

Low-dose intermittent interferon-alpha therapy for HCV-related liver cirrhosis after curative treatment of hepatocellular carcinoma

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HCC was also higher in the non-IFN group than IFN group (6.7% and 27% vs 0 and 0% at 1- and 3-year, $P = 0.048$, respectively).

CONCLUSION: Low-dose intermittent IFN-alpha therapy for patients with HCV-related compensated cirrhosis after curative HCC treatment was effective by making patients tolerant to medical or surgical treatment for recurrent HCC in the later period of observation.

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Key words: Hepatitis C virus; Hepatocellular carcinoma; Interferon therapy; Liver cirrhosis; Liver function; Recurrence; Survival

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Abstract

AIM: To assess the efficacy of low-dose intermittent interferon (IFN) therapy in patients with hepatitis C virus (HCV)-related compensated cirrhosis who had received curative treatment for primary hepatocellular carcinoma (HCC).

METHODS: We performed a prospective case controlled study. Sixteen patients received 3 MIU of natural IFN-alpha intramuscularly 3 times weekly for at least 48 wk (IFN group). They were compared with 16 matched historical controls (non-IFN group).

RESULTS: The cumulative rate of first recurrence of HCC was not significantly different between the IFN group and the non-IFN group (0% vs 6.7% and 68.6% vs 80% at 1- and 3-year, $P = 0.157$, respectively). The cumulative rate of second recurrence was not also significantly different between the IFN group and the non-IFN group (0% vs 6.7% and 35.9% vs 67% at 1- and 3-year, $P = 0.056$, respectively). Although the difference in the Child-Pugh classification score between the groups at initial treatment of HCC was not significant, the score was significantly worse at the time of data analysis in the non-IFN group than IFN group (7.19 ± 1.42 vs 5.81 ± 0.75 , $P = 0.0008$). The cumulative rate of deviation from objects of any treatment for recurrent

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms worldwide. Approximately 80% of Japanese patients with HCC have a history of hepatitis C virus (HCV) infection, and most such patients have liver cirrhosis^[1-3]. Although recent advances in imaging techniques and treatment of HCC have improved prognosis of patients with HCV-related HCC, the outcome is still unsatisfactory; the 5-year survival rate is only 50% to 70% even after curative treatment such as hepatic resection and local ablation^[4]. The reasons for this unfavorable prognosis is considered to include high intrahepatic tumor recurrence rates and biochemical deterioration by sustained hepatic damage, both resulting from persistent HCV infection^[5]. Even after curative hepatic resection for HCV-related HCC, the rate of intrahepatic tumor recurrence within 1 year is 20% to 40%, rising to about

80% by 5 years^{14,15}. Intrahepatic recurrences of HCC may result from intrahepatic metastasis originating from the primary HCC or from ongoing multicentric carcinogenesis related to chronic HCV infection. In addition, sustained underlying HCV-related hepatic damage may compromise hepatic functional reserve, worsening clinical outcome. Thus, prevention of HCC recurrence and preservation of liver function are both highly important priorities in improving prognosis of patients with HCV-related HCC.

Interferon (IFN) therapy for patients with HCV infection is effective as evident by reduction of serum alanine transaminase (ALT) activity and eradication of HCV. Accordingly, IFN is valuable in minimizing hepatic necrosis, inflammation, and fibrosis, as well as reducing the likelihood of hepatocarcinogenesis¹⁶⁻¹⁸. The primary goal of treatment of patients with HCV infection is elimination of the virus. Several studies have reported recently that IFN therapy provided after curative treatment for HCV-related HCC prevents HCC recurrences and improves survival¹⁷⁻²³. Such improvement of prognosis is more predominant when IFN therapy results in elimination of HCV RNA²⁴. However, most patients with HCV-related HCC also have liver cirrhosis. Many centers do not advocate IFN therapy of patients with compensated cirrhosis, mainly because of the disappointing sustained virological response (SVR) rates in such patients²⁵. Several studies indicated that the response of cirrhotic patients to antiviral therapy is low²⁶⁻²⁸. The reasons for the low SVR rate in such patients include inability to administer IFN at recommended doses due to adverse effects and dose-limiting cytopenia. On the other hand, several investigators suggested that the use of low-dose IFN therapy for viral elimination was as effective in the treatment of cirrhotic patients with HCV as it is in non-cirrhotic patients^{29,30}. Furthermore, they indicated that the same therapy could improve the underlying liver histology. There is evidence to suggest that low-dose IFN therapy might be beneficial in HCV-related cirrhosis, not only because it prevents the progression of liver disease, but also because it reduces the risk of hepatocarcinogenesis^{31,32}. In this regard, low-dose IFN therapy seems to be tolerable without significant life-threatening adverse effects than the standard dose of IFN.

However, it is not known whether low-dose IFN after curative treatment of primary HCC could slow disease progression or reduce the rate of clinical decompensation in cirrhotic patients, in addition to prevention of HCC recurrence. Several studies used the standard dose of IFN after HCC treatment^{17,23,33}, and studies using low-dose IFN therapy for HCV-related cirrhosis after HCC treatment also reported that such regimen may reduce late recurrence of HCC³⁴.

In this prospective case controlled trial, we assessed the efficacy of low-dose intermittent IFN therapy on HCV-related liver cirrhosis after curative treatment of primary HCC in terms of overall survival, HCC recurrence, and liver function.

MATERIALS AND METHODS

Patients

A total of 176 consecutive patients received their initial

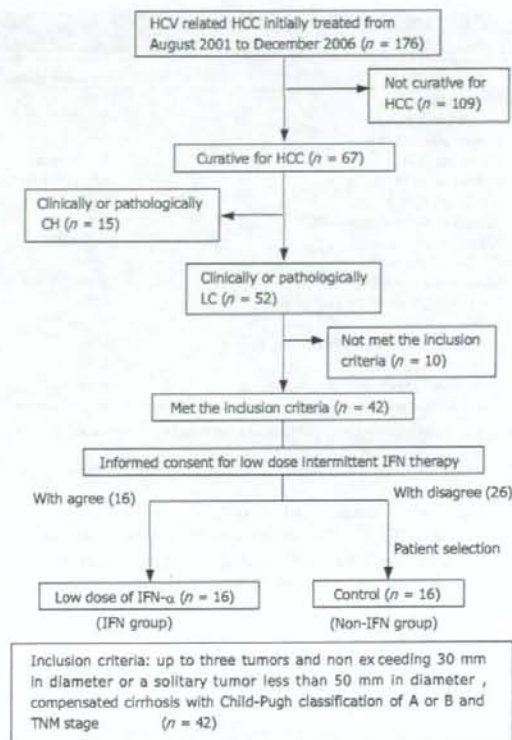


Figure 1 Schematic flow chart of enrolled patients.

treatment for HCV-related primary HCC at Hiroshima University Hospital between August 2001 and December 2006. Of these, 67 patients with HCC underwent first medical or surgical therapeutic intervention with curative intent (defined as complete tumor eradication with no visible residual tumor in computed tomographic images, or resection of all evident tumor tissue). Medical treatments included percutaneous radiofrequency (RF) ablation and ethanol injection, while surgical procedures included hepatic resection and RF ablation under laparotomy. Among these 67 patients, 52 patients with liver cirrhosis (LC), which was diagnosed clinically and pathologically, were considered for this prospective study. Figure 1 shows our study flow. Among these 52 patients with HCV-related LC, we assessed 42 patients who met the following inclusion criteria: (1) the presence of up to three tumors with none exceeding 30 mm in diameter or a solitary tumor less than 50 mm in diameter; (2) tumor-node-metastasis (TNM) stage of ; (3) detectable serum HCV RNA; (4) all seronegativity for hepatitis B marker including hepatitis B surface antigen, hepatitis B anti-core antibody and hepatitis B surface antibody; (5) compensated cirrhosis with a Child-Pugh class A or B; (6) platelet count $40000/\mu\text{L}$; and (7) absence of local recurrence during the follow-up period and of any ectopic intrahepatic recurrence within 12 wk after treatment for primary HCC. We used the TNM classification system

Table 1 Characteristics of participating patients

	Interferon group	Non-interferon group	P value
No. of patients	16	16	
Age in years (range)	68.5 ¹ (53-73)	67.5 ¹ (58-75)	NS
Gender (Male/Female)	10/6	11/5	NS
Albumin (g/dL)	3.7 ¹ (3.0-4.8)	3.7 ¹ (3.0-4.5)	NS
Platelet count ($\times 10^4/L$)	8.0 ¹ (4.5-14.2)	8.4 ¹ (4.6-14.3)	NS
ICG R-15 (%)	17.3 ¹ (6.1-40.8)	18.2 ¹ (5-45)	NS
Alanine aminotransferase (IU/L)	59 ¹ (35-99)	58 ¹ (21-143)	NS
Alpha fetoprotein (ng/mL)	54 ¹ (5.3-293.6)	38 ¹ (5.0-121.7)	NS
Child-Pugh score (A/B)	13/3	13/3	NS
Main tumor size (mm)	15 ¹ (10-50)	18 ¹ (10-40)	NS
No. of HCC tumors (single/multiple)	9/7	10/6	NS
Stage (/ /)	8/3/5	7/5/4	NS
Treatment (medical/surgical)	8/8	9/7	NS
HCV genotype (1/2)	12/4	14/2	NS
Viral loads (low/high)	6/10	5/11	NS

ICG-R15: Indocyanine green retention at 15 min; Low viral loads: HCV RNA < 100 KIU/mL, high viral loads: HCV RNA \geq 100 KIU/mL. ¹median.

of the Liver Cancer Study Group of Japan as a staging system for HCC^[31]. The underlying liver condition leading to LC was identified by histopathological examination of resected tissue samples. When this was not available, laboratory tests were performed including serum albumin, platelet, prothrombin time and indocyanine green retention at 15 min (ICG-R15), and radiological examination such as ultrasonography and computed tomography.

Of the 42 patients with LC who met the above eligibility criteria, 16 patients received low-dose IFN therapy after signing a written informed consent (IFN group). Of the remaining 26 patients who rejected IFN therapy, we selected 16 patients as the control (non-IFN group). These 16 patients, who met the eligibility criteria mentioned above, were matched by age, gender, tumor size, number of tumors, TNM stage of HCC, serum albumin level, platelet counts, ICG-R15 and Child-Pugh class with patients of the IFN group. Thus, a total of 32 patients (16 in the IFN group and 16 in the non-IFN group) were enrolled in this study. All agreed to participate in the research protocol, which was approved by the hospital research ethics board. Table 1 shows the baseline characteristics of patients of the two groups. The data indicates no significant differences between the groups for age, gender, liver function, tumor characteristics, and therapeutic methods used against primary HCC.

IFN therapy

In the IFN group, patients received 3 MIU of natural IFN-alpha (human lymphoblastoid IFN; Sumiferon, Daiippon Sumitomo Pharmaceuticals, Osaka, Japan) intramuscularly three times weekly for at least 48 wk as long as possible. IFN therapy commenced within 12 wk after initial treatment for HCC. Patients received post-treatment IFN therapy up to the detection of HCC recurrence, and then patients who could have curative treatment for recurrent HCC restarted IFN therapy when possible. However, patients who had advanced liver dysfunction or untreatable progressive HCC did not receive IFN therapy. In the control group, none of the patients received IFN therapy after curative treatment of HCC; instead, they

were on ursodeoxycholic acid (UDCA) and stronger neomiphagen C (SNMC).

Follow-up

After curative treatment for primary HCC, all patients underwent liver function tests, serum tumor marker assays such as alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist (PIVKA)-II, every month, abdominal ultrasonography every 3 mo, and dynamic computed tomography (CT) every 6 mo. If recurrences of HCC were suspected, additional examinations including CT during arteriography or tumor biopsy were performed. Recurrence of HCC was defined as any new nodules appearing as hyperattenuation by CT during hepatic arteriography or as hypodattenuation in CT performed during arteriography. Hypovascular HCC was confirmed histopathologically by fine-needle aspiration biopsy. Patients with recurrent HCC were treated medically or surgically, with curative intent if possible. Patients without curative treatment of recurrent HCC then received transcatheter chemoembolization. After repeated transcatheter chemoembolization, patients were finally unable to receive any treatment for recurrent HCC.

End points

We analyzed the outcome of this prospective study in December 2006. We compared the rate of HCC recurrence and the survival rate between IFN group and control group. We assessed whether low-dose of IFN therapy was effective in inhibiting recurrence of HCC, preserving liver function and prolonging survival. In addition, we also assessed the cumulative rate of deviation from objective of any treatment against recurrent HCC due to progression of HCC and/or underlying liver dysfunction.

Statistical analysis

The Chi-square and Fisher exact tests were used for categorical variables, while Student's *t*-test and the Mann-Whitney *U* test were used for continuous and ordinal variables, as appropriate. The Kaplan-Meier method used to assess cumulative survival and recurrence rates calculated from the date of diagnosis to the date of

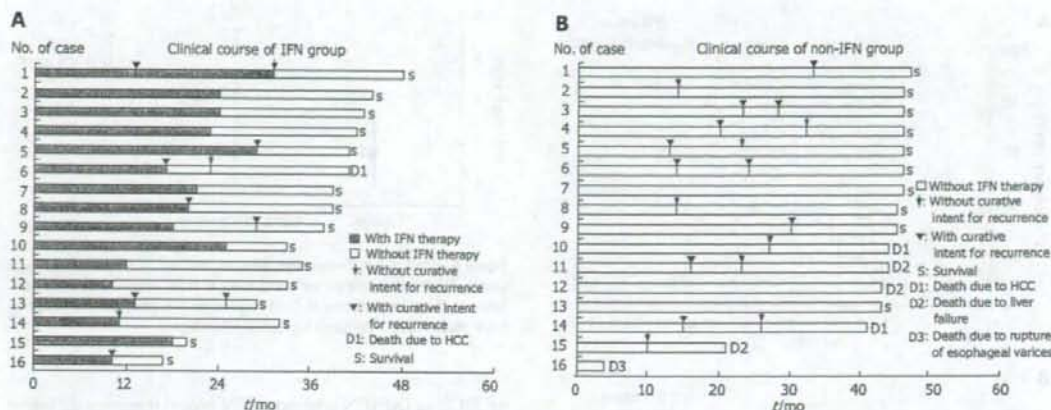


Figure 2 A: Clinical course of the interferon group. Patients who had a curative treatment for primary HCC received 3 MIU of natural interferon- α three times weekly for at least 48 wk as long as possible except Cases 12, 14 and 16. Recurrent HCCs were treated with or without curative treatment; B: Clinical course of the non-interferon group. Patients who had a curative treatment of primary HCC did not receive IFN therapy. Recurrent HCCs were also treated with or without curative treatment.

disease recurrence or death. Surviving patients and patients who died of causes unrelated to the liver were defined as censored cases, while patients who died of causes related to the liver were defined as noncensored cases. The log-rank test was used to compare survival and recurrence curves. *P* values below 0.05 were considered to indicate statistical significance. The JMP version 5.1 statistical software package (SAS Institute, Cary, NC) was used for analysis of data.

RESULTS

Clinical course of IFN group

Figure 2A shows the clinical course of 16 patients of the IFN group from the initial treatment of primary HCC to the date of data analysis. The duration of low-dose IFN therapy ranged from a minimum of 10 mo to a maximum of 25 mo (median 16 mo). Although 8 patients did not have HCC recurrence, HCC recurred in 8 patients after initial treatment of HCC during a median follow-up period of 37 mo. Of the recurred patients, 7 developed HCC recurrence during IFN therapy (Cases 1, 5, 6, 8, 13, 14 and 16) except 1 patient (Case 9) who had HCC recurrence after discontinuation of IFN therapy. Of the 8 patients with HCC recurrence, 4 were treated with surgical resection therapy (Cases 5, 9, 13 and 16), 3 patients with percutaneous RF ablation therapy (Cases 1, 6 and 8) and 1 patient transcatheter chemoembolization (Case 14). Of these patients, a patient with transcatheter chemoembolization (Case 14) could not have curative treatment and repeated transcatheter chemoembolization. He was excluded from the study concerning the next recurrence. Of the 7 patients with curative treatment for HCC recurrence, 2 restarted IFN therapy, one continued IFN therapy until next recurrence (Case 1), which was not curative, and the other continued until intolerant generalized fatigue (Case 8). The remaining 5 patients (Cases 5, 6, 13, 14 and 16) were followed without IFN therapy because of rejection of

IFN therapy. Although one of these 5 patients was not curative for first recurrence (Case 14), he was tolerant to repeated transcatheter chemoembolization and was still alive at the date of data analysis. Two patients without curative treatment at the second recurrence (Cases 1 and 6) were also relatively tolerant to the repeated medical treatment such as transcatheter chemoembolization. Of these patients, one died of progression of HCC in spite of repeated transcatheter chemoembolization and hepatic arterial infusion (Case 6), another was alive at the date of data analysis (Case 1). Of 3 patients without curative treatment of HCC, two survivors' status of HCC were not progressive (stage and stage) and underlying liver function could be tolerant to the treatment such as transcatheter chemoembolization because of relatively preserved function (Cases 1 and 14).

The 16 patients who received IFN therapy included 2 patients with virological response (Cases 2 and 3) and 14 patients who did not get SVR [3 transient responders (Cases 8, 9 and 11), and 11 non-responders (Cases 1, 4, 5, 6, 7, 10, 12, 13, 14, 15 and 16)]. Among the 14 patients who did not show SVR, 8 were biochemical responders with normalized ALT (Cases 1, 4, 5, 7, 9, 10, 13 and 16), including 4 transient responders and 4 non-responders. Two sustained virological responders who received IFN therapy for 96 wk have viral characteristics of genotype 1 and low viral load. Among the patients who did not show SVR, 7 discontinued IFN treatment because of recurrence of HCC, while 2 patients restarted IFN therapy after the curative treatment of recurrent HCC. None of the patients who received IFN therapy developed life-threatening side effects.

Clinical course of non-IFN group

Among the non-IFN group, the first recurrence of HCC occurred in 13 patients during a median follow-up period of 45 mo (Figure 2B). HCC recurred in 6 of the 7 non-IFN patients who had a sustained normalized ALT. Of the 13 patients with recurrent HCC among the non-IFN group, 4 were treated with hepatic resection (Cases 1, 4, 9 and 11),

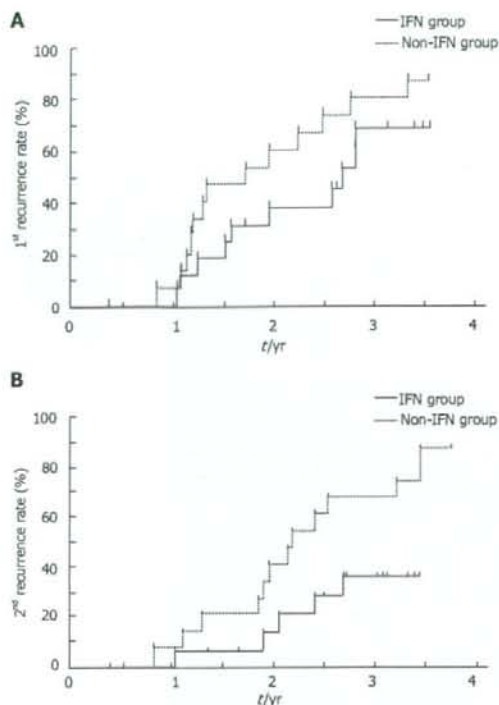


Figure 3 A: Cumulative rate of first recurrence. Rates of first recurrence for the IFN and non-IFN groups. The rate of first recurrence of HCC in the IFN group was not significantly different from that of the non-IFN group ($P = 0.157$); B: Cumulative rate of second recurrence. Rates of second recurrence for the IFN and non-IFN group. The rate of second recurrence of HCC in the IFN group was not significantly different from that of the non-IFN group ($P = 0.056$).

6 with local ablation including percutaneous RF ablation or ethanol injection (Cases 3, 5, 6, 7, 10 and 14) and 3 with transcatheter chemoembolization (Cases 2, 8 and 15). Of the 13 recurrent patients, 5 patients (2 received ethanol injection and 3 transcatheter chemoembolization) could not be treated curatively and was excluded from the study concerning the next recurrence. These 5 patients were treated repeatedly with transarterial chemoembolization after first recurrence. Among the remaining 8 patients who were treated curatively for first recurrence, 7 developed a second recurrence (Cases 3, 4, 5, 6, 9, 11 and 14). Among these 7 patients with second recurrence, 2 were treated curatively for HCC [1 with RF ablation (Case 3) and 1 with hepatic resection (Case 6)], while the remaining 5 patients were not (4 patients due to uncontrolled multiple HCC and one patient due to underlying liver dysfunction). The latter group of 5 patients received transarterial chemoembolization repeatedly after second recurrence.

Comparison of the first and second recurrence rates of HCC

We compared the overall cumulative rates for first and second recurrence between IFN and non-IFN groups (Figure 3). The 1-, 2- and 3- year rates of first recurrence

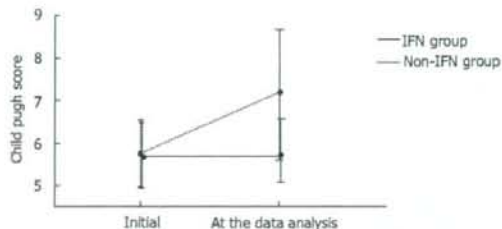


Figure 4 Effect of IFN therapy after curative treatment of HCC on Child-Pugh scores. IFN-treated patients were less likely to show deterioration of hepatic function. The average scores of Child-Pugh of the IFN group were significantly better preserved than the non-IFN group ($P = 0.0008$).

of HCC in the IFN and non-IFN group were not different (0% vs 6.7%, 38.1% vs 60% and 68.6% vs 80%, respectively, Figure 3A, $P = 0.156$). The 1-, 2- and 3-year rates of second recurrence in the IFN and non-IFN groups were 0% vs 6.7%, 13.5% vs 33.3% and 35.9% vs 67%, respectively (Figure 3B, $P = 0.056$).

Liver function

Patients of the IFN group were less likely to develop worsening of hepatic dysfunction compared with the non-IFN group. We compared the average score determined for Child-Pugh classification at initial treatment of HCC with that at the time of data analysis (Figure 4). Although the difference in the Child-Pugh classification score between the two groups at initial treatment of HCC was not significant, the score was significantly worse at the time of data analysis in the non-IFN group than IFN group ($P = 0.0008$).

Deviation from objects of any treatments for recurrent HCC

At the date of data analysis, patients who developed recurrent HCC were treated repeatedly, as possible, for the purpose of curative treatment including surgical resection and ablative therapy such as RF ablation and ethanol injection. Patients who were difficult to treat with curative intent received transcatheter chemoembolization or hepatic arterial infusion. Although patients with recurrent HCC received repeated treatments, some patients finally could not be treated because of excessive progression of HCC or liver dysfunction. Figure 5 shows that the cumulative rate of deviation from objects of any treatment for recurrent HCC between the IFN group and non-IFN group. In the IFN group, one patient could not receive treatment due to progressively advanced HCC in later period. On the other hand, 8 patients in the non-IFN group could not receive treatment because of underlying liver dysfunction ($n = 2$) and progressively advanced HCC ($n = 6$). The 1-, 2- and 3- year rates of deviation from objects of any treatment for recurrent HCC in the IFN and non-IFN group were 0% vs 6.7%, 0% vs 20% and 0% vs 27%, respectively ($P = 0.048$). Thus, the IFN group tended to be treatable for recurrent HCC compared with the non-IFN group.

Survival of patients

At the date of data analysis, 1 patient among the IFN

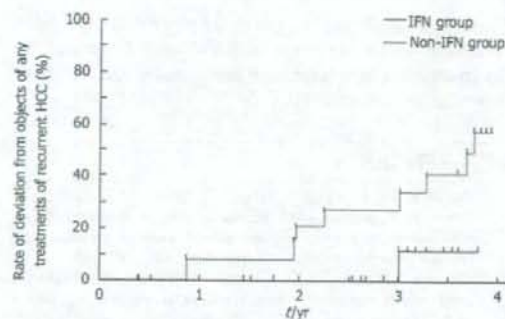


Figure 5 Cumulative rate of deviation from objects of any treatment of recurrent HCC. Recurrent HCC tended to be treatable later in the IFN group than non-IFN group ($P = 0.048$).

group and 6 patients among the non-IFN group had died of liver disease. Of the 8 recurrence patients among the IFN group, 1 died of advanced multiple HCC and none died of liver failure. On the other hand, of the 13 recurrence patients among the non-IFN group, 2 died of advanced HCC and 2 died of liver failure in spite of the relatively early stage of HCC. Among the 3 patients without recurrent HCC of the non-IFN group, 1 died of liver dysfunction and 1 died of ruptured esophageal varices.

With regard to the cumulative survival rates of the IFN and non-IFN groups (Figure 6), the respective rates of survival were 100% vs 93.7% at 1 year, 100% vs 87.5% at 2 years, 100% vs 87.5% at 3 years and 83.3% vs 61.4% at 4 years. Thus, the cumulative survival rate was not significantly different between the two groups for first 4 years after curative treatment of HCC ($P = 0.45$). The median survival time following the first treatment of HCC was 37 mo (range, 17 to 45) for the IFN group and 45 mo (range, 4 to 47) for the non-IFN group.

DISCUSSION

HCC recurrence is still a risk even if HCV-related HCC is treated with curative intent. Most of such patients with HCC have underlying liver cirrhosis, and deterioration of underlying hepatic function may be a hindrance to treatment of recurrent HCC and be associated with prognosis. The present prospective case controlled study of cirrhotic patients shows that low-dose intermittent IFN therapy after curative treatment of HCC could preserve liver function and increase the chance of treatment for recurrent tumor.

Previous studies indicated that IFN therapy after curative treatment of HCC was effective in inhibiting or delaying the development of recurrent HCC^{17,23,34,36}. Although several recent studies have reported the efficacy of chemoprevention with IFN therapy after treatment of HCV-related HCC, the basis of the benefit was not clear. Shiratori *et al.*^{23,33} and Ikeda *et al.*¹⁷ reported that IFN therapy in cirrhotic patients reduced recurrence of HCC and improved prognosis. Although they used standard IFN dosage per time, there are no other reports on the effect of

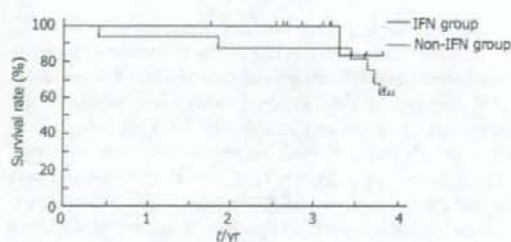


Figure 6 Cumulative survival rate. Comparison of the cumulative survival rates of the IFN and non-IFN groups. The cumulative survival rate was not significantly different between the two groups ($P = 0.45$).

low-dose IFN therapy after curative treatment of primary HCC in cirrhotic patients. Sakaguchi *et al.*²¹ reported that low-dose, long-term, intermittent IFN therapy in patients who had curative HCV-related HCC suppressed recurrence of HCC and improved survival, though it was not clear whether their patients had underlying liver cirrhosis or not. On the other hand, Mazzaferro *et al.*³⁴ indicated that low-dose intermittent IFN therapy seemed to reduce late recurrence in patients with HCV-related cirrhosis after resection of HCC. Considered together, these results suggest that low-dose IFN therapy is potentially useful for cirrhotic patients when used as long as possible. However, our results of low-dose intermittent IFN therapy showed no significant difference in recurrence between those who received IFN therapy and those who did not. Unfortunately, since the difference in treatment outcome between the above three studies might be due to the use of different IFN regimens (e.g. dosage and frequency), and background characteristics of cirrhotic patients (e.g., performance status), the results varied and no standard IFN regimen to pursue after curative treatment of HCV-related HCC could be advocated.

The design of the present study was not randomized controlled type, and differed in details of the IFN protocol and characters of patients from the other studies. Although there was no significant difference in the recurrence rate between the IFN and non-IFN groups, the recurrence rate in the later period of observation including second recurrence appeared to be lower in patients with IFN therapy. Furthermore, the recurrent HCC in patients on IFN therapy did not seem to be aggressive compared with that in patients without IFN therapy, probably because they could be treated with curative intent during the observation period. Thus, low-dose intermittent IFN therapy seemed to have delayed or reduced the chance of development of recurrent HCC in the later period of observation, although IFN did not completely inhibit HCC recurrence in our cirrhotic patients.

Most cirrhotic patients cannot receive a standard full-dose IFN regimen due to underlying liver dysfunction and unfavorable complication such as cytopenia. Hence, it could be difficult to achieve SVR in most cirrhotic patients on low-dose intermittent IFN therapy. Valla *et al.*³⁷ performed a randomized, controlled trial of IFN-alpha 2b but the results showed a lack of any benefits in terms of sustained biochemical response, liver function test

results, histology, occurrence of decompensation or HCC, or prolongation of survival. On the other hand, Everson and coworkers^[29,30] suggested that the use of low-dose IFN therapy for viral elimination was as effective in the treatment of cirrhotic patients with HCV as it is in non-cirrhotic patients. Several recent studies have reported that IFN therapy following HCC treatment improved liver function of patients with HCV-related HCC, although it is not clear which specific IFN action is important for these benefits. We also demonstrated that preservation of liver function was significantly better in the IFN group than in the non-IFN group even when HCV was not completely eradicated. Thus, hepatic functional preservation increases the chance of treatment for recurrent. Therefore, the cumulative rate of deviation from objects of any treatment for recurrent HCC might be lower in patients with IFN therapy than in patients without IFN therapy as we showed that low-dose IFN resulted in less advanced recurrence and hepatic functional preservation. Although the survival rates were not significantly different between the two groups in our observation period, we need a longer observation to determine differences in survival rates. Although we also assessed the correlation between the observed beneficial effects of the low-dose intermittent IFN therapy and HCV genotype, we could not reach the clear conclusion due to small sample size. In the future, the study with large sample size may be needed to conclude.

In our study, only about 12.5% (2/16) of patients who received IFN therapy had sustained viral elimination. And there were no significant difference in population of patients with normalized ALT between the IFN and non-IFN group ($n = 10$, $n = 7$, respectively). In spite of these results, patients treated with low-dose intermittent IFN therapy have a hepatic functional preservation greater than IFN untreated patients who received continuous medication with UDCA or SNMC after curative treatment of HCC. Although the mechanism of this reason is not well known, we suggested that the anti-inflammatory activity by low-dose intermittent IFN therapy may be stronger than medication with UDCA or SNMC and induce regression or retardation of underlying hepatic fibrosis, and finally, inhibits the progression of hepatic dysfunction.

Adverse effects such as reduction in blood counts by low-dose of IFN- α were not observed in our study, although neutropenia and/or thrombocytopenia were identified before IFN therapy. Furthermore, none of the patients required dose reduction in our study. Although 4 patients discontinued IFN therapy because of generalized fatigue, 2 of these patients restarted IFN therapy after that. Therefore, low-dose intermittent IFN- α therapy can be used relatively safely for cirrhotic patients with thrombocytopenia. However, patients who can not receive even low-doses of IFN also exist due to severe cytopenia or advanced liver cirrhosis. Medication with UDCA or SNMC or phlebotomy may be useful in decreasing ALT level for those patients.

Most cirrhotic patients who had received curative treatment for primary HCC have a limited hepatic reserve or thrombocytopenia. Therefore, low-dose intermittent IFN therapy might be effective for better prognosis. However, further studies of larger samples followed-up for

longer periods should be conducted to establish a definite conclusion about the effect of low-dose IFN therapy for the prevention of progressive liver disease and effect of treatment for recurrent HCC.

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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 μ L of serum by the SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μ L of H₂O for DNA analysis or 8.8 μ L of ribonuclease-free H₂O 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 μ L of nucleic acid extract and heated at 65°C for 5 minutes. The samples were set on ice for 5 minutes. Then 4 μ L of 5 \times reverse transcription (RT) buffer, 2 μ L of 10 mM dNTPs, 2 μ L 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- μ L reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 200 nM of forward primer (5'-TTTGGGGCATGGACAT-TGAC-3', nucleotides 1893-1912), 200 nM of reverse primer (5'-GGTGAACAATGGTCCGGAGAC-3', nucleotides 2029-2049), and 1 μ L 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 μ g/ μ L 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

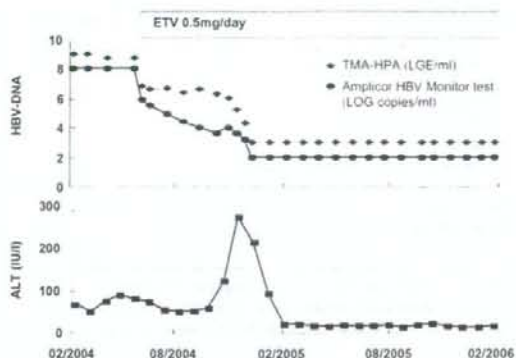


Fig. 1. Time courses of serum HBV DNA and ALT levels of patients treated with ETV. Expression of HBV nucleic acids determined by the TMA-HPA was higher than that determined by the Amplicor HBV Monitor test soon after beginning administration of ETV. The discrepancy was less marked when both measurements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

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-

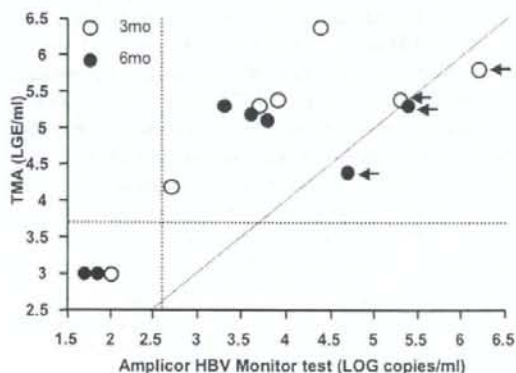


Fig. 2. Correlation of HBV nucleic acid levels determined by the TMA-HPA with HBV DNA levels determined by the Amplicor HBV Monitor test during ETV therapy. Serum samples obtained from the 2 patients who received low-dose ETV (0.01 mg) are indicated by arrows. The vertical and horizontal dotted lines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

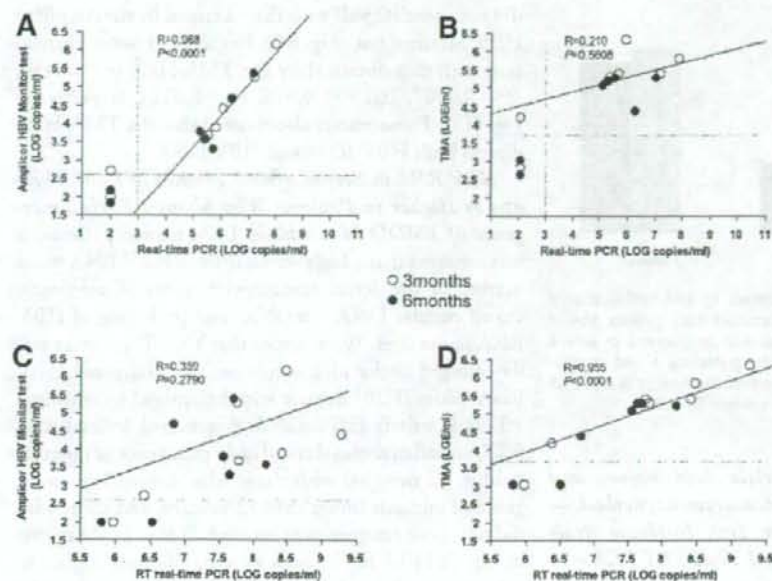


Fig. 3. Correlation between HBV nucleic acid and HBV DNA measurements after 3 and 6 months of ETV therapy. (A) Correlation between Amplicor HBV Monitor test and that determined by in-house real-time PCR. (B) Correlation of HBV nucleic acid level determined by the TMA-HPA and of HBV DNA determined by 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/ μ L, we detected them by nested PCR using 1 μ L 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).

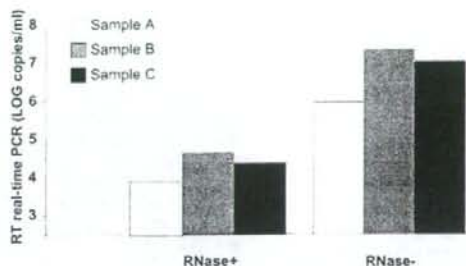


Fig. 4. Presence of HBV RNA confirmed by RNA treatment of 3 nucleic acid samples (samples A-C) obtained from patients after 3 months of LAM therapy. Extracted nucleic acid samples with or without RNase digestion were further digested by proteinase K and ethanol-precipitated after phenol/chloroform extraction. 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

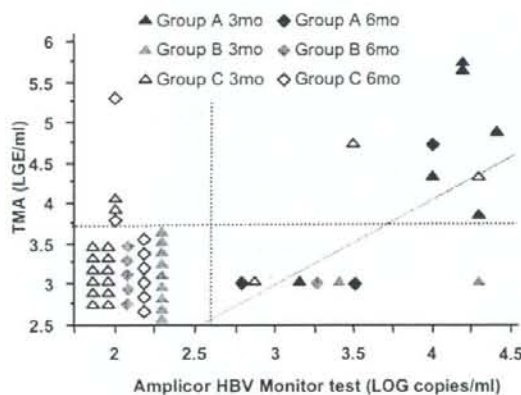


Fig. 5. Correlation of HBV nucleic acid levels determined by the TMA-HPA with HBV DNA levels determined by the Amplicor HBV Monitor test during LAM therapy. During ETV therapy the TMA-HPA showed higher expression of HBV DNA in patients regardless of the presence of the mutation than did the Amplicor 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.

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.

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

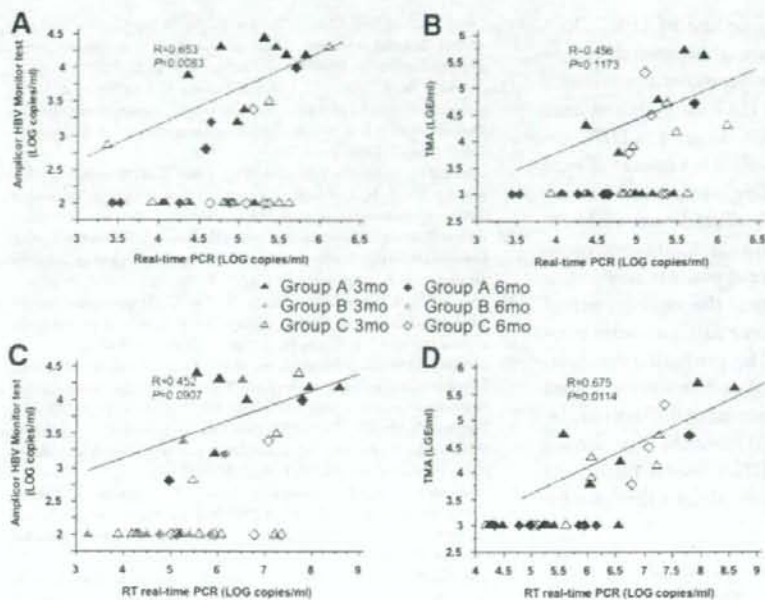


Fig. 6. Correlations between HBV nucleic acid and HBV DNA measurements after 3 and 6 months of LAM therapy. (A) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with that determined by in-house real-time PCR. (B) Correlation of HBV nucleic acid level determined by the TMA-HPA 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.

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

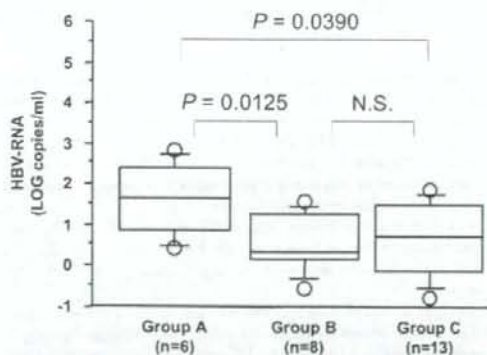


Fig. 7. Box plots of HBV RNA levels of patients in group A (patients who showed emergence of the mutants within 1 year), group B (those who developed resistance after 1 year of LAM therapy), and group C (patients who did not show resistance to LAM therapy). HBV RNA level represents the difference between 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).

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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Dual effect of APOBEC3G on *Hepatitis B virus*

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G to A hypermutation of *Hepatitis B virus* (HBV) and retroviruses appears as a result of deamination activities of host APOBEC proteins and is thought to play a role in innate antiviral immunity. Alpha and gamma interferons (IFN- α and - γ) have been reported to upregulate the transcription of APOBEC3G, which is known to reduce the replication of HBV. We investigated the number of hypermutated genomes under various conditions by developing a quantitative measurement. The level of hypermutated HBV in a HepG2 cell line, which is semi-permissive for retrovirus, was 2.3 in 10^4 HBV genomes, but only 0.5 in 10^4 in permissive Huh7 cells. The level of APOBEC3G mRNA was about ten times greater in HepG2 cells than in Huh7 cells. Treatment of HepG2 cells with either IFN- α or - γ increased the transcription of APOBEC3G and hypermutation of HBV. These mRNAs and hypermutation of HBV genomes were induced more prominently by IFN- γ than by IFN- α . Both IFNs decreased the number of replicative intermediate of HBV. Overexpression of APOBEC3G reduced the number of replicative intermediate of HBV and increased hypermutated genomes 334 times, reaching 968 in 10^4 genomes. Deamination-inactive APOBEC3G did not induce hypermutation, but reduced the virus equally. Our results suggest that APOBEC3G, upregulated by IFNs, has a dual effect on HBV: induction of hypermutation and reduction of virus synthesis. The effect of hypermutation on infectivity should be investigated further.

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INTRODUCTION

Hepatitis B virus (HBV) is a small, enveloped DNA virus with partially double-stranded DNA as a genome (Ganem & Schneider, 2001; Seeger & Mason, 2000). The virus replicates through transcription of pregenome RNA and reverse transcription, like retroviruses (Skalka & Goff, 1993; Summers & Mason, 1982). Infection with HBV causes chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma (Wright & Lau, 1993; Bruix & Llovet, 2003; Ganem & Prince, 2004).

Recent reports have shown that a cytidine deaminase, APOBEC3G, which is packaged in human immunodeficiency virus (HIV) virions in non-permissive cells, induces G to A hypermutation to a nascent reverse transcript of HIV and serves as part of the innate antiviral activity (Mangeat *et al.*, 2003; Zhang *et al.*, 2003; Lecossier *et al.*, 2003; Harris

et al., 2003). Recent studies have demonstrated that a small number of HBV DNA in serum samples of patients with chronic HBV infection contains hypermutated genomes (Gunther *et al.*, 1997; Suspene *et al.*, 2005a; Noguchi *et al.*, 2005). We reported previously that there are small numbers of hypermutated genomes in serum samples of the majority of patients with chronic HBV infection and that G to A hypermutation could be induced in cultured liver cells derived from HepG2 cell lines (Noguchi *et al.*, 2005) using a peptide nucleic acid-mediated PCR clamping method. Suspene *et al.* (2005a) developed the more sensitive differential DNA denaturation (3D)-PCR method to detect hypermutated genomes and found that some APOBEC proteins induce G to A, and in some cases C to T, hypermutations in HBV DNA (Suspene *et al.*, 2005a). Why only a very small proportion of the HBV genome is hypermutated is unknown at present. Furthermore, the

mechanism that controls the level of APOBEC protein expression and degree of hypermutation has not been fully investigated. Recently, Tanaka *et al.* (2006) identified an interferon (IFN)-stimulated response element (ISRE) in the promoter region of APOBEC3G and showed that IFN- α upregulates transcription of APOBEC3G. Peng *et al.* (2006) also reported that IFN- α and - γ upregulate mRNA transcription of APOBEC proteins. However, these reports did not analyse whether increased numbers of APOBEC proteins actually increase hypermutation. More recently, Bonvin *et al.* (2006) demonstrated that IFN induces transcription of APOBEC proteins and increases hypermutation of HBV.

IFNs are cytokines that play a major role against many pathogens (Samuel, 2001; Colonna *et al.*, 2002; Grandvaux *et al.*, 2002). We also reported in a previous study that both IFN- α and - γ reduce virus replication in stably HBV-transfected cell lines without inducing a remarkable increase in G to A hypermutation (Noguchi *et al.*, 2005). However, the method used in previous experiments for detection of hypermutation was not as sensitive as the method of Suspene *et al.* (2005a, b) and not quantitative. To assess the level of hypermutation, a reliable measurement of hypermutated genome is needed. In the present study, we developed a new and sensitive method for the measurement of hypermutated genome levels. Using this method, we show here that both IFN- α and - γ increased the levels of hypermutated genomes in cultured cell lines. Furthermore, both IFNs increased the mRNA level of APOBEC3G. We also performed overexpression experiments to examine whether APOBEC3G and its inactive mutants increase the levels of hypermutation and reduce HBV replication.

METHODS

Plasmid constructs. The expression vector for haemagglutinin (HA)-tagged human APOBEC3G, pcDNA3/HA-A3G, was constructed as described previously (Kobayashi *et al.*, 2004). APOBEC3F cDNA was obtained by modifying APOBEC3F like (IMAGE clones from Open Biosystems) to have the same sequence as human APOBEC3F transcript variant 1 (GenBank NM_145298) and cloned into pcDNA3/HA (Invitrogen). APOBEC3G mutants were constructed using a QuikChange mutagenesis kit (Stratagene). The construction of wild-type HBV 1.4 genome length, pTRE-HBwt, has been described previously (GenBank accession no. AB206816) (Tsuge *et al.*, 2005).

Cell culture and transfection. Huh7 and HepG2 cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum at 37 °C in 5% CO₂. Cells were seeded to semi-confluence in six-well tissue culture plates. Transient transfection of the plasmids into HepG2 and Huh7 cell lines was performed using TransIT-LT1 (Mirus) according to the instructions provided by the supplier. A plasmid encoding a secreted form of human placental alkaline phosphatase (SEAP) was co-transfected to adjust the transfection efficiency. The SEAP assay in the culture medium was performed using the Great Escape SEAP Reporter System 3 (BD Bioscience).

T23 cells are HepG2 cells stably transfected with the plasmid pTRE-HBwt. They were cultured using a method described previously

(Tsuge *et al.*, 2005). Cells were seeded to semi-confluence in six-well tissue culture plates and then treated with medium containing either IFN- α (Hayashibara Biochemical Laboratories) or IFN- γ (Shionogi & Co.). The cells were harvested 12–72 h after IFN treatment. Core-associated HBV DNA was extracted from the cells for HBV DNA quantification and quantitative analysis of G to A hypermutated genomes (Noguchi *et al.*, 2005).

Analysis of core-associated HBV DNA. The cells were harvested 4 days after transfection and lysed with 250 μ l lysis buffer [10 mM Tris/HCl pH 7.4, 140 mM NaCl and 0.5% (v/v) NP-40] followed by centrifugation for 2 min at 15 000 g. The core-associated HBV genome was immunoprecipitated from the supernatant by mouse anti-core monoclonal antibody anti-HBc determinant α (Institute of Immunology, Tokyo, Japan) and subjected to quantitative analysis after SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems). The primers used for amplification were #1, 5'-ACTTCAAGCCCAACAMRRATCA-3' (nt 2978–2999) [numbers are those of HBV subtype C reported by Norder *et al.* (1994)] and #2, 5'-AGAGYTTGKTGGAATGKTGGA-3' (nt 24–1), where M is A/C, R is G/A, Y is T/C and K is G/T. The probe was a 6-carboxy-fluorescein (FAM)-labelled minor-groove binder (MGB) probe, 5'-(FAM)-TTAGAGGTGGAGAGATGG-(MGB)-3' (nt 3184–3167). Real-time PCRs were set up in 25 μ l TaqMan Universal Master Mix with 1 μ l DNA solution, 0.9 μ M each primer and 0.25 μ M probe. The amplification conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 62 °C for 90 s).

Amplification and analysis of hypermutated HBV genomes by 3D-PCR. HBV DNA was extracted from 100 μ l serum obtained from a chronic HBV carrier (genotype C) by SMITEST (MBL International) and was dissolved in 20 μ l H₂O. Hypermutated genomes were detected by modified 3D-PCR using primers #1 and #2 and DNA solution from serum containing 8.0×10^7 or 2.3×10^5 copies of core-associated HBV DNA in 25 μ l of 100 mM Tris/HCl pH 8.3, 50 mM KCl, 15 mM MgCl₂, 0.2 mM each dNTP, 10 pmol each primer and 1.25 U Taq DNA polymerase (GeneAmp, Nippon Gene Co.), together with 0.25 μ g anti-Taq high (TOYOBO Co.). The amplification conditions included an initial denaturation step at 83–95 °C for 5 min, followed by 45 cycles of denaturation at 83–95 °C for 1 min, annealing at 50 °C for 30 s, extension at 72 °C for 30 s followed by 10 min of final extension. Amplicons were separated by electrophoresis on 2% (w/v) agarose gel, cloned and sequenced in an ABI PRISM 3130 Genetic Analyzer with a BigDye Terminator version 3.1 cycle sequencing ready reaction kit (Applied Biosystems). The PCR products were also analysed on Hanse Analytik (HA)-yellow gel as described previously (Suspene *et al.*, 2005b; Tsuge *et al.*, 2005; Abu-Daya *et al.*, 1995).

Quantitative analysis of hypermutated genomes by real-time PCR. Hypermutated genomes were quantified by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems) and the above primers and probes. The amplification conditions included activation at 95 °C for 10 min followed by initial denaturation at 88 °C for 20 min and 45 cycles of amplification (denaturation at 88 °C for 15 s, annealing at 50 °C for 30 s and extension at 62 °C for 90 s). We chose 88 °C as this temperature is appropriate for detection of about 20% hypermutated genomes. There are 200–300 such hypermutated genomes in 10^4 genomes present in HepG2 cells transiently transfected with APOBEC3G. The buffer comprised 10 mM Tris/HCl pH 8.3, 50 mM KCl, 3 mM MgCl₂, 10 mM EDTA, 60 nM Passive Reference 1 (Applied Biosystems), 0.2 mM each dNTP, 0.9 μ M each primer, 0.25 μ M probe, 5×10^6 copies of HBV DNA

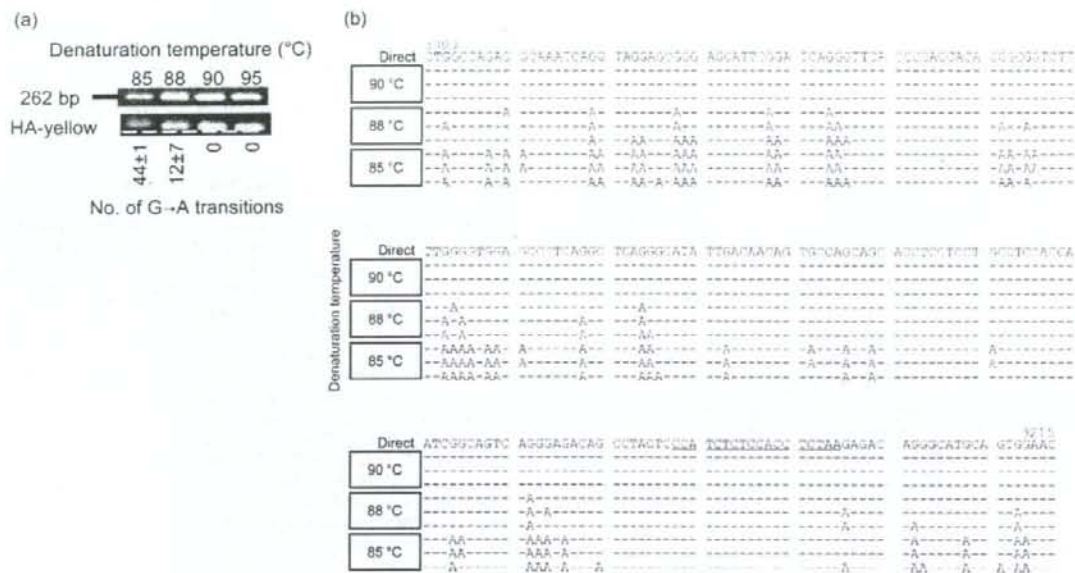


Fig. 1. Amplification of HBV DNA by 3D-PCR. (a) Detection of hypermutated genomes by HA-yellow agarose gel electrophoresis. The numbers of G to A transitions are expressed as means \pm SD generated from the sequence analysis of five independent clones from PCR products. The white dotted line was added to help visualize the retardation of AT-rich DNA in HA-yellow agarose gel. (b) Nucleotide sequences of HBV amplified by 3D-PCR. The nucleotide sequences obtained by direct sequencing are used as a reference sequence. The nucleotide sequences where the probe hybridizes are underlined. Note that the number of G to A mutations correlates with denaturation temperature.

and 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a final volume of 25 μ l. A standard curve was constructed by the simultaneous amplification of serial dilutions of the 3D-PCR products.

Western blot analysis. Cell lysates were prepared as described above, resolved on 10% (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Whatman) via electro-blotting. The membranes were incubated with anti-haemagglutinin fusion epitope monoclonal antibody (Roche) or with anti- β -actin monoclonal antibody (Sigma-Aldrich) followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit antibody or sheep anti-mouse immunoglobulin (Amersham Biosciences). Proteins were visualized via the ECL system (Amersham Biosciences).

Quantification of mRNA of APOBEC3G or APOBEC3F by reverse transcription and real-time PCR. Total RNA was extracted from HepG2 cell lines by using an RNeasy Mini kit (Qiagen). The RNA was reverse transcribed with random primers and Moloney murine leukemia virus reverse transcriptase (ReverTra Ace, TOYOBO Co.) at 42 °C for 60 min according to the instructions provided by the manufacturer. Quantitative analysis of APOBEC3G and APOBEC3F cDNA was performed by real-time PCR using TaqMan Gene Expression assays (Applied Biosystems). To confirm that the APOBEC3G and -3F PCR primers specifically amplify the target genes, quantitative PCR on the expression plasmids encoding human APOBEC3G and -3F, used as templates, was performed. No cross amplification was observed, even when we used 10^7 copies of APOBEC3G plasmid in the amplification reaction of

APOBEC3F and vice versa. A standard curve was constructed by the amplification of serial dilutions of the known number of plasmids containing human APOBEC3G and APOBEC3F. The target cDNA was normalized to the endogenous RNA level of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers and FAM-labelled probe used to quantify GAPDH were purchased from Applied Biosystems.

Infectivity of luciferase reporter viruses produced from HepG2 and Huh7 cell lines. Luciferase reporter viruses with or without viral infectivity factor (Vif) were prepared by co-transfection of pNL43/ Δ Env-Luc (wild-type) or pNL43/ Δ EnvVif-Luc (Δ Vif) plus pVSV-G together with a mock vector or expression vectors for A3G by Lipofectamine (Invitrogen) as described previously (Janini *et al.*, 2001; Shindo *et al.*, 2003). Productive infection was measured by luciferase activity. Values were presented as percentage of infectivity relative to the value of each virus without expression of APOBEC3G proteins.

RESULTS

Quantitative analysis of hypermutated genome by real-time PCR

Using serum samples from a patient with a high viral load, we amplified a large number of hypermutated genomes by 3D-PCR and detected them by HA-yellow agarose gel electrophoresis (Fig. 1a). Nucleotide sequence analysis

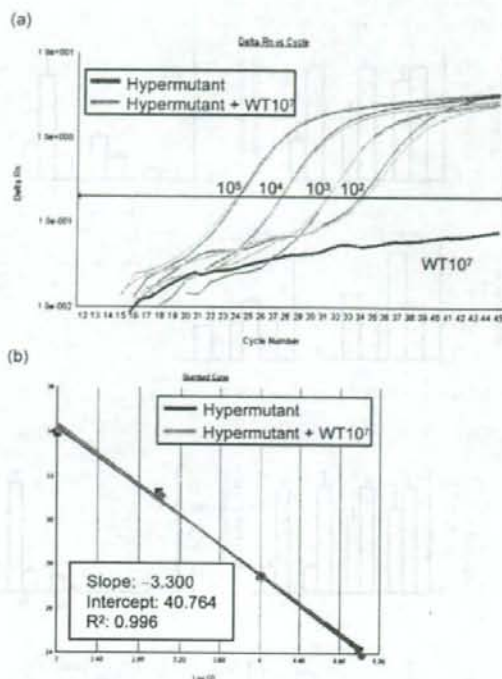


Fig. 2. Quantitative measurement of hypermutated HBV DNA using 3D-PCR combined with real-time PCR. The indicated numbers (10^2 – 10^5) of hypermutated genomes alone (orange lines) and a mixture of wild-type plus hypermutated genomes (green lines) were amplified by 3D-PCR. 3D-PCR did not result in amplification of wild-type sequence (purple line). Denaturation temperature was 88 °C.

showed detection of more heavily hypermutated genomes at lower denaturation temperatures (Fig. 1b). To develop quantitative measurement, we selected sequences with many G residues, designed primers that contained only a small number of G residues and used degenerate primers. A probe sequence was designed without a G residue. Using this primer and probe set, we could amplify only hypermutated genomes (Fig. 2). When hypermutated and non-mutated genomes were co-amplified, only hypermutated genomes were successfully amplified using the above primer and probe set (Fig. 2b). Non-hypermutated genomes (10^7 copies) were not amplified, although conventional PCR amplified both mutated and non-mutated genomes equally (data not shown). We also tried to detect only slightly (four of the 58 G residues) mutated genomes by 3D-PCR, but could not detect such genomes. It should thus be noted that the quantitative measurement we developed in this study detects only hypermutated genomes.

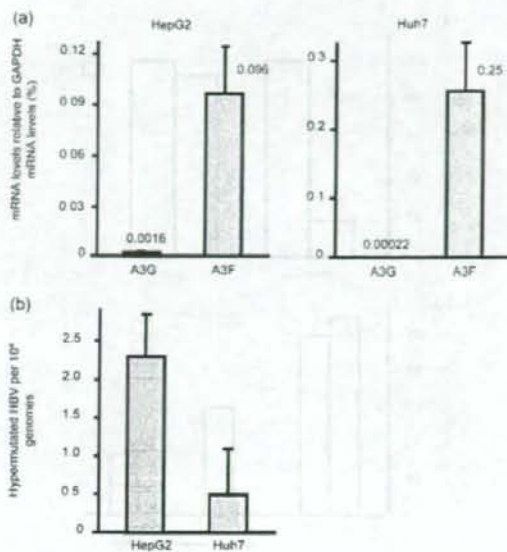


Fig. 3. Expression levels of APOBEC3G and -3F protein mRNAs in HepG2 and Huh7 cell lines. (a) mRNAs were extracted from cultured cell lines and the number of mRNA was quantified by real-time PCR with a probe for APOBEC3G and -3F. The expression levels were expressed as a percentage of GAPDH mRNA. (b) Number of hypermutated HBV genomes measured by real-time 3D-PCR in HepG2 and Huh7 cell lines transiently transfected with pTRE-HBV-wt. Results are means \pm SD values of three independent experiments.

Detection of APOBEC3G mRNA and hypermutated genomes in semi-permissive and permissive cell lines

In retrovirus studies, it is known that some cell lines allow production of infectious retrovirus virions with Vif deficiency (permissive cells) while others do not. The difference between semi-permissive and permissive cell lines is the expression of APOBEC3G (Mangeat *et al.*, 2003; Zhang *et al.*, 2003; Lecossier *et al.*, 2003; Harris *et al.*, 2003; Shirakawa *et al.*, 2006). Thus, we examined the expression of APOBEC3G in both HepG2 and Huh7 cell lines. The APOBEC3G mRNA level detected by real-time PCR was very low (approx. 0.002% relative to GAPDH mRNA) and about ten times greater in HepG2 cells than in Huh7 cells (Fig. 3a).

The number of hypermutated genomes in HepG2 cells transiently transfected with pTRE-HB-wt was about five times that in Huh7 cells (Fig. 3b). Vif-deficient HIV-1 virions produced from HepG2 cell exhibited very low infectivity compared with wild-type (Fig. 4a). In contrast, the infectivity of HIV-1 virions produced by Huh7 was