay is needed for the proper treatment of patients using different nucleotide and nucleoside analogues. Mechanisms that control transcription of HBV from cccDNA deserve further investigation in order to develop more effective therapies for HBV infection.

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Short Communication

Accordion Index: A new tool for the prediction of the efficacy of peg-interferon-α-2b and ribavirin combination therapy for chronic hepatitis C

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Aim: An optimal treatment regimen based on individual virological response is essential to maximize the efficiency of interferon (IFN) therapy for chronic hepatitis C.

Methods: Using indicators of the virological response and the treatment intensity, we developed the Accordion Index as a new tool for the efficacy prediction of peg-IFN and ribavirin (RBV) combination therapy. For the Accordion Index, the IFN-AC ratio and RBV-AC ratio were defined as follows: IFN-AC ratio = (total IFN dose given during the entire treatment period)/(total IFN dose required to achieve hepatitis C virus [HCV]–RNA negativity), RBV-AC ratio = (total RBV dose given during the entire treatment period)/(total RBV dose required to achieve HCV–RNA negativity).

Results: The analysis of the association between the Accordion Index and the sustained virological response (SVR) revealed that of 25 patients who had HCV–RNA negativity during treatment, all 10 patients with an IFN-AC ratio and RBV-AC ratio of at least 4.0 achieved SVR, while only four of 15 patients with an IFN-AC ratio or RBV-AC ratio of less than 4.0 achieved SVR. With the cut-off value for both the IFN-AC ratio and RBV-AC ratio at 4.0 or higher, the quality of SVR prediction was as follows: the positive predictive value was 100%, the negative predictive value was 73.3%, and accuracy was 84.0%.

Conclusion: The Accordion Index will thus be a useful tool for planning optimal treatment regimens for individual patients.

Key words: Accordion Index, interferon-AC ratio, peg-interferon, ribavirin-AC ratio, ribavirin, sustained virological response

INTRODUCTION

COMBINATION THERAPY WITH peg-interferon (PEG-IFN) and ribavirin (RBV) is the current first-line therapy used to eliminate hepatitis C virus (HCV) in patients with chronic hepatitis C.[1] The duration of treatment is determined based on viral genotype, with treatment administered for 48 weeks in patients with genotype 1 and 24 weeks in patients with genotypes 2 or 3. Approximately 30% of patients with HCV genotype 1 who are HCV–RNA-negative at the end of treatment relapse after the discontinuation of treatment, and the sustained virological response (SVR) rate is 42–52%.[2–4] The SVR rate in patients with HCV genotype 2 is 81–84%,[4,5] and some of these patients may be overdosed.[6]

Several studies have shown that the SVR rate correlates with the duration of treatment required to achieve HCV–RNA negativity.[7,8] Current treatment regimens are designed in accordance with expected sensitivity of individual patients to treatments to reduce potential adverse drug reactions and to increase the possibility of SVR. Specifically, the treatment duration might be shortened in patients who achieve HCV–RNA negativity at an early stage of treatment,[9] while the treatment duration should be extended in patients with slow virological response.[10] Franciscus introduced this concept of the treatment design as an "accordion" effect at the Digestive Disease Week Conference Highlights held in Los Angeles in 2006 (Franciscus A., 2006, unpublished data). By further advancing the concept, we developed a new tool for SVR prediction, the Accordion Index (the IFN-AC

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ratio and the RBV-AC ratio). In the present study, we analyzed the association between the Accordion Index and treatment efficacy, and evaluated the usefulness of this index in determining doses and treatment duration for individual patients.

METHODS

Patients and methods

BETWEEN JANUARY 2005 and December 2006 at the National Hospital Organization Kokura Hospital, 82 patients received combination therapy with PEG-IFN-α-2b and RBV for the treatment of chronic hepatitis C, and efficacy evaluation was possible in 44 patients. Of these 44 patients, the 25 patients who achieved HCV-RNA negativity during treatment (11 males and 14 females; mean age: 56.0 ± 12.8 years) were included in the study. Pre-treatment serum HCV-RNA levels were quantified using the Amplicor HCV monitor test, version 2.0 (Roche Diagnostics, Tokyo, Japan). A serum HCV qualitative assay using the Amplicor HCV test, version 2.0 (Roche Diagnostics, Tokyo, Japan) was conducted at least every 4 weeks after the start of treatment to determine the time of HCV-RNA negativity. Patients who remained HCV-RNA negative at 6 months after the end of treatment were judged to be SVR, and patients who achieved HCV-RNA negativity during treatment, but had recurrence after the end of treatment, were considered to have a transient response (TR).

For the Accordion Index, the IFN-AC ratio and RBV-AC ratio were defined as follows: IFN-AC ratio = (total IFN dose given during the entire treatment period)/(total IFN dose required to achieve HCV-RNA negativity), RBV-AC ratio = (total RBV dose given during the entire treatment period)/(total RBV dose required to achieve HCV-RNA negativity).

The study protocol was approved by the institutional ethics committee of the National Hospital Organization Kokura Hospital, and all of the patients gave their informed consent to participate in this study. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice.

RESULTS

Study patients

OF THE 25 patients in the study, 14 (seven males and seven females; mean age: 51.1 ± 12.7 years) were in the SVR group. Ten had HCV serogroup 1, and four had serogroup 2. The pretreatment HCV-RNA load was $1.4 \pm 2.6 \times 10^6$ IU/mL. The TR group consisted of 11 patients (four males and seven females; mean age: 61.5 ± 10.1 years) of whom seven had HCV serogroup 1, and four had serogroup 2. The pretreatment HCV-RNA load was $2.3 \pm 2.7 \times 10^6$ IU/mL.

Association between the Accordion Index and treatment efficacy

The IFN-AC ratio and RBV-AC ratio for individual patients in the SVR and TR groups are shown in Figure 1. All 10 patients whose IFN-AC ratio and RBV-AC ratio were 4.0 or higher achieved SVR, while only four of 15 patients whose IFN-AC ratio or RBV-AC ratio was less than 4.0 achieved SVR. With the cut-off value for both the IFN-AC ratio and RBV-AC ratio at 4.0 or higher, the quality of the SVR prediction was as follows: the positive predictive value was 100%, the negative predictive value was 73.3%, and accuracy was 84.0%.

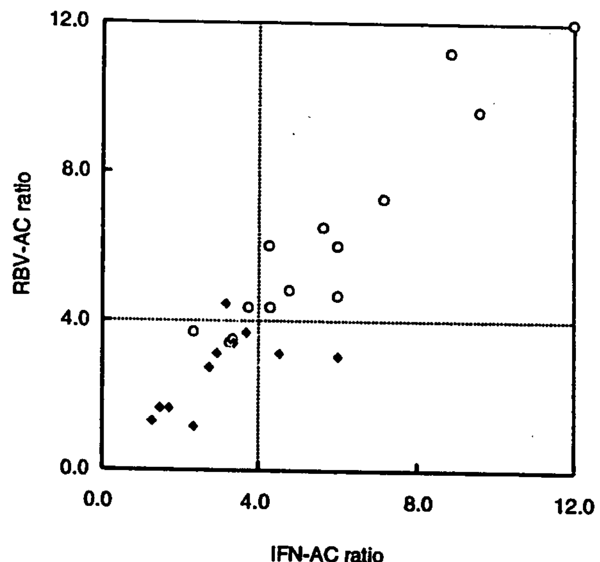

Figure 1 Interferon (IFN)-AC ratio and ribavirin (RBV)-AC ratio in sustained virological response (O SVR) and transient response (◆ TR) patients. IFN-AC ratio and RBV-AC ratio were calculated by the following formulas: IFN-AC ratio = (total IFN dose given during the entire treatment period)/(total IFN dose required to achieve hepatitis C virus [HCV]-RNA negativity), RBV-AC ratio = (total RBV dose given during the entire treatment period)/(total RBV dose required to achieve HCV-RNA negativity).

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DISCUSSION

THE PRESENT STUDY shows that the Accordion Index is closely associated with SVR and that the Index is a useful tool for determining optimal doses and treatment duration for individual patients scheduled to receive combination therapy with PEG-IFN and RBV.

There is no established theory referring to how long treatment should be continued following HCV-RNA negativity to achieve SVR. Drusano *et al.* developed a hypothesis that the longer the absence of HCV-RNA, the more likely SVR is achieved. Their study showed that the SVR rate was 80% with a 32-week absence of HCV-RNA and 90% with a 36-week absence of HCV-RNA.[11] The model developed by Drusano *et al.*, however, was not consistent with the subsequent study outcome. The SVR rate among rapid virological responders (HCV-RNA negative at week 4 of treatment) with HCV genotype 1 was approximately 90% after 24 weeks of treatment,[9] while approximately 40% of slow virological responders (HCV-RNA positive at week 12 and HCV-RNA negative at week 24) had recurrence after 72 weeks of treatment.[10] These studies suggest that rapid virological responders can achieve SVR with short-term treatment after achieving HCV-RNA negativity, while slow virological responders require a much longer treatment period to achieve SVR.

The original "accordion" theory is a hypothesis describing the correlation between the treatment period required to achieve HCV-RNA negativity and the subsequent treatment duration required to achieve SVR. We believe, however, that the doses given to the patient must be taken into consideration, together with the treatment duration. Increasing the doses of PEG-IFN and RBV is known to promote viral reduction at an early stage of treatment and to improve the SVR rate.[12,13] Doses are often reduced in combination treatment with PEG-IFN and RBV due to various adverse drug reactions, such as decreased platelets, decreased neutrophils, anemia, and malaise. It is inappropriate to analyze the treatment period after dose reduction with the same weighting as the treatment period before dose reduction in such cases. We therefore integrated the drug doses and treatment duration to develop the Accordion Index. In the formulas of the Accordion Index, the total drug dose required to achieve HCV-RNA negativity is representative of the individual virological response, while the total dose given during the entire treatment period is an indicator of the treatment intensity. Specifically, IFN-AC is the ratio of the total IFN dose given during the entire treatment period to the total IFN dose required to achieve HCV-RNA negativity, and RBV-AC is the ratio of the total RBV dose given during the entire treatment period to the total RBV dose required to achieve HCV-RNA negativity.

Our analysis identifies the close association of the IFN-AC ratio and RBV-AC ratio with SVR, indicating a sufficient positive predictive value, negative predictive value, and accuracy for SVR prediction with the cut-off value for both the IFN-AC ratio and RBV-AC ratio at 4.0 or higher. The study outcomes suggest that individualized treatment regimens may be planned based on the virological response of patients by calculating the total doses of PEG-IFN and RBV so far given at the time HCV-RNA negativity is achieved and determining the subsequent doses and treatment duration to ensure that the total doses at the end of treatment will be more than four times the doses required to achieve HCV-RNA negativity.

The number of elderly patients with chronic hepatitis C is increasing in Japan. Treating high-risk patients who have underlying heart disease or anemia with fixed doses of PEG-IFN or RBV frequently results in treatment discontinuation due to adverse drug reactions.[14] The virological responses of patients who receive combination therapy with PEG-IFN and RBV vary greatly. While some patients achieve HCV-RNA negativity with short-term treatment and low doses, others do not respond even with long-term treatment and high doses. Physicians therefore need to plan optimal treatment regimens for individual patients by finely adjusting drug doses and treatment periods in accordance with their response and tolerability.

The Accordion Index is expected to help physicians plan optimal individual regimens of PEG-IFN and RBV combination therapy. Further analyses in a larger sample size and a prospective study should be conducted following the current pilot study.

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Distinct responses of two hepatocellular carcinoma cell lines of a similar origin to immunotherapies targeting regulatory or effector T cells

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Abstract. Balance between effector T cells (Teff) and regulatory T cells (Treg) appears to be very crucial for effective anti-tumor immunotherapy. The therapeutic efficacies of enhancement of Teff and suppression of Treg were compared between two murine hepatoma cell lines of a similar origin, MH129 and MH134. Enhancement of Teff was achieved by infection of tumor cells with adenovirus expressing glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), and suppression of Treg, by depletion of $CD4^+CD25^+$ naturally occurring Treg by administration of anti-CD25 monoclonal antibody (PC61) or low-dose cyclophosphamide. Our data show that MH129 cells were susceptible to Treg depletion but resistant to GITR expression, and *vice versa* for MH134 cells. Thus, in MH129 cells, injection of PC61 prior to or after tumor cell inoculation completely or partially, respectively, eradicated tumor growth. Low-dose cyclophosphamide administered after tumor cell inoculation also delayed tumor growth. However, GITR expression either *in vitro* or *in vivo* exhibited little effect. In contrast, in MH134 cells, PC61 induced partial tumor growth delay only when injected prior to tumor cell inoculation, and low-dose cyclophosphamide showed no effect, but GITR, particularly when administered *in vitro*, inhibited tumor growth. An additive effect of PC61 and GITR was observed only in MH134 cells. The ratios of peripheral $CD4^+CD25^+$ to $CD4^+$ T cells remained unaltered during the experimental course in both tumor models. From these results we speculate that this different sensitivity may be due to a difference in relative induction levels of Teff versus Treg, not due to different immunogenicity or different kinetics of peripheral Treg, between the two tumor models. Future studies identifying antigen(s) or epitope(s) specific for Teff and Treg in these tumor cell lines are necessary as analysis of the immune response to such antigen(s) or epitope(s) may in general help predict the relative efficacy of different immunotherapies against distinct tumors.

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Key words: effector T cells, $CD4^+CD25^+$ regulatory T cells, GITRL, cyclophosphamide, PC61

Introduction

It is well known that most tumor cells possess tumor-associated antigens, many of which are found as non-mutated self-components (1). These tumor antigens however generally fail to elicit a significant anti-tumor immune response. This is because, although autoreactive effector T cells (Teff) are present in the periphery of virtually all the subjects, they are kept in check by regulatory T cells (Treg) (2). Therefore, the balance between the number and/or the function of Teff and Treg appears to be highly crucial for the outcome of anti-tumor immunotherapy. In other words, an effective anti-tumor immune response can be obtained not only by enhancing Teff function but also by attenuating the suppressor function of Treg.

Among the different types of Treg identified so far, $CD4^+CD25^+$ Treg have been evaluated most extensively. It has become increasingly clear that Treg play a critical role in autoimmunity, transplantation and also in tumor immunity (3). Thus *in vivo* depletion of $CD4^+CD25^+$ Treg with anti-CD25 monoclonal antibody (PC61) has been shown to eradicate some but not all tumors in animal models (4-9). Additionally, our recent study has demonstrated that low-dose cyclophosphamide (20 mg/kg) also depletes Treg selectively and induced a significant anti-tumor immune response in a murine hepatoma cell line MH129 (10).

Glucocorticoid-induced tumor necrosis factor receptor (TNFR)-related protein (GITR), a member of the TNFR superfamily, is highly expressed in Treg (11). Although GITR expression is dispensable for Treg function (12), agonistic monoclonal antibody against GITR (DTA-1) has previously been shown to abrogate the suppressor function of Treg and consequently enhance the immune response (13,14). Therefore GITR was originally considered a potential target for

suppression of Treg function. Later, however, it was discovered that GITR is also expressed in resting CD4+CD25- and CD8+ T cells at low levels and was up-regulated upon activation, and the signal through GITR co-stimulated both CD4+CD25- and CD8+ T cells particularly with suboptimal T cell receptor stimulation (12,15). Importantly, the co-culture experiments with CD4+CD25- and CD4+CD25+ T cells from wild-type or GITR knockout mice revealed that ligation of GITR on Teff is required to abrogate suppression by Treg (16). Thus it is currently believed that the signal through GITR mainly activates Teff rather than suppressing Treg (11,17).

In this study we evaluated the relative therapeutic efficacy of enhancement of Teff function through GITR signaling by adenovirus-mediated expression of GITR ligand (GITRL) and inhibition of suppressor function of Treg by depletion with PC61 or low-dose cyclophosphamide in two murine hepatoma cell lines, MH129 and MH134, of a similar origin.

Materials and methods

Cell lines and mice used. MH129 and MH134 cells were CCl_4-induced murine hepatoma cell lines from C3H/He strain (18), and were maintained in RPMI-1640 medium with 10% FCS and appropriate antibiotics. *In vitro* growth of each cell line was evaluated with the trypan blue exclusion test.

Six-week-old female C3H/HeN mice were purchased from Charles River (Tokyo, Japan) and kept in a specific pathogen-free facility. All experiments were conducted in accordance with the principles and procedures outlined in the Guideline for the Care and Use of Laboratory Animals in Nagasaki University.

Preparation of anti-CD25 monoclonal antibody. Anti-CD25 monoclonal antibody was purified from ascites of nude mice intraperitoneally injected with hybridoma PC61 using HiTrap™ protein G HP column (Amersham, Piscataway, NJ, USA). PC61 hybridoma was from Dr K. Yui, Nagasaki University, Nagasaki, Japan.

Construction of adenovirus expressing mouse GITRL. The plasmid containing cDNA for mouse GITRL (pTG16491) was kindly provided by Dr B. Calmels, Transgene, Strasburg, France (19). GITRL cDNA was excised by digestion with *Nhe*I and *Sal*I, and ligated into *Nhe*I- and *Sal*I-digested bicistronic vector pIRES2-AcGFP1 (Takara, Tokyo, Japan). The DNA fragment containing the GITRL cDNA, IRES and AcGFP was then released by *Nhe*I- and *Not*I-digestion and ligated into *Nhe*I- and *Not*I-digested adenovirus shuttle vector pHMCMV6 (20). The resulting plasmid pHM-GITRL-IRES-GFP was cut with *PI-Sce*I and *I-Ceu*I, and ligated into pAdHM15 (20). pAdHM-GITRL-IRES-GFP was linearized with *Pac*I, and transfected into 293 human embryonic kidney cells to yield Ad-GITRL-IRES-GFP. The adenovirus obtained was amplified in 293 cells and purified using two rounds of CsCl gradient centrifugation as previously described (21). Determination of plaque-forming unit (pfu) was also performed as previously described (21).

Expression of GITRL and GFP was confirmed by FACScan flow cytometry and Cell Quest software program (BD Biosciences, Mountain View, CA, USA) after staining the MH129 cells infected with Ad-GITRL-IRES-GFP at an MOI of 100 with PE-conjugated anti-GITRL antibody (e-Bioscience, San Diego, CA, USA).

Infectivity of MH129 and MH134 cells to adenovirus infection. The cells were infected with Ad-EGFP (a kind gift from Dr H. Yamasaki, Nagasaki University) at MOIs of 30, 100 and 300. On the next day, expression of EGFP was determined by flow cytometry as described above.

Expression of GITR on MH129 and MH134 cells. Expression of GITR on the cells was analyzed by staining the cells with PE-anti-GITR antibody (e-Bioscience) as described above.

In vivo experiments. The cells ($5x10^5$ cells/mouse) were subcutaneously injected into the flanks of mice. Tumor sizes were determined from caliper measurement using the standard formula (length x width^2/2) and expressed as the mean ± SE.

In the first series of experiments with cyclophosphamide and PC61, groups of mice were treated with either an intraperitoneal injection of cyclophosphamide (20 or 200 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) or PC61 (500 μg/mouse) four days before or after tumor cell inoculation. *In vivo* depletion of CD25+ cells by PC61 was confirmed by flow cytometric analysis as described above after staining splenocytes four days after an intraperitoneal injection of antibody with FITC-conjugated-anti-CD4 and PE-anti-CD25 antibodies that recognize a different epitope of CD25 (7D4) (e-Bioscience) (10).

In the second series of experiments with GITRL, groups of mice were injected with the cells infected with Ad-GITRL-IRES-GFP or Ad-EGFP at an MOI of 100 for 24 h. Alternatively, other groups of mice were first injected with uninfected tumor cells, and when tumor sizes reached ~5 mm in diameter, $5x10^8$ pfu Ad-GITRL-IRES-GFP or Ad-EGFP in 50 μl PBS was injected intratumorally.

Finally, the combined effect of PC61 and Ad-GITRL-IRES-GFP was also studied. Groups of mice were injected on day 0 with the cells infected with Ad-GITRL-IRES-GFP at an MOI of 100 for 24 h, and were injected with PC61 on day +4 (MH129) or day -4 (MH134).

Results

As evident from our recent report (10) and Figs. 1 and 2 in this study, MH129 cells are susceptible to depletion of CD25+ cells. Thus, injection of PC61 prevented tumor formation completely or partially when administered four days before or after tumor cell inoculation, respectively (Fig. 1A). CD4+CD25+ T cell depletion by low-dose cyclophosphamide (20 mg/kg) also significantly suppressed tumor growth (Fig. 2A). In contrast, MH134 cells were resistant to depletion of CD25+ cells. As shown in Fig. 1B, injection of PC61 prior to tumor cell inoculation only partially delayed tumor growth, and injection following tumor cell inoculation contributed no effect. Low-dose cyclophosphamide also showed no significant therapeutic benefit (Fig. 2B). As we have recently demonstrated (10) and illustrated in Fig. 2, high-dose

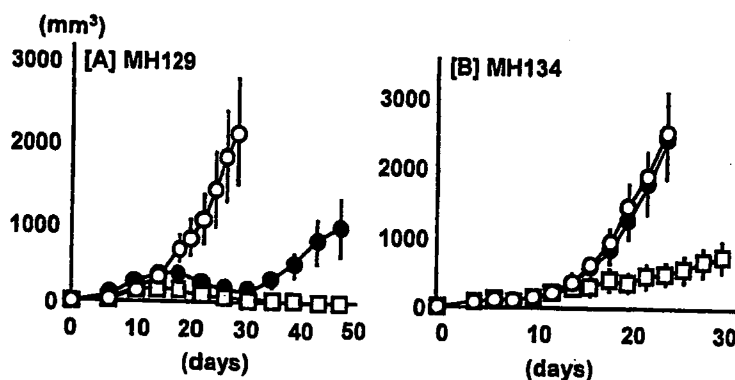

Figure 1. Anti-tumor effect of PC61 on MH129 and MH134 tumor cells in C3H/HeN mice. The mice were inoculated with 5×10^5 MH129 (A) or MH134 (B) cells on day 0. Groups of mice were untreated (○) or treated with 500 μg PC61 on day -4 (□) or +4 (●). The data are the means ± SE (n=6). The same experiments were repeated at least twice with similar results.

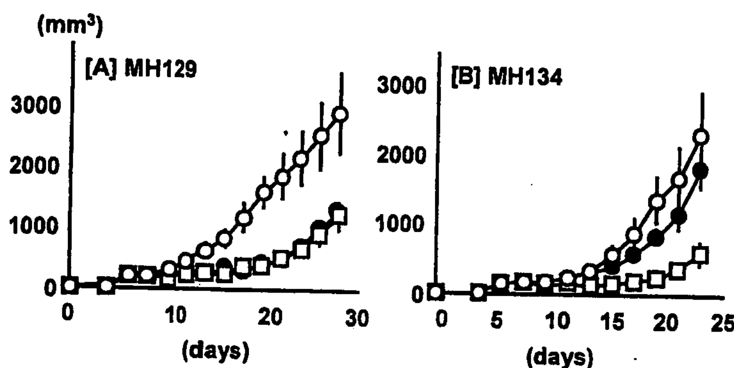

Figure 2. The anti-tumor effect of low (20 mg/kg)- and high (200 mg/kg)-dose cyclophosphamide on MH129 and MH134 tumor cells in C3H/HeN mice. The mice were inoculated with 5×10^5 MH129 (A) or MH134 (B) cells on day 0. Groups of mice were untreated (○) or treated with high-dose (□) or low-dose (●) cyclophosphamide on day +4. The data are the means ± SE (n=6). The same experiments were repeated at least twice with similar results.

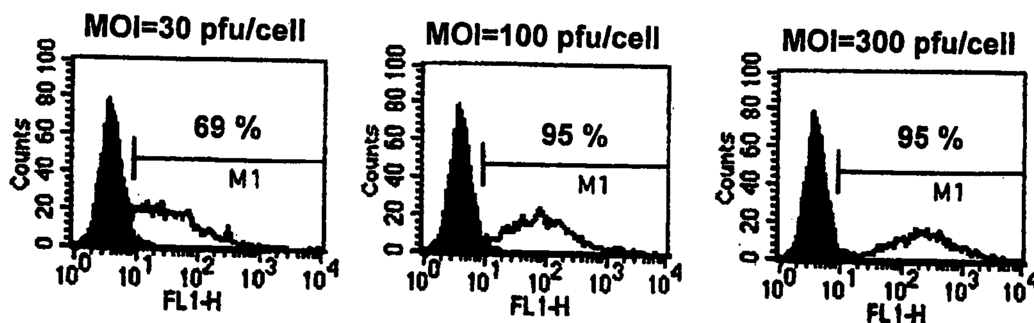

Figure 3. Flow cytometric analysis of MH129 cells infected with Ad-GITRL-IRES-GFP. The cells were untreated or infected with Ad-GITRL-IRES-GFP at an MOI of 100 pfu/cell. One day later, expression of GITRL and GFP was examined as described in Materials and methods.

cyclophosphamide was equally effective in both cell lines through its direct cytotoxic effect.

Since this difference, despite the similar origin of the two cell lines, may be attributed to different kinetics of peripheral $CD4^+CD25^+$ T cells in the two tumor models, the percentages of T cell subpopulations in splenocytes were studied. The ratios of $CD4^+CD25^+$ to $CD4^+$ T cells in the spleen remained unchanged during the experimental course (8.7±3.3% in the control; 9.2±1.2% in MH129-bearing mice at day 7, 6.2±2.5% in MH129-bearing mice at day 21; 7.2±1.9% in MH134-bearing mice at day 7; and 8.44±0.72% in MH134-bearing mice at day 21; mean ± SD).

The relative therapeutic effect of GITRL enhancement of Teff function in the two cell lines was next compared. To do this, recombinant, bicistronic adenovirus expressing GITRL and GFP was constructed. Expression of both molecules was confirmed by flow cytometry (Fig. 3). Ninety-five percent infectivity was observed at an MOI of 100 (Fig. 4). *In vitro* cell growth rates of both cells were not affected by infection with Ad-GITRL-IRES-GFP, and neither cells expressed GITR (data not shown). The *in vivo* effect of GITRL expression was evaluated by two different methods; one was by inoculation of adenoviral infected cells, and the other by injection of adenovirus into established tumors.

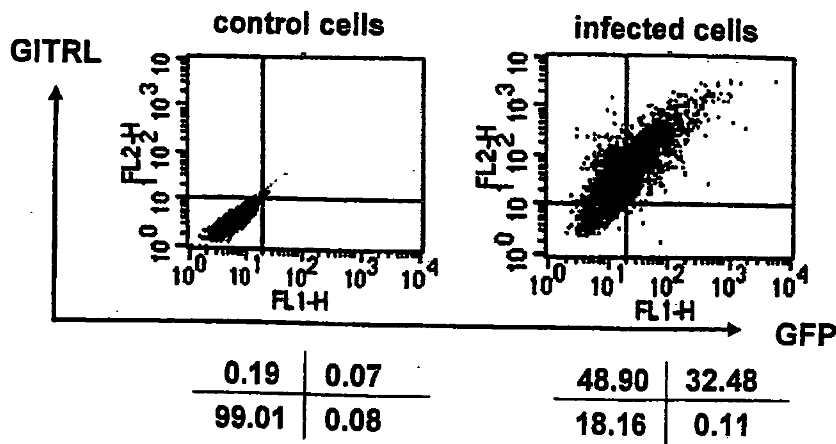

Figure 4. Flow cytometric analysis of adenovirus infectivity in MH129 cells. The cells were infected with Ad-EGFP at MOIs of 30, 100 and 300 pfu/cell. One day later, expression of EGFP was examined as described in Materials and methods.

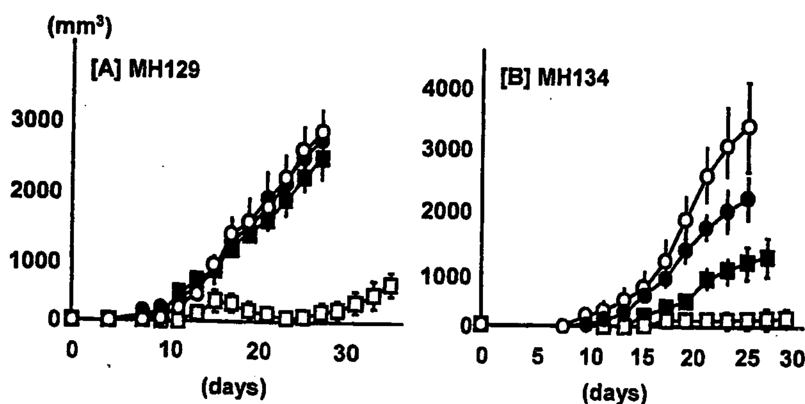

Figure 5. The anti-tumor effect of Ad-GITRL-IRES-GFP on MH129 and MH134 tumor cells in C3H/HeN mice. The mice were inoculated with 5x10^{5} MH129 (A) or MH134 (B) cells infected for 24 h with Ad-EGFP (○) or Ad-GITRL-IRES-GFP (■) on day 0. Alternatively the mice were inoculated with 5x10^{5} uninfected cells, and 5x10^{8} pfu Ad-EGFP (data not shown) or Ad-GITRL-IRES-GFP (•) were injected intratumorally when tumors became ~5 mm in diameter. Furthermore, the mice were also inoculated with Ad-GITRL-IRES-GFP-infected cells and treated with PC61 four days after or before tumor cell inoculation in the MH129 and MH134 cells, respectively (□). The data are the means ± SE (n=6-12). The same experiments were repeated at least twice with similar results.

In vivo growth of MH129 tumors was not affected by GITRL expression for either method (Fig. 5A). However, MH134 tumor growth was blunted by GITRL, particularly when the cells were infected with Ad-GITRL-IRES-GFP before inoculation (Fig. 5B). Thus, the relative responses of two hepatoma cell lines to Treg depletion and Teff enhancement are quite different; MH129 cells are susceptible to the former but resistant to the latter, and *vice versa* for MH134 cells.

Finally, the combined effect of PC61 and GITRL was also studied. The tumor cells were infected with Ad-GITRL-IRES-GFP prior to tumor cell inoculation, and PC61 was injected four days after or before tumor cell inoculation in MH129 and MH134, respectively. The additive effect of PC61 and GITRL was observed only in MH134 cells ($p<0.05$, Figs. 1 and 5) in the combination study.

Discussion

Effective therapeutic outcome of anti-tumor immunotherapy may be obtained by attenuating Treg suppressor function and/or enhancing Teff, for example, by depletion of Treg with PC61 or low-dose cyclophosphamide and stimulation of Teff by GITR signal or DTA-1, respectively. The therapeutic efficacies of PC61 and GITRL or DTA-1, alone or in combination with other antigen-specific immunotherapies, have recently been documented in animal models, showing that the effects of PC61 and DTA-1 vary in different types of tumor cells. For example, Meth A fibrosarcoma cells are sensitive but B16 melanoma cells are resistant to both antibodies (4,5,17,22,23). Thus it is generally accepted that Treg depletion and Teff enhancement can increase antitumor immunity against highly immunogenic tumors but has little or no effect against poorly to non-immunogenic tumors (17). However our present study clearly shows that the relative responsiveness to GITRL and PC61 is different even in two murine hepatoma cells of a similar origin.

The exact mechanism(s) of this difference are at present unclear, but may not be explained by a difference in immunogenicity of these cells, because each tumor cell line responded to at least one therapeutic approach. Also a different kinetics of peripheral Treg between these tumor models is also unlikely, because there was no difference in peripheral Treg number between these models. Instead, a difference in relative induction levels of Teff versus Treg by each cell line

may explicate our results. From our results we suggest the following scenario. It is clear that the balance between Teff and Treg tipped toward Treg in these two tumor models, because both tumors grew well in syngeneic immunocompetent mice. However, MH129 tumor cells substantially activated both Teff and Treg (but Treg > Teff). The depletion of Treg could reverse this balance, thereby leading to tumor shrinkage; however, Teff were already activated fully and GITRL was therefore ineffective. In contrast, the activation of Treg was considerably high, but that of Teff trifling in MH134 cells. The depletion of Treg by PC61 could therefore not enhance tumor immunity because of negligible Teff activity; while activated Teff by GITRL overweighed Treg and enhanced anti-tumor immunity. In these cases, activation of Treg does not necessarily mean proliferation of Treg, because the number of $CD4^+CD25^+$ Treg was unaltered after tumor cell inoculation in our study. An additive effect of PC61 and GITR in MH134 cells, not in MH129, fits our hypothesis.

Notably, a recent study has shown that tumor antigen(s) prime both Teff and Treg even in the same regional lymph nodes (24), and moreover another study has also shown that certain antigen(s) selectively induce Treg (25).

To confirm our hypothesis, future studies identifying tumor antigen(s) or epitope(s) specific for Teff and Treg in these tumor cells will be required. Analysis of immune response to such antigen(s) or epitope(s) may help predict the relative efficacy of different immunotherapies against distinct tumors.

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CASE REPORT

Clearance of hepatitis C virus after living-donor liver transplantation in spite of residual viremia on end date of interferon therapy before transplantation

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Abstract

Interferon (IFN) therapy is the only treatment strategy for hepatitis C virus (HCV) infection after liver transplantation (LT), but prophylactic and treatable IFN therapy after LT has been shown to be insufficient due to the adverse effects of IFN and rivabirin. In this paper, we describe the disappearance of HCV after LT without IFN therapy in the presence of residual viremia on the day of LT. We herein report our findings since this is considered an important case for the anti-HCV strategy of post LT. A 60-year old woman with LC and HCC was referred to Nagasaki University Hospital in August 2004. After she underwent LT on February 18, 2005, we injected peg-IFN-α-2a the 11th time at 18 wk and HCV-RNA was still positive in the serum at LT. The serum HCV-RNA was negative one month after operation and subsequently dissolved 15 mo after operation without IFN therapy. As a result, we speculate that if HCV-RNA is positive while HCV core antigen is negative before LT, then it may lead to clearance of HCV after LT. Therefore long acting peg-IFN-α-2a is thus considered a potentially effective agent for the treatment of HCV-related cirrhosis before LT.

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Key words: Pegylated interferon; Liver transplantation; Hepatitis C virus

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INTRODUCTION

Living donor liver transplantation (LDLT) has become a common treatment strategy for hepatocellular carcinoma (HCC) and end stage liver cirrhosis (LC) in Japan[1]. However, hepatitis C virus (HCV) infection, the most common cause of LDLT, is found in nearly all re-infected graft livers, thus leading to a rapid progression to LC and re-liver transplantation[2]. Interferon (IFN) treatment for HCV infection after LT is the only treatment strategy at present, but its effects are still incomplete[3]. Because the titer of HCV is the most decay in early transplant phase of liver transplantation[4], anti-HCV therapy could thus be considered at this time[5], but prophylactic and treatable IFN therapy after LT has so far been ineffective due to the adverse effects of IFN and rivabirin[6].

Recently, pegylated interferon (peg-IFN), utilizing polyethyleneglycol moiety attached to IFN *via* an amide bond, has been used in the treatment of chronic HCV infection. Peg-IFN-α-2a characterized by a prolonged absorption half-life (50 h), a restricted volume of distribution (8-12 L), and a decreased clearance (94 mL/h)[7], has been found to be safe and tolerable after LT[8]. We thus consider peg-IFN-α-2a a potentially useful treatment of LC due to HCV infection in patients awaiting liver transplantation.

In this case, we made an attempt to achieve HCV clearance from a graft liver after LDLT. For this purpose, peg-IFN-α-2a mono-therapy was performed for 13 wk until LDLT. We observed the disappearance of HCV after LDLT without IFN therapy in the presence of residual viremia on the day of LDLT. The titer of HCV disappeared in early post-LDLT due to the administration of peg-IFN-α-2a. We herein report our findings.

CASE REPORT

A 60-year old woman with LC and HCC was referred

to Nagasaki University Hospital in August 2004. She was diagnosed having diabetes and HCV-related LC in 1995 and 1999, respectively, and had no history of blood transfusion, alcohol abuse and intra-venous drug use. After the diagnosis of LC, she was treated with IFN-α, but this medication was stopped due to depression. In August 2003, a tumor was detected measuring 3.5 cm in diameter in the caudate lobe of the liver, she underwent trans-arterial chemoembolization (TACE) therapy twice in September 2003 and February 2004. The HCC decreased and no new HCC was detected. However, she suffered from hepatic encephalopathy (disorientation and flapping tremor) in June 2004 and thus was hospitalized. Consequently she and her family decided to undergo living donor liver transplantation at our hospital.

She entered our hospital for evaluation of LDLT in September 2004 (Figure 1). On admission, she had no ascites or hepatic encephalopathy. Laboratory data revealed 2.0 mg/dL total bilirubin, 97 IU/L AST, 88 IU/L ALT, 855 IU/L ALP, 6.7 mg/dL total protein, 3.1 mg/dL albumin, 76% prothrombin time and 81000/μL platelets, she was thus evaluated to be Child-Pugh grade B. Her HCV genotype was 1b, and the viral load in serum was 1860 KIU/mL by Amplicor PCR or 3320 fmol/L by HCV core antigen assay. HCC remained unchanged on admission. She underwent peg-IFN-α-2a treatment with the goal of virus clearance, because LDLT might be unnecessary depending on the status of her liver function. We concluded that LDLT should be performed after disappearance of HCV-RNA in her serum. She received peg-IFN-α-2a, 90 μg once a week from December 12, but we had to discontinue it due to neutropenia (under 500/μL) after the first week of therapy. As a result, we modified the regimen of peg-IFN-α-2a treatment from once a week to once every two weeks.

At five weeks after initiation of peg-IFN-α-2a treatment, HCV core antigen was not detectable in her serum, but HCV-RNA was still detectable by qualitative PCR(nested PCR) even at 12 wk after initiation of therapy. We therefore decided to perform LDLT with her daughter as donor, since the complete disappearance of HCV-RNA was thought to be impossible. She underwent LDLT on February 18, 2005, and we used peg-IFN-α-2a for the 11th time at 18 wk and HCV-RNA was still positive in the serum at this time.

Surgery was successfully performed. Histopathological examination revealed that the explanted liver exhibited mixed macro and micro nodular cirrhosis, and three tumors were observed in the caudate lobe, one of them showed complete necrosis by TACE while the others were viable and diagnosed with well-differentiated HCC, and another well-differentiated HCC was found in the anterior segment of the explanted liver. There was no-evidence of vascular invasion of HCC. The patient was given tacrolimus and predonisolone as immunosuppressants. On postoperative day (POD) 2, she underwent re-anastomosis of the hepatic artery, because of decreased arterial flow caused by an intimal tear. On POD 5, she underwent the third laparotomy, because of hematoma around the portal vein and thrombosis in the portal vein. Due to food aspiration, methicillin resistance staphylococcus aureus (MRSA) caused pneumonia and a systemic infection. At

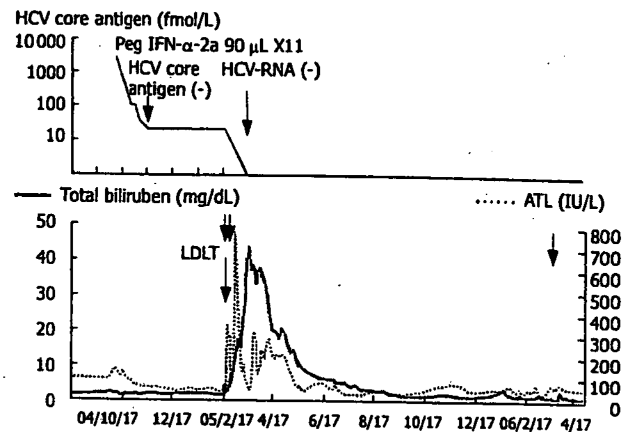

Figure 1 Clinical course of the disease at our hospital. The upper diagram showing the course of the virus titter, in which HCV is the core antigen (fmol/L), and expediently negative HCV core antigen is denoted as 20 fmol/L and negative qualitative HCV-RNA as 1 fmol/L. The lower diagram showing the course of total bilirubin and AST. Three small arrows show abdominal operation after LDLT and a large arrow demonstrates LDLT.

this time, icterus was worsening and the total bilirubin in serum rose to 30 mg/dL on POD 30. She was treated with intensive care, tracheostomy, artificial respiration, MRSA specific antibiotics, hyperbaric oxygen therapy and steroid pulse therapy. Finally, at post operative month (POM) 4, she was weaned off artificial respiration and her total bililubin decreased to 3.1 mg/dL and she showed a good recovery at POM 8. At POM 13, she was operated on for anastomotic stricture of the common bile duct and was then treated on an out-patient basis.

Serum HCV-RNA was negative at POM 1 and subsequently dissolved at POM 15. Liver biopsies performed at POD 2, 5, 32 and POM 13 revealed no findings of HCV reactivation.

DISCUSSION

Crippin JS and colleagues[9] tried IFN-α-2b (1 or 3 MU/d three time a week) +/- ribavirin (400 mg) within 12 weeks, and a loss of detectable HCV-RNA was seen in 5/15 (33%) patients. Thomas RM and colleagues[10] reported that 12 cases (60%) responded to IFN-α-2b 5 MU/d therapy before LT with a clearance of serum HCV-RNA, four of the 12 cases did not show any evidence of HCV recurrence after LD. Forns X and colleagues[11] used IFN-α-2b 3 MU/d + ribavirin 800 mg/d and 9 cases (30%) demonstrated a clearance of serum HCV-RNA, 6 of the 9 cases did not have any evidence of HCV recurrence after LD. Recently, Everson GT and colleagues[12] described that low dose IFN treatment, peg-IFN-α-2b 0.5 μg/kg per week or IFN-α-2b 1.5 MU/d + ribavirin 600 mg/d per six months for genotypes 2 and 3 or 1 year for genotype 1, was performed for advanced HCV patients, and 12 of 15 cases showing a clearance of HCV-RNA before LT remained HCV-RNA negative 6 or more months after transplantation and 32 cases who were positive for HCV-RNA before remained HCV-RNA positive. In a previous report[13], IFN therapy before LT was shown

to be an effective treatment for the clearance of HCV with advanced LC, but sustained HCV clearance after LT was never acquired in patients with a detectable level of HCV before LT. In contrast, our case demonstrated a clearance of HCV-RNA regardless of the fact that HCV-RNA in serum was positive before LDLT. After liver transplantation, we did not prescribe cyclosporine A and mycophenorate mofetil exerting anti-HCV effects *in vitro*. We hypothesize that peg-IFN-α-2a as a long acting IFN, when injected immediately prior to LDLT, may thus induce an anti-viral activity in the anhepatic phase and also soon after LDLT. The HCV titer is the lowest in the anhepatic phase and the immediately early post LDLT[4], therefore this period requires treatment in order to achieve a clearance of HCV. In our case, HCV showed trace quantities due to the negative HCV core antigen and positive HCV-RNA. We thus speculate that trace quantities of HCV in the graft respond to the continuing presence of peg-IFN-α-2a, though a similar case has not been reported up to now.

In cases of a sustained viral response to IFN therapy after LT, hepatic fibrosis does not progress except for other reasons of hepatic injury, rejection and stenosis of bile[14,15]. HCV relapse post SVR after LT has been reported at 7, 8 and 15 mo after treatment[15,16]. For the reasons stated above, we must pay attention to advanced liver fibrosis and HCV-RNA in the serum.

Based on our findings, we hypothesize that positive HCV-RNA is positive and negative HCV core antigen before LT may lead to a clearance of HCV after LT, and long acting peg-IFN-α-2a may be a potentially effective agent for HCV infection before LT. This hypothesis should be further confirmed.

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