Knudson's two hit theory, may be involved in the carcinogenesis. In addition, further studies on genetic alterations or immunohistochemical staining of *GMDS* protein or both may uncover the precise stage(s) and phenotype(s) of colorectal tumors in which the deregulated *GMDS* function is involved and answer the question whether two hits or haploinsufficiency plays a role in tumorigenesis. Indeed, certain kinds of colon cancer tissues showed negative staining for AAL on immunohistochemical analysis (manuscript in preparation), suggesting that a deficiency of fucosylation because of the mutation of fucosylation-related genes, including *GMDS*, could account for the absence of AAL.

The development of cancer-specific therapies with little or no toxic side effects is one of the goals of molecular oncology and translational research. TRAIL is being evaluated presently as a selective anticancer agent, but many human cancer cell lines are resistant to TRAIL therapy, suggesting that many patients will be nonresponders. Elucidation of the mechanisms underlying the resistance is actively underway. The levels of TRAIL receptors do not generally show a direct correlation with sensitivity to apoptosis stimulation.[41] In this study, the gene, protein, and cell surface expression levels of TRAIL receptors, DR4 and DR5, were not different between mock and *GMDS*-rescued cells (data not shown). Recently, Wagner et al[42] reported that the *O*-glycosylation of TRAIL receptors on cancer cells modulates the sensitivity to TRAIL. This study, for the first time, showed the importance of glycosylation in TRAIL-induced apoptosis. The present study showed that the restoration of fucosylation in fucosylation-deficient cancer cells leads to high susceptibility to TRAIL. Although treatment with benzyl-GalNAc, a general inhibitor of *O*-glycosylation, resulted in resistance to TRAIL in several TRAIL-susceptible cancer cells,[42] the susceptibility to TRAIL in the *GMDS*-rescued cells was not suppressed by treatment with it (data not shown). These results show that the susceptibility to TRAIL of the *GMDS*-rescued cells is independent of *O*-glycosylation, indicating that many cancer cell lines acquire resistance to TRAIL-induced apoptosis in a cell type-specific manner.

To determine whether the less susceptibility to TRAIL by the decrease of fucosylation was observed in other cell lines, we investigated the effect on the susceptibility to TRAIL by *GMDS* small interfering RNA (siRNA)-mediated knockdown. The transfection of *GMDS* siRNA could not lead to a decrease of cellular fucosylation regardless of a decrease of *GMDS* expression. Thus, the susceptibility to TRAIL did not change by the transfection of *GMDS* siRNA. The knockdown efficiency by siRNA transfection had limitations. Almost complete loss of *GMDS* activity would be required for the significant decrease of fucosylation.

Although the pathologic role of fucosylation in cancer cells remains to be elucidated in detail, the findings we reported here show that a deficiency of fucosylation because of a *GMDS* mutation leads to escape from NK cell–mediated tumor surveillance through the acquisition of resistance to TRAIL, followed by tumor progression and metastasis. *GMDS* is a novel regulatory molecule for TRAIL-induced apoptosis and the immune system.

## Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.04.002.

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*Conflicts of interest*

**The authors disclose no conflicts.**

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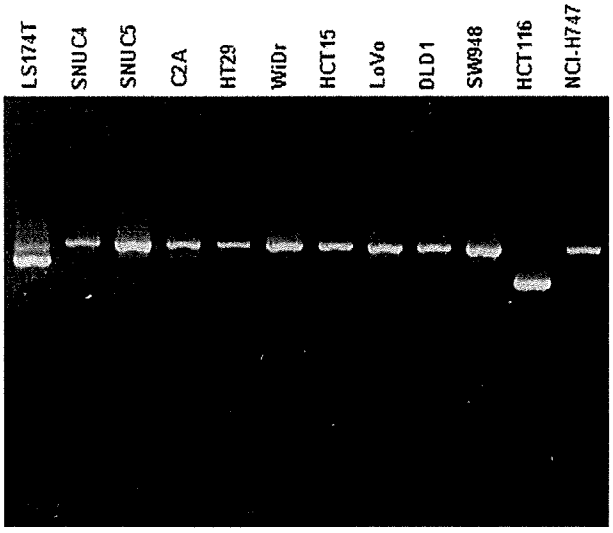
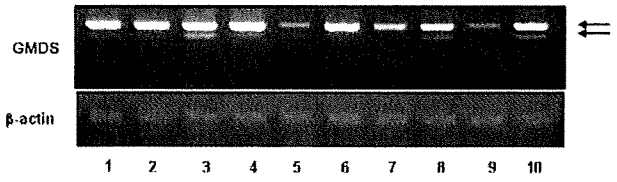
**Supplementary Table 1.** Primer Sequences

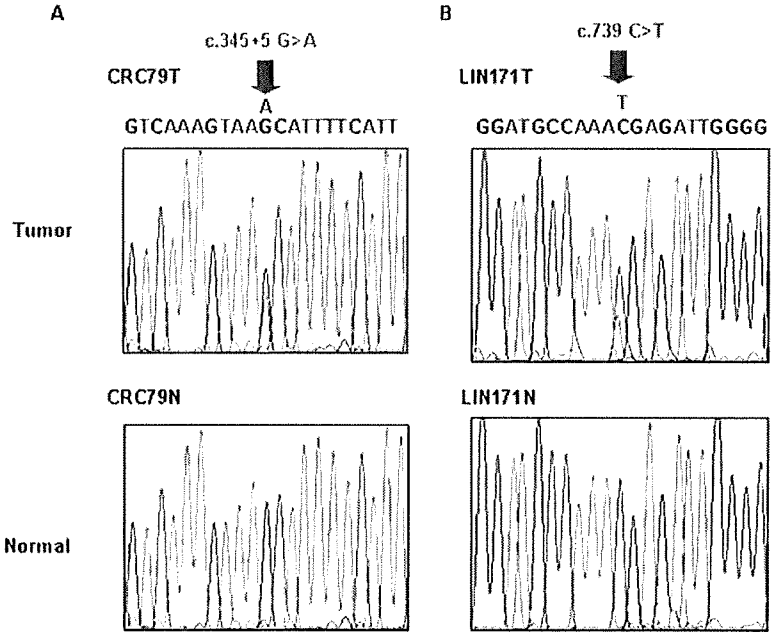
| Primer | 5′-Sequence-3′ |
|---|---|
| Primers used for PCR | |
| *GMDS*-forward | GCAAGCTTAAAATGGCACACGCACCGGCAC |
| *GMDS*-reverse | GCGGATCCTCAGGCATTGGGGTTTGTC |
| FX-forward | ATGGGTGAACCCCAGGGATCC |
| FX-reverse | TCACTTCCGG GCCTGCTCGTA |
| GAPDH-forward | AACGGGAAGCTTGTCATCAAT |
| GAPDH-reverse | GCCAGTGAGCTTCCCGTTCA |
| *GMDS*-exon1 forward | TGTAAAACGACGGCCAGTCCTCCTTCCTCTTCCCTGAG |
| *GMDS*-exon1 reverse | CAGGAAACAGCTATGACCGAGAGGGCTGTTCCTGGAG |
| *GMDS*-exon2 forward | TGTAAAACGACGGCCAGTCTCAAAACTCTTCATCTAGACC |
| *GMDS*-exon2 reverse | CAGGAAACAGCTATGACCGTTTCAGTGGAAAACACACTC |
| *GMDS*-exon3 forward | TGTAAAACGACGGCCAGTTGGGGAGAAAGTAAGCAGTG |
| *GMDS*-exon3 reverse | CAGGAAACAGCTATGACCTTATCTGAACATAGGAACATCTG |
| *GMDS*-exon4 forward | TGTAAAACGACGGCCAGTCTCCAGAACCATCAGTACAGC |
| *GMDS*-exon4 reverse | CAGGAAACAGCTATGACCTACTACACTGAGAAGCAGCAG |
| *GMDS*-exon5 forward | TGTAAAACGACGGCCAGTGACAGCACCTTTGTGCTATG |
| *GMDS*-exon5 reverse | CAGGAAACAGCTATGACCACAGATAACGCCACAC |
| *GMDS*-exon6 forward | TGTAAAACGACGGCCAGTGAAGATGGTGAGAATCAC |
| *GMDS*-exon6 reverse | CAGGAAACAGCTATGACCATACACCAAATAGACATGCAAC |
| *GMDS*-exon7 forward | TGTAAAACGACGGCCAGTCGGGTTTGGTTCACCAAACTG |
| *GMDS*-exon7 reverse | CAGGAAACAGCTATGACCACTTTTTCACTGTACGCTTG |
| *GMDS*-exon8 forward | TGTAAAACGACGGCCAGTCCATATCTGATTATGTGCAC |
| *GMDS*-exon8 reverse | CAGGAAACAGCTATGACCACTCTACACTGAAACTTTCTG |
| *GMDS*-exon9 forward | TGTAAAACGACGGCCAGTAATAATCAGCTGGAGCATCAG |
| *GMDS*-exon9 reverse | CAGGAAACAGCTATGACCACTGACAGCTCCAAAGGCTG |
| *GMDS*-exon10-11 forward | TGTAAAACGACGGCCAGTGAACGCGCACAGCAGGGACC |
| *GMDS*-exon10-11 reverse | CAGGAAACAGCTATGACCGTCTGCACCGGAGACTCTG |
| Primers used for mutagenesis | |
| Exon 2-4 deletion-forward | CAGGCCAGATTTCCTTTGACCTCGCT |
| Exon 2-4 deletion-reverse | AAGGAAATCTGGCCTGTGATACCGGT |
| Exon 5-7 deletion-forward | ACGTCAAAGCTATGTGGTTGATGTTG |
| Exon 5-7 deletion-reverse | CACATAGCTTTGACGTGGCTCTGGGC |
| Exon 7 deletion-forward | AGAAGAGGCTATGTGGTTGATGTTGCAGAATGAT |
| Exon 7 deletion-reverse | CCACATAGCCTCTTCTGGGACTCTCATGATTGAA |
| c.739 C>T-forward | TGCCAAATGAGATTGGGGCCATGCCA |
| c.739 C>T-reverse | CAATCTCATTTGGCATCCAGATTTCC |
| Primers used for sequencing | |
| M13F | TGTAAAACGACGGCCAGT |
| M13R | CAGGAAACAGCTATGACC |
| T7 | TAATACGACTCACTATAGGG |
| SP6 | ATTTAGGTGACACTATAGAA |




**Supplementary Figure 1.** Additional mutations of *GMDS* in human colon cancer cell lines. RT-PCR analysis was performed with RNA derived from several colon cancer cell lines. In LS174T and HCT116 cells, short transcripts of *GMDS* were observed.




**Supplementary Figure 3.** Additional mutations of *GMDS* in human ovarian cancer tissues. RT-PCR analysis was performed with RNA derived from 10 microdissected ovarian cancer tissues. In numbers 3, 4, 5, 8, and 10, a short transcript of *GMDS* was observed. *Arrowheads* indicate the wild-type (*top*) or mutant (*bottom*) *GMDS*.




**Supplementary Figure 2.** Additional mutations of *GMDS* in human colon cancer tissues. PCR and subsequent direct sequencing analyses were performed in 100 human colon cancer tissues. Two types of point mutations, c.345+5 G>A (*A*) and c.739 C>T (*B*), found in this experiment were indicated. The primer sequences used for the PCR and direct sequencing are summarized in Supplementary Table 1.

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***Original Article***

# Effect of interferon α-2b plus ribavirin therapy on incidence of hepatocellular carcinoma in patients with chronic hepatitis

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*Aim:* The objective of this study was to elucidate the long-term effects of interferon (IFN)α-2b plus ribavirin combination therapy and to clarify whether this therapy can reduce the incidence of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C.

*Methods:* A total of 403 patients infected with hepatitis C virus (HCV) were enrolled in a multicenter trial. All patients were treated with a combination of IFN-α-2b plus ribavirin therapy. We examined the incidence of HCC after combination therapy and analyzed the risk factors for liver carcinogenesis.

*Results:* A sustained virological response (SVR) was achieved by 139 (34%) of the patients. The cumulative rate of incidence of HCC was significantly lower in SVR patients than in non-SVR patients ($P = 0.03$), while there was no difference in the cumulative incidence of HCC between the transient response (TR) group and the no response (NR) group. Cox's regression analysis indicated the following risk factors as independently significant in relation to the development of HCC: age being > 60 years ($P = 0.006$), advanced histological staging ($P = 0.033$), non-SVR to IFN therapy ($P = 0.044$). The cumulative incidence rate of HCC was significantly lower in patients who had average serum alanine aminotransferase (ALT) levels of < 40 IU/L than in those who showed average serum ALT levels of ≧ 40 IU/L after the combination therapy ($P = 0.021$).

*Conclusions:* These results suggest that the attainment of SVR or continuous normalization of ALT levels after IFN therapy can affect patients apart from HCC development.

**Key words:** chronic hepatitis C, continuous normalization of ALT, hepatocellular carcinoma, interferon plus ribavirin combination therapy, sustained virological response

## INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most common malignancies in Japan and its incidence has been increasing over the last 30 years. Recently, various treatments such as transcatheter arterial embolization/chemoembolization, radio frequency ablation and hepatic resection have been reported to yield significant improvements in overall patient survival,[1–3] but HCC relapse has thus far been observed in a majority of treated patients due to the highly malignant potential of the liver. In general, approximately 70–80% of Japanese HCC patients are also diagnosed with type C chronic hepatitis or cirrhosis.[4] It has also been shown that the chronic hepatitis C (CHC) liver slowly but steadily progresses to cirrhosis[5,6] and the risk of HCC increases according to the degree of liver fibrosis.[7,8] In this regard, the success of treatment

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for chronic hepatitis C virus (HCV) infection is expected to prevent the patient's liver from progressing to cirrhosis and to reduce the risk of development of HCC. Interferon (IFN) has been proven to be effective in reducing and in eliminating HCV from the circulation; in decreasing serum alanine aminotransferase (ALT) levels; and in improving the histological appearance of the liver in patients with CHC.[9–11] Moreover, it has been demonstrated that IFN monotherapy in CHC patients is associated with reducing the incidence of HCC, especially in those patients who achieved a sustained virological response (SVR).[12–14] Recently, many investigators have reported that combination therapy using IFN-α-2b or pegylated IFN (Peg-IFN) N plus ribavirin is more effective for eradicating HCV than IFN monotherapy.[15–17] However, it has not been accurately evaluated whether or not the combination therapy using Peg-IFN plus ribavirin could reduce HCC development in patients infected with HCV.

In this study, we evaluated the long-term effect of IFN-α-2b plus ribavirin therapy on the incidence of HCC in HCV-infected patients treated with the combination therapy by retrospective examination of the clinical outcomes.

## METHODS

### Patients

THIS STUDY WAS a multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum in Japan. A total of 459 patients with HCV infection were treated with a combination of IFN-α-2b (Intron; Schering-Plough Corporation, Kenilworth, NJ, USA) plus ribavirin (Rebetol; Schering-Plough, Auxerre, France) between June 2002 and March 2005. All patients were treated with 6 MU of IFN-α-2b subcutaneously thrice a week and with oral ribavirin daily. Ribavirin was given at a total daily dose of 600 mg for patients who weighed < 60 kg and 800 mg for patients who weighed ≧ 60 kg. Patients who were positive for hepatitis B surface antigen, anti-human immunodeficiency virus antibody or those with other liver diseases (alcoholic liver disease, autoimmune liver disease, etc) were excluded from this study. Also excluded were patients with a history of HCC and those who developed HCC within the first 6 months of the follow-up period after the end of IFN therapy, because of the possibility that microscopic HCC had been present before initiation of the treatment. The remaining 403 patients infected with HCV were enrolled and followed in this study. The observation term was terminated upon the start of the next IFN therapy, such as Peg-IFN plus ribavirin after a combination of IFN-α-2b plus ribavirin therapy. Responses to IFN therapy were divided into the following three groups based on the viral load: sustained virological response (SVR) was defined as the absence of detectable serum HCV-RNA at 24 weeks after completion of IFN therapy. Transient response (TR) was defined as the absence of HCV-RNA from the serum at the end of treatment but detectable at 24 weeks after completion of therapy. Those categorized as having no response (NR) did not meet these criteria.

This study protocol followed the ethical guidelines of the 1975 Declaration of Helsinki, and informed consent was obtained from each patient.

### Blood tests

Serum samples were stored frozen at −80°C. HCV-RNA levels were analyzed by quantitative reverse transcription (RT)–PCR assay (Amplicor-HCV version 2.0; Roche Diagnostic Systems, Tokyo, Japan). The lowest detection limit of this assay was 50 IU/mL. All patients were examined for serum HCV-RNA level and underwent hematological and biochemical tests just before therapy, every 4 weeks during treatment and every 12 weeks thereafter until the end of treatment.

Normal serum ALT is defined as < 40 IU/L. In addition, the biological response to IFN therapy was defined based on "the average serum ALT level", which was calculated from all data of ALT levels after completion of IFN therapy.

### Histological evaluation

The patients underwent liver biopsies within 6 months before the start of therapy. Histopathological interpretation of specimens was done by experienced liver pathologists who had no clinical information. The histological appearance of the liver sample sections was evaluated according to METAVIR's histological score.[18] Fibrosis stage was evaluated on a scale from 0 to 4.

### Diagnosis and follow up of HCC

Ultrasonography was carried out before IFN therapy and every 3 to 6 months during the follow-up period. New space-occupying lesions detected or suspected at the time of ultrasonography were further examined by computed tomography (CT) or hepatic angiography. HCC was diagnosed by the presence of typical hypervascular characteristics on angiography, in addition to the findings from CT. If no typical image of HCC was observed, fine-needle aspiration biopsy was carried out with the

patient's consent, or the patient was carefully followed until a diagnosis was possible with a definite observation by CT or angiography.

### Statistical analysis

Quantitative variables were expressed as mean ± SD. The Kaplan–Meier method was used to calculate the cumulative incidence of HCC. The prognostic relevance of clinical variables and HCC incidence was evaluated by univariate analysis with log-rank test and by multivariate Cox's regression analysis. A value of $P < 0.05$ (two-tailed) was considered to indicate significance. All calculations were performed with SPSS version 15.0J (SPSS, Chicago, IL, USA).

## RESULTS

### Baseline characteristics in patients treated with interferon therapy

THE BASELINE CLINICAL features of the enrolled patients are shown in Table 1. The mean age of the patients was 55.8 ± 10.9 years, and 64% of the total cases were male. Two hundred and sixty-one patients (73%) were infected with HCV genotype 1 and had a viral load of more than $10^5$ IU/ml. Liver biopsy was done for 320 cases and the ratio of patients with severe fibrosis (F3–4) diagnosed by the HAI score was more than 31%. The mean platelet count was 14.8 ± 5.1 × $10^4$/μl, and the ALT level was 96.0 ± 62.6 IU/L. A sustained virological response (SVR) was achieved by 139 patients (34%) by combination therapy of IFN-α-2b plus ribavirin. According to an intent-to-treat analysis, 20% (51/261) of patients with HCV genotype 1 and a high viral load (≧ 100KIU/mL) achieved SVR by the combination therapy, whereas 75% (73/97) of the patients with HCV genotype 2 or a low load showed SVR. The median observation period for all patients was 36.5 ± 14.8 months with a range of 6 to 62 months from the end-point of IFN treatment.

Table 1 Baseline characteristics in patients treated with interferon therapy

| | All cases |
|---|---|
| Number of patients | 403 |
| Age | 55.8 ± 10.9 |
| Gender (male/female) | 257/145 |
| Genotype and viral load (1H/non-1H) | 261/97 |
| Fibrosis (F0/1/2/3/4) | 15/149/56/92/8 |
| WBC (/μl) | 5113 ± 1487 |
| Platelet (× $10^4$/μl) | 14.8 ± 5.1 |
| ALT (IU/l) | 96.0 ± 62.6 |
| IFN effect (SVR/TR/NR/cessation) | 139/109/110/45 |

Data are number of patients, mean ± standard deviation. Fibrosis stage is evaluated on a scale from 0 to 4 according to METAVIR's histological score. 1H, Genotype 1 and high viral load; non-1H, all except for 1H; ALT, alanine aminotransferase; IFN, interferon; NR, no response; SVR, sustained virological response; TR, transient response; WBC, white blood cells.

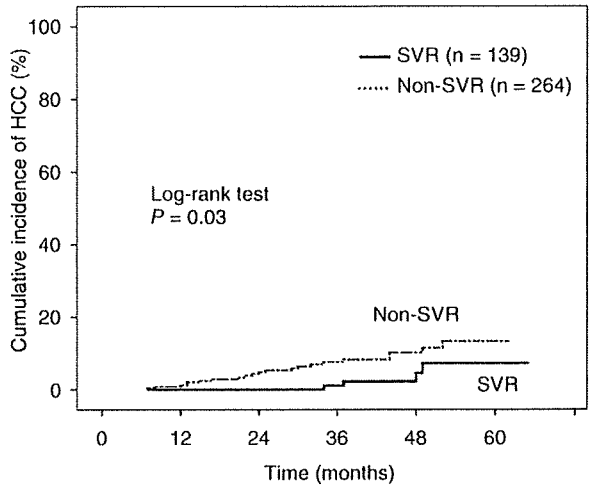


Figure 1 Cumulative incidence of development of hepatocellular carcinoma (HCC) according to treatment effect: (—) sustained virological response; (.....) non-sustained virological response.

### Cumulative incidence of development of HCC according to the treatment effect (SVR vs. non-SVR)

Figure 1 shows the Kaplan–Meier estimates of the cumulative HCC incidence according to the treatment effect (SVR vs. non-SVR). Twenty-five (6%) of the 403 enrolled patients developed HCC; four (2.9%) of the SVR group and 21 (8.0%) of the non-SVR group. The cumulative incidence rate of HCC was significantly lower in patients of the SVR group than in those of the non-SVR group ($P = 0.03$).

### Cumulative incidence of HCC development according to the treatment effect (SVR vs. TR vs. NR vs. cessation)

Figure 2 shows the Kaplan–Meier estimates of the cumulative HCC incidence according to the treatment effect (SVR vs. TR vs. NR vs. cessation). Five patients (4.6%) of the TR group, nine (8.2%) of the NR group and seven (15.6 %) of the cessation group developed

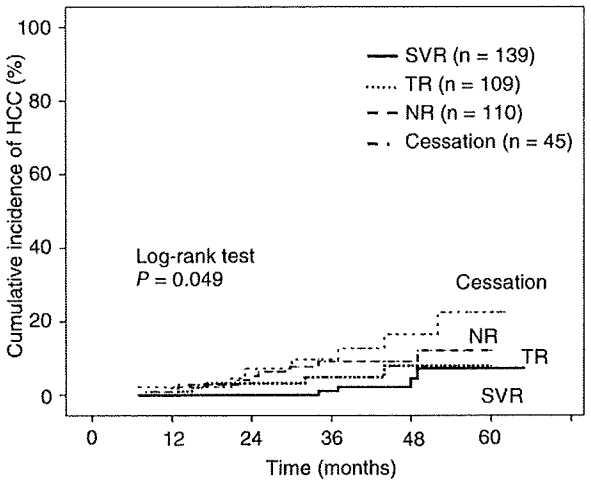


**Figure 2** Cumulative incidence of hepatocellular carcinoma (HCC) development according to treatment effect: (—) sustained virological response; (.....) transient response group; (- -) no response; (- ·) cessation.

HCC. There was no significant difference in the cumulative incidence of HCC between the TR and NR groups ($P = 0.394$). In contrast, the cumulative incidence rate of HCC was significantly lower in patients of the SVR group than in those of the NR group ($P = 0.05$). These results indicate that treatment of the TR group with IFN-α-2b plus ribavirin therapy did not reduce HCC development when compared to the NR group.

## Risk factors for cumulative incidence of HCC development

Univariate analysis with the log-rank test showed that the following were significant risk factors for the development of HCC; older age (> 65 years) ($P = 0.01$), severe fibrosis ($P = 0.006$), high platelet count ($> 14 \times 10^4/\mu l$) ($P = 0.017$) and non-SVR ($P = 0.03$). Stepwise multivariate analyses of these four variables were performed for all patients treated with combination therapy of IFN-α-2b plus ribavirin by Cox's regression analysis, as shown in Table 2. The analysis indicated the following factors as independent significant risk factors related to the development of HCC: older age (risk ratio, 3.23; 95% CI, 1.37–8.56; $P = 0.006$), fibrosis staging (risk ratio, 1.69; 95% CI, 1.04–2.67; $P = 0.033$) and non-SVR to IFN therapy (risk ratio, 3.57; 95% CI, 1.04–12.36; $P = 0.044$).

## Cumulative incidence of HCC development according to average serum ALT levels after combination therapy

The average serum ALT levels in 134 patients (96.4%) of the SVR group were < 40 IU/L after completion of the combination therapy, while 63 patients (24.4%) of the non-SVR group showed serum ALT levels of ≧ 40 IU/L. Figure 3 shows Kaplan–Meier estimates of the cumulative HCC incidence according to the average serum ALT levels after combination therapy. The cumulative incidence rate of HCC was significantly lower in patients with average serum ALT levels of < 40 IU/L than with average serum ALT levels of ≧ 40 IU/L ($P = 0.021$).

## Cumulative incidence of HCC development according to the treatment effect (SVR vs. non-SVR) in patients showing less than 40 IU/L average ALT levels after the combination therapy

Figure 4 shows Kaplan–Meier estimates of the cumulative HCC incidence according to the treatment effect (SVR vs. non-SVR) in patients who showed less than 40 IU/L average ALT levels after the combination therapy. There was no significant difference in the cumulative incidence rate of HCC between the SVR and non-SVR groups ($P = 0.37$).

**Table 2** Risk factors for cumulative incidence of HCC development

| Variable | Category | Risk ratio | *P* value | 95% CI |
|---|---|---|---|---|
| Gender | male | 1 | | |
| | female | 0.34 | 0.053 | 0.11–1.01 |
| Age (years) | 65 < | 1 | | |
| | 65 ≥ | 3.23 | 0.006 | 1.37–8.56 |
| Fibrosis | F0/1/2/3/4 | 1.69 | 0.033 | 1.04–2.67 |
| IFN therapy | Non-SVR | 1 | | |
| | SVR | 0.28 | 0.044 | 1.04–12.36 |

CI, confidence interval; IFN, interferon; SVR, sustained virological response.

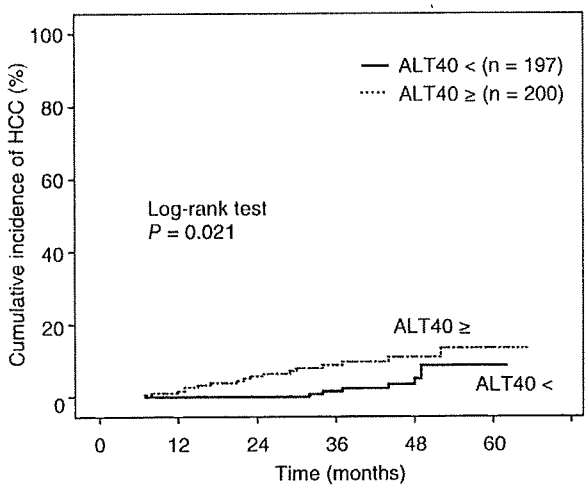


**Figure 3** Cumulative incidence of HCC development according to average alanine aminotransferase (ALT) levels after the combination therapy. (—) ALT < 40 IU/ml; (.....) ALT > 40 IU/ml.

## DISCUSSION

COMBINATION THERAPIES USING IFN-α-2b or Peg-IFN plus ribavirin have been proven to be more effective in treating for HCV infection than IFN monotherapy.[15–17] However, it has not been accurately evaluated whether the combination therapies using IFN-α-2b or Peg-IFN plus ribavirin could reduce the development of HCC, and what the risk factors of HCC incidence were in patients infected with HCV. In this study, we retrospectively examined the incidence of HCC with IFN-α-2b plus ribavirin therapy to clarify the indicators of combination therapy for reducing HCC in patients infected with HCV. We also evaluated whether or not SVR or continuous normalization of ALT levels could reduce the risk of development of HCC.

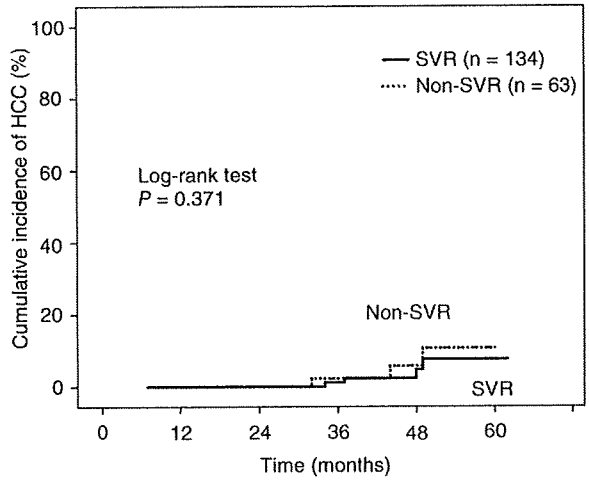


**Figure 4** Cumulative incidence of hepatocellular carcinoma (HCC) development according to the treatment effect in patients who showed less than 40 IU/L average alanine aminotransferase (ALT) levels after the combination therapy. (—) Sustained virological response; (.....) non-sustained virological response.

Previous studies have demonstrated that IFN monotherapy has a preventive effect on the development of HCC, especially in patients with SVR.[12–14] In this study, using the combination of IFN-α-2b plus ribavirin, we obtained almost the same result for the SVR group treated with IFN-α-2b plus ribavirin therapy, which showed a significantly lower possibility of HCC development over a long-term period when compared with the non-SVR group. In contrast, we found no difference in the cumulative incidence of HCC between the TR and NR groups, while Kasahara *et al.* reported that the cumulative incidence of HCC in patients who achieved TR by IFN monotherapy was significantly lower than those with NR.[13] Recent reports have demonstrated that the combination therapy of IFN-α-2b plus ribavirin is able to induce a SVR in a significant proportion of patients with IFN monotherapy-resistant chronic hepatitis C,[19,20] suggesting that a viral relapse after IFN therapy is efficiently suppressed by combination with ribavirin. Since the combination therapy was a more effective treatment for HCV infection than IFN monotherapy[15–17] and there are fewer TR patients with combination therapy than with monotherapy, we speculate that not all, but quite a few patients of the TR group given IFN monotherapy corresponded to the SVR group given the combination therapy, and that the TR group given the combination therapy might have been included in the NR group of IFN monotherapy. This would mean that the "TR group given combination therapy" should be distinguished from the "TR group given IFN monotherapy", and might explain why the results of this study were inconsistent with previous reports of the cumulative incidence of HCC in the TR group given IFN monotherapy being significantly lower than those with NR.[13]

The Kaplan–Meier method showed that older age (> 65 years), severe fibrosis (F2–4), high platelet count ($> 14 \times 10^4$) and non-SVR were significantly associated with the development of HCC. The Cox's regression analysis indicated that older age, fibrosis staging and non-SVR to IFN therapy were significant risk factors related to the development of HCC. These results were

almost comparable with those of previous reports using IFN monotherapy[12–14,21] and IFN plus ribavirin combination therapy,[22–24] suggesting that the factors associated with the development of HCC are common among these treatments and that patients of older age, with advanced fibrosis and showing non-SVR to IFN therapy should be followed up carefully for longer periods, even if IFN therapy could be performed completely. In addition, four of the SVR group patients developed HCC at more than 6 months after the treatment, which means these patients need careful follow-up even if SVR has been achieved.[25]

The incidence of HCC has been reported to be lower in patients with normal ALT levels, even if serum HCV-RNA was positive 6 or 12 months after IFN monotherapy, when compared to those without a biochemical response,[13,26,27] suggesting that the aim of IFN therapy for patients infected with HCV should be not only HCV eradication, but also the achievement of a biochemical response in order to reduce the incidence of HCC. In this study, we divided the patients into two groups, one with persistently normal serum ALT levels and the other with elevated serum ALT levels based on "the average serum ALT levels" after completion of IFN therapy. We then evaluated the cumulative HCC incidence of each group using the Kaplan–Meier estimation. Our data showed that patients with continuous normalization of ALT levels have a lower possibility of HCC development than those showing elevated ALT after the combination therapy, suggesting that continuous normalization of ALT levels after the combination therapy is an important factor for reducing HCC development. Interestingly, based on the Kaplan–Meier estimates of the cumulative HCC incidence according to the treatment effect in patients who showed less than 40 IU/L average ALT levels after the combination therapy, we found no difference in HCC incidence rates between the SVR group and non-SVR group. Figure 1 shows that the combination therapy is strongly associated with a reduced incidence of HCC in the patients who attain SVR, which seems to be a means for achieving normalization of serum ALT levels in HCV patients. However, it was also shown that, even in the non-SVR group, patients with persistently normal serum ALT levels achieved a reduced risk of HCC development. Taken together, our aim of treatment for patients infected with HCV is to primarily completely eradicate HCV. Next, for the non-SVR group patients, we would speculate that maintaining normalization of ALT levels by some other treatments may prevent HCC development in HCV-infected patients with abnormal serum ALT levels even if SVR is not achieved. Other treatments should be used to decrease serum ALT levels to below the upper limit of the normal range. Hopefully, the new treatments such as those with protease inhibitors can be helpful for these patients.[28]

Although IFN monotherapy in CHC patients has been demonstrated to be associated with reducing the incidence of HCC, especially in patients who attain SVR,[12–14] what actually occurs in IFN plus ribavirin combination therapy has not been clarified and the indicator for reducing HCC in patients infected with HCV has not been defined. We showed that this combination therapy could reduce the incidence of HCC and that older age, severe fibrosis and non-SVR were risk factors for HCC development. This therapy can increase the SVR patient ratio, and SVR or continuous normalization of ALT levels after combination therapy using IFN-α-2b plus ribavirin reduce the incidence of HCC in patients with HCV infection. Therefore, this therapy can not only avert the advance of the disease toward liver cirrhosis, but also decrease the risk of HCC. IFN plus ribavirin combination therapy is beneficial for HCV patients from both aspects. In conclusion, the present study shows that the attainment of SVR or continuous normalization of serum ALT levels induced by the combination therapy has a significantly beneficial effect on the clinical course of HCV patients by decreasing the incidence of HCC.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# Factors contributing to antiviral effect of adefovir dipivoxil therapy added to ongoing lamivudine treatment in patients with lamivudine-resistant chronic hepatitis B

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**Abstract**
*Purpose* The antiviral effect of adefovir dipivoxil (ADV) added to ongoing lamivudine (LAM) treatment for LAM-resistant chronic hepatitis B (CHB) differs among patients. We investigated clinical factors affecting the response to ADV therapy in LAM-resistant CHB.
*Methods* The subjects were 75 LAM-resistant CHB patients treated with ADV in addition to LAM. Virological response (VR) was defined as HBV DNA clearance (<2.6 logcopies/ml) at 12 months after the start of ADV therapy. Clinical factors contributing to VR were examined by univariate and multivariate analyses.
*Results* Lower HBV DNA at baseline and negative hepatitis B e antigen (HBeAg) were significant factors affecting VR in univariate analysis. In multivariate analysis, lower HBV DNA at baseline ($P = 0.005$), negative HBeAg ($P = 0.009$), and higher ALT ($P = 0.036$) were significant independent factors contributing to VR. In HBeAg-positive patients, HBV DNA clearance was more frequently observed during ADV therapy in patients with baseline HBV DNA ≤7.0 logcopies/ml than in those with baseline HBV DNA >7.0 logcopies/ml. By contrast, the link of lower HBV DNA at baseline to better therapeutic response was not evident in HBeAg-negative patients.

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*Conclusion* In ADV therapy added to ongoing LAM treatment for LAM-resistant CHB, lower baseline HBV DNA and negative HBeAg contributed to a better antiviral effect. Addition of ADV should be done promptly before marked increase in HBV DNA, especially in CHB patients showing LAM resistance positive for HBeAg.

**Keywords** Adefovir dipivoxil · Lamivudine resistance · Chronic hepatitis B

## Introduction

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV) [1]. Chronic HBV infection can cause liver cirrhosis and hepatocellular carcinoma (HCC), resulting in hepatic disease-related deaths of 500,000 to 1.2 million persons [2, 3]. To prevent disease progression and improve the prognosis of patients with chronic HBV infection, HBV DNA replication must be continuously suppressed as much as possible by antiviral therapy. For this purpose, nucleos(t)ide analogs are currently used for a wide range of patients with chronic HBV infection because of their strong antiviral activities and fewer side effects.

Lamivudine (LAM) is the first approved nucleos(t)ide analog for chronic hepatitis B (CHB) patients, but the increasing incidence of LAM resistance during long-term LAM therapy is a serious problem. The emergence rate of the LAM-resistant virus has been reported to be 24% at 1 year and 70% at 4 years of treatment [4]. Almost all LAM resistance is caused by rtM204V/I mutation occurring in the reverse transcriptase domain of the HBV polymerase gene [5].

To counteract this resistance, adefovir dipivoxil (ADV) was considered as it exerts antiviral effects not only on nucleos(t)ide analog-naïve CHB patients but also on LAM-resistant ones [6–9]. ADV-resistant mutation has been reported to be detected in 11% of patients at 3 years and 29% at 5 years for nucleos(t)ide analog-naïve CHB patients [10]. ADV resistance results from rtA181V/T and/or rtN236T mutation [10]. Either switching from LAM to ADV or adding ADV to LAM has been shown to be effective for LAM-resistant CHB patients. In the case of switching from LAM to ADV, ADV resistance has been reported to appear in 18% of patients at 1 year, which is more frequent than in the case of ADV monotherapy for nucleos(t)ide analog-naïve patients [11]. On the other hand, in the case of ADV administration in addition to LAM, the emergence of resistant virus for both LAM and ADV has been reported to be rare for at least 3 years of treatment [12]. Therefore, ADV therapy added to ongoing LAM treatment is currently accepted as the main therapeutic regimen for LAM-resistant CHB patients rather than a switch from LAM to ADV. However, the antiviral effect of ADV therapy in addition to LAM treatment differs among patients with LAM-resistant CHB.

In this study, we investigated clinical factors influencing the therapeutic efficacy of ADV therapy added to ongoing LAM treatment in LAM-resistant CHB patients.

## Patients and methods

### Patients

The participating centers were 12 institutions in the Osaka area of Japan (Otemae Hospital, Sumitomo Hospital, Osaka Police Hospital, NTT Nishinihon Osaka Hospital, Higashiosaka City General Hospital, Suita Municipal Hospital, Osaka Rousai Hospital, Kinki Central Hospital, Ikeda Municipal Hospital, National Hospital Organization Osaka National Hospital, Itami City Hospital, and Osaka University Hospital). The subjects were 75 consecutive CHB patients showing LAM resistance. Before the preceding LAM therapy, they all had had hepatitis B surface antigen (HBsAg) for more than 6 months and levels of HBV DNA detectable by the polymerase chain reaction (PCR) method [13]. None of them tested positive for hepatitis C virus antibody or human immunodeficiency virus antibody, nor was there evidence of other forms of liver diseases, such as alcoholic liver disease, drug-induced liver disease, or autoimmune hepatitis.

### Anti-HBV treatment

All patients were administered 100 mg of LAM daily. Thirteen (17%) patients had had a history of interferon (IFN) therapy. LAM resistance was judged by detection of rtM204V/I mutation (for 37 patients) or by the existence of virological breakthrough (for 38 patients). Virological breakthrough was defined as the reappearance of detectable HBV DNA of more than 1 log increase in HBV DNA from the nadir on repeated occasions. The median duration of the preceding LAM therapy was 38 (range, 11–83) months. After the emergence of LAM resistance, all patients received 10 mg of ADV daily in addition to ongoing LAM therapy. After the commencement of ADV therapy, liver function and HBV DNA tests were conducted monthly for the first 6 months and every 2 months thereafter. Hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) were checked every 2 months. The median follow-up duration of ADV therapy was 22 (range 12–51) months. HBV DNA clearance (<2.6 logcopies/ml) at 12 months after the beginning of ADV therapy was defined as a virological response (VR).

### Baseline characteristics of the patients

The baseline characteristics of the patients at the commencement of ADV therapy were as follows. They were 59 males and 16 females, with a median age of 54 (range 27–79) years. Forty-one (55%) tested positive for HBeAg, and anti-HBe developed in 34 patients. The virus was genotyped for 13 patients, all of whom were infected with HBV of genotype C. The HBV DNA ranged from 3.1 to >7.6 (median 7.1) logcopies/ml, and the median ALT level ranged from 15 to 500 (median 105) IU/L. The median levels of total bilirubin and albumin were 0.8 (range 0.4–3.9) mg/dl and 3.9 (range 2.1–4.8) g/dl, respectively. The median platelet counts were 11.7 (range 3.5–25.5) $\times$ $10^4/mm^3$. Of the 75 patients, 27 (36%) showed features of cirrhosis by liver biopsy and/or imaging procedures. Five patients (7%) developed HCC as detected by imaging modalities.

### HBV testings

HBsAg, HBeAg, and anti-HBe were examined by chemiluminescent immunoassay. HBV DNA was measured by the PCR-based method (Amplicor HBV monitor, Roche Diagnostics, Tokyo, Japan) [13], with a lower detection limit of 2.6 logcopies/ml. The LAM-resistant rtM204V/I mutation was examined by PCR-enzyme-linked minisequence assay [14]. HBV genotype was determined based on PCR-direct sequencing of portions of core and polymerase genes. The primers used for this study were BF1s (5′-TTT TTC ACC TCT GCC TAA TCA-3′, nt 1821–1841), BR3 (5′-TTC CCG AGA TTG AGA TCT TC-3′, nt 2440–2421), BF6 (5′-CCT CCA ATT TGT CCT GGC TA-3′, nt 350–369), and BR8 (5′-TTG CGT CAG CAA ACA CTT GG-3′, nt 1195–1176) [15, 16].

### Statistical analysis

Group comparisons were carried out by the chi-square test, Student's $t$ test and Mann–Whitney's $U$ test. Independent factors contributing to VR during ADV therapy added to ongoing LAM treatment were estimated using multivariate multiple logistic regression analysis in combination with stepwise regression analysis. A $P$-value of less than 0.05 (two-tailed) was considered to indicate a significant difference. All statistical analyses were performed using the SPSS version 15.0J software (SPSS, Chicago, IL).

## Results

### Virological and biochemical response to ADV therapy added to ongoing LAM in CHB patients showing LAM resistance

Of the 75 CHB patients showing LAM resistance who underwent ADV therapy added to ongoing LAM treatment, HBV DNA clearance was achieved in 29 (39%) of 75 at 6 months, 35 (47%) of 75 at 12 months, and 34 (72%) of 47 at 24 months. Among the HBeAg-positive patients, HBeAg loss was observed in 8 (20%) of 41 at 6 months, 7 (18%) of 39 at 12 months, and 6 (22%) of 27 at 24 months. As for the biochemical response, ALT normalization ($\leq$40 IU/l) was seen in 57 (76%) of 75 at 6 months, 56 (75%) of 75 at 12 months, and 40 (85%) of 47 at 24 months of treatment.

### Pretreatment clinical factors associated with therapeutic response to ADV in addition to LAM treatment

We first investigated pretreatment clinical factors associated with the therapeutic efficacy of ADV added to ongoing LAM treatment by univariate analysis. The baseline characteristics of patients at the beginning of ADV therapy in addition to LAM in the presence or absence of VR are shown in Table 1. Patients showing VR had significantly lower HBV DNA at baseline than patients who did not achieve VR [median 6.3 (range 3.1 to >7.6) vs. 7.3

**Table 1** Patient clinical characteristics at the beginning of ADV therapy in addition to LAM in LAM-resistant CHB patients in the presence or absence of virological response (VR)

| Clinical characteristics | VR ($n$ = 35) | Non-VR ($n$ = 40) | $P$ value |
|---|---|---|---|
| Gender (male/female) | 26/9 | 33/7 | 0.386 |
| Age (years) | 52 (28–67) | 55 (27–79) | 0.896 |
| Duration of prior LAM therapy (months) | 38 (12–83) | 37 (13–64) | 0.856 |
| Positive HBeAg | 12 (34%) | 29 (73%) | 0.001 |
| HBV DNA (logcopies/ml) | 6.3 (3.1 to >7.6) | 7.3 (3.9 to >7.6) | 0.002 |
| ALT (IU/l) | 106 (16–500) | 75 (15–455) | 0.136 |
| Total bilirubin (mg/dl) | 0.9 (0.4–3.9) | 0.7 (0.4–3.9) | 0.664 |
| Albumin (g/dl) | 4.0 (2.4–4.8) | 3.8 (2.1–4.6) | 0.351 |
| Platelet count ($\times 10^4/mm^3$) | 12.2 (4.8–24.1) | 11.5 (3.5–25.5) | 0.854 |
| Liver disease (chronic hepatitis/cirrhosis) | 20/15 | 28/12 | 0.247 |
| Presence of HCC (%) | 2 (6%) | 3 (8%) | 0.757 |

Continuous variables are expressed as median (range)

**Table 2** Baseline factors affecting virological response (logistic regression analysis, stepwise method)

| Factors | Category | Odds ratio | 95% CI | *P* |
|---|---|---|---|---|
| Gender | Male/female | | | NS |
| Age (years) | By 1 year | | | NS |
| Duration of prior LAM therapy (months) | By 1 month | | | NS |
| HBeAg | Negative/positive | 5.766 | 1.855–36.62 | 0.009 |
| HBV DNA (logcopies/ml) | By 1 logcopy/ml | 2.362 | 1.335–5.178 | 0.005 |
| ALT (IU/l) | By 1 IU/l | 1.006 | 1.000–1.011 | 0.036 |
| Total bilirubin (mg/dl) | By 1 mg/dl | | | NS |
| Albumin (g/dl) | By 1 g/dl | | | NS |
| Platelet count ($\times 10^4$/mm$^3$) | By 1 $\times 10^4$/mm$^3$ | | | NS |
| Liver disease | Chronic hepatitis/cirrhosis | | | NS |
| Presense of HCC (%) | No/yes | | | NS |

*CI* Confidence interval, *NS* not significant

(range 3.9 to >7.6), $P = 0.002$]. HBeAg was detected in only 12 (34%) of 35 patients with VR, compared with 29 (73%) of 40 patients without VR ($P = 0.001$). Gender ratio, age, duration of preceding LAM therapy, ALT, total bilirubin, albumin, platelet counts, disease severity, and presence of HCC did not differ between VR and non-VR patients.

Factors affecting the therapeutic response to ADV therapy in addition to ongoing LAM were also evaluated by multivariate analysis (Table 2). Eleven pretreatment clinical factors were applied to the analysis as variables. Two factors, lower baseline HBV DNA ($P = 0.005$, odds ratio: 2.362, 95% confidence interval: 1.335–5.178) and negative HBeAg ($P = 0.009$, odds ratio: 5.766, 95% confidence interval: 1.855–36.62), were selected as significant independent factors affecting VR, as was the case for univariate analysis. In addition, higher baseline ALT was also chosen as a significant independent factor ($P = 0.036$, odds ratio 1.006, 95% confidence interval: 1.000–1.011). As for the biochemical response to ADV therapy added to LAM, no pretreatment clinical factors showed a significant relationship with the occurrence of ALT normalization in our 75 LAM-resistant CHB patients.

## HBV DNA clearance during ADV therapy in addition to ongoing LAM treatment according to HBeAg status

Next, we investigated HBV DNA clearance during ADV therapy added to ongoing LAM treatment in LAM-resistant CHB patients positive or negative for HBeAg (Fig. 1). In HBeAg-positive patients, HBV DNA was cleared in 8 (20%) of 41 at 6 months, 12 (29%) of 41 at 12 months, and 16 (59%) of 27 at 24 months. On the other hand, HBV DNA clearance was seen in 21 (62%) of 34 at 6 months, 23 (68%) of 34 at 12 months, and 18 (90%) of 20 at 24 months in HBeAg-negative patients. A significant difference ($P < 0.05$) in the frequency of HBV DNA clearance was

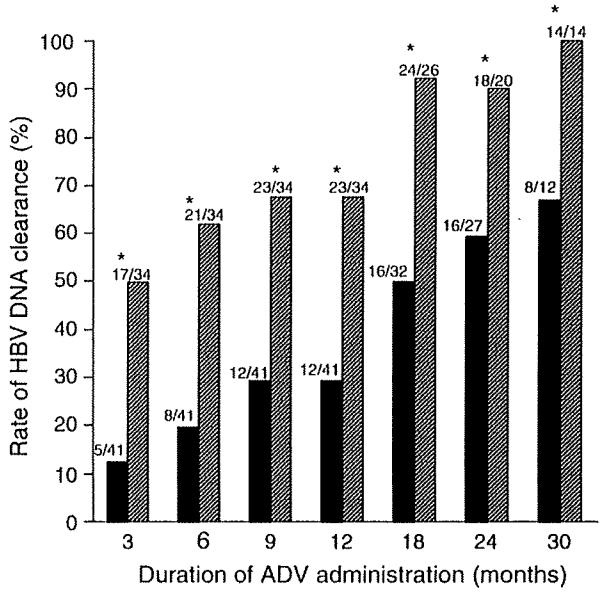


**Fig. 1** Rates of HBV DNA clearance in CHB patients positive or negative for HBeAg during ADV therapy in addition to LAM. $*P < 0.05$ between HBeAg-positive and HBeAg-negative patients. *Solid bars* HBeAg-positive patients, *hatched bars* HBeAg-negative patients

observed between HBeAg-positive and HBeAg-negative patients at 3, 6, 9, 12, 18, 24, and 30 months of treatment. Thus, patients negative for HBeAg tended to respond to ADV therapy added to ongoing LAM treatment better than those positive for it in LAM-resistant CHB.

## HBV DNA clearance during ADV therapy in addition to ongoing LAM treatment in relation to HBeAg status and baseline HBV DNA

We examined HBV DNA clearance during ADV therapy in addition to ongoing LAM treatment in HBeAg-positive and

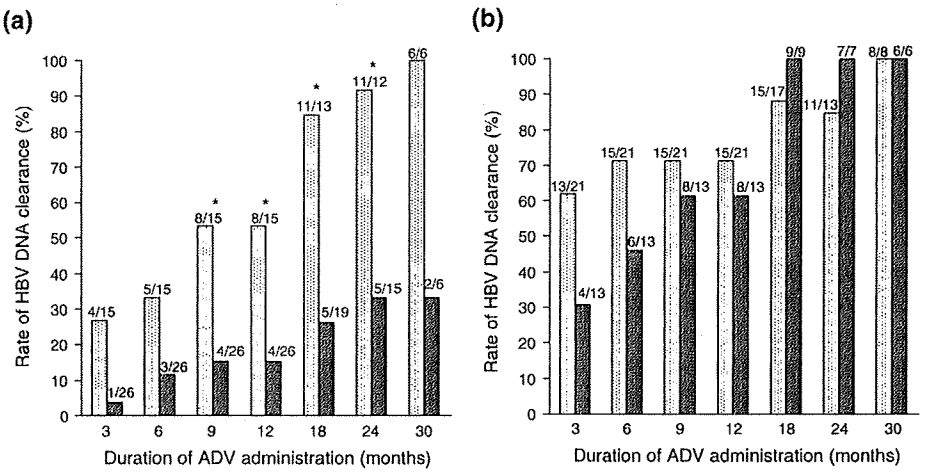


**Fig. 2** Rates of HBV DNA clearance during ADV therapy in addition to LAM according to HBV DNA at baseline in **a** HBeAg-positive CHB patients and **b** HBeAg-negative CHB patients. *$P < 0.05$ between patients with low (≤7.0 logcopies/ml) and high (>7.0 logcopies/ml) HBV DNA. *Dotted bars* Patients with HBV DNA ≤7.0 logcopies/ml at baseline, *hatched bars* patients with HBV DNA >7.0 logcopies/ml at baseline

HBeAg-negative CHB patients in relation to baseline HBV DNA. In the case of HBeAg-positive CHB patients (Fig. 2a), the rates of HBV DNA clearance were 33% (5/15) at 6 months, 53% (8/15) at 12 months, and 92% (11/12) at 24 months in patients with low viremia (baseline HBV DNA ≤7.0 logcopies/ml). By contrast, the frequencies of HBV DNA clearance were only in 12% (3/26) at 6 months, 15% (4/26) at 12 months, and 33% (5/15) at 24 months in patients with high viremia (baseline HBV DNA >7.0 logcopies/ml). A significant difference ($P < 0.05$) in the frequency of HBV DNA clearance was observed between patients with low and high viremia at 9, 12, 18, and 24 months of treatment. In the case of HBeAg-negative patients (Fig. 2b), the rates of HBV DNA clearance were 71% (15/21) at 6 months, 71% (15/21) at 12 months, and 85% (11/13) at 24 months in patients with low viremia (baseline HBV DNA ≤7.0 logcopies/ml). The frequencies of HBV DNA clearance were 46% (6/13) at 6 months, 62% (8/13) at 12 months, and 100% (7/7) at 24 months in patients with high viremia (baseline HBV DNA >7.0 logcopies/ml). No significant differences were observed in the frequency of HBV DNA clearance between patients with low and high viremia. According to these findings, the relevance of lower baseline HBV DNA for achieving a better antiviral effect was evident only in HBeAg-positive patients, but not in HBeAg-negative ones in ADV therapy added to LAM treatment for LAM-resistant CHB.

## Discussion

This study investigated factors affecting the antiviral efficacy of ADV therapy added to ongoing LAM treatment in LAM-resistant CHB patients. Therapeutic efficacy was assessed as the presence or absence of VR. Both univariate and multivariate analyses revealed that lower baseline HBV DNA and negative HBeAg were strong factors associated with a better therapeutic response. Another significant factor revealed by multivariate analysis was high ALT, although it was weaker than the other two factors. In previous investigations, female gender, lower baseline HBV DNA, negative HBeAg, higher ALT, and genotype D rather than A have been reported to contribute to better VRs to ADV therapy in nucleos(t)ide-naïve and LAM-resistant CHB patients [17–21]. Our results agreed partially with them. The present study, as well as previous studies [18, 19], also revealed that a high baseline ALT may be a determining factor for a better response to ADV therapy in addition to LAM treatment in LAM-resistant CHB. This may be because the host immune response against viral antigens induced by active breakthrough hepatitis has a favorable antiviral effect during ADV therapy. In this study, however, a low baseline viremic level was shown to be a stronger factor than high baseline ALT. The baseline ALT level was the third factor contributing to VR. Therefore, in LAM-resistant CHB, ADV administration should be started before the flare-up of ALT elevation, especially in patients with severe liver disease such as cirrhosis.

In LAM-resistant patients, the HBV DNA level is low during the initial phase, but increases with time, leading to the onset of breakthrough hepatitis. Thus, in ADV therapy added to LAM treatment for LAM-resistant-CHB, the baseline HBV DNA level varies with the observation period after the emergence of LAM resistance. A previous report on Italian HBeAg-negative CHB patients showing LAM resistance revealed that patients with low viremia and normal ALT tended to respond to ADV therapy in addition to LAM treatment better than those with high viremia and abnormal ALT [17]. In the present study conducted in Japan, a genotype C-endemic area, such a close relationship between lower baseline HBV DNA and better therapeutic response was remarkable in

HBeAg-positive patients but not in HBeAg-negative ones. Our finding suggests that, in LAM-resistant CHB, ADV should be added before the HBV DNA begins to increase markedly, especially in HBeAg-positive patients.

In this study, none of the 75 patients showed virological breakthrough after the beginning of ADV administration. All displayed more than 1 log reduction of HBV DNA at 12 months of ADV treatment. This indicates that our patients may not have produced viruses resistant to both LAM and ADV. The emergence of resistant viruses has been reported to be rare in combination therapy using LAM and ADV for LAM-resistant CHB patients, although recent studies have found the existence of a virus resistant to both drugs [22, 23]. The rtA181V/T/S mutation has been reported to confer cross resistance to LAM and ADV [22, 23]. In ADV monotherapy for nucleos(t)ide analog-naïve CHB patients, the absence of HBV DNA reduction to <4 logcopies/ml at 24 weeks of treatment has been reported to be related to the higher emergence of a ADV-resistant virus [24], as is the case in LAM monotherapy [25]. In ADV therapy added to LAM treatment in LAM-resistant CHB patients, the poor response during the initial phase may lead to the development of virus resistance to LAM and ADV as well. From this point of view, the addition of ADV to ongoing LAM treatment before the elevation of HBV DNA may be beneficial in LAM-resistant CHB patients to avoid the development of a multi-drug-resistant virus. Recently, some investigators have reported that tenofovir disoproxil fumarate is effective against a virus resistant to both LAM and ADV [22, 23], but it has not yet been approved for clinical use.

Our results conclusively showed that, with ADV therapy added to LAM treatment for LAM-resistant CHB patients, lower baseline HBV DNA and negative HBeAg contributed to a better antiviral effect. After the emergence of LAM resistance, ADV should be added before the marked elevation of HBV DNA in order to attain better antiviral efficacy, especially in HBeAg-positive patients.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# Lamivudine-to-entecavir switching treatment in type B chronic hepatitis patients without evidence of lamivudine resistance

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**Abstract**
*Purpose* A considerable number of chronic hepatitis B (CH-B) patients remain under continuous lamivudine treatment, although switching treatment to entecavir could be beneficial. We investigated the antiviral efficacy of switching treatment to entecavir in CH-B patients without apparent evidence of lamivudine resistance during the preceding lamivudine treatment.
*Methods* Forty-four CH-B patients, who underwent lamivudine treatment for more than 6 months and showed no evidence of lamivudine resistance, switched to entecavir. Serial changes in hepatitis B virus (HBV) DNA were correlated with the patients' baseline HBV DNA at the commencement of entecavir administration. The entecavir-resistant substitution was examined by PCR-direct sequencing. The median follow-up period of entecavir treatment was 20 (10–23) months.
*Results* All 31 patients with baseline HBV DNA <2.6 logcopies/ml maintained HBV DNA-negative status during entecavir treatment. Of seven patients having HBV DNA of 2.6–<4.0 logcopies/ml, all achieved undetectable HBV DNA at the end of follow-up. As for six patients having HBV DNA ≥4.0 logcopies/ml, three patients achieved undetectable HBV DNA, whereas virological breakthrough was observed in one patient at month 15. An entecavir-resistant virus having rtM204V, rtL180M and rtS202G substitutions was detected in this patient.
*Conclusions* The lamivudine-to-entecavir switching treatment may be generally recommendable in CH-B patients without evidence of lamivudine resistance during

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the preceding lamivudine treatment. However, great care should be taken with respect to the emergence of entecavir-resistance, especially in patients who do not respond well to the preceding lamivudine treatment.

**Keywords** Chronic hepatitis B · Lamivudine resistance · Entecavir-resistance

## Introduction

Nucleos(t)ide analogs have been accepted as useful agents for suppressing hepatitis B virus (HBV) replication and disease progression in patients with type B chronic hepatitis (CH-B). Lamivudine, the first approved nucleoside analog, has been shown to provide short-term benefit for CH-B patients with respect to the reduction of HBV DNA, normalization of alanine aminotransferase (ALT) and improvement of liver histology [1, 2]. However, a serious shortcoming of lamivudine is the high incidence of drug resistance during long-term treatment. The detection rate of lamivudine resistance has been reported to be 24% at 1 year and 70% at 4 years of treatment [3]. Lamivudine resistance is caused by an rtM204V/I substitution within the reverse transcriptase domain of HBV polymerase gene [4–6]. An rtL180M substitution frequently emerges as a "replication-compensatory" one with the "resistance-causative" rtM204V/I substitution [4–7]. The emergence of lamivudine-resistant mutant HBV leads to the elevation of HBV DNA ("virological breakthrough") and the subsequent increase of ALT ("breakthrough hepatitis"), resulting in disease progression. Adefovir dipivoxil and tenofovir disoproxil fumarate have been shown to be effective in both nucleos(t)ide analog-naïve and lamivudine-resistant CH-B patients [8–13].

Recently, entecavir has been demonstrated to exert antiviral efficacy in both nucleos(t)ide analog-naïve and lamivudine-refractory CH-B patients [14–16]. The frequency of entecavir-resistance has been reported to be less than 1% at 4 years of treatment in nucleos(t)ide analog-naïve CH-B patients [17]. On the other hand, in switching treatment to entecavir for lamivudine-refractory CH-B patients, most of whom developed lamivudine resistance during the preceding lamivudine therapy, the cumulative probability of entecavir-resistance has been reported to be no less than 40% at 4 years of treatment [17]. Entecavir-resistance has been shown to be established by amino acid substitution(s) at rt184, rt202 and/or rt250 along with the lamivudine-resistant rtM204V and rtL180M substitutions [18]. In the case of nucleos(t)ide analog-naïve patients, the requirement of at least three amino acid substitutions serves as a high genetic barrier to entecavir-resistance. By contrast, in the case of lamivudine-resistant patients, a lower genetic barrier results in higher incidence of entecavir-resistance because two amino acid substitutions, rtM204V and rtL180M, already exist from the preceding lamivudine treatment. The reduced susceptibility to entecavir of the lamivudine-resistant virus compared with the wild-type virus is also a reason for the higher emergence rate of entecavir-resistance in lamivudine-resistant patients than in nucleos(t)ide analog-naïve ones [19].

Although lamivudine is not currently recommended as a first-line drug for nucleos(t)ide analog-naïve CH-B, a considerable number of CH-B patients are under continuous treatment with lamivudine. In these patients, the switch to entecavir treatment could be advantageous over continuation of lamivudine treatment by offering stronger antiviral efficacy and less chance of drug resistance. With respect to the manner of emergence of entecavir-resistance, switching a patient's treatment may be more appropriate before the appearance of lamivudine resistance than after its development. However, the usefulness of lamivudine-to-entecavir switching treatment has not been assessed in CH-B patients without apparent evidence of lamivudine resistance.

This led us to investigate the antiviral efficacy and emergence of entecavir-resistance in CH-B patients who showed no evidence of lamivudine resistance during the preceding lamivudine treatment and underwent the switching treatment to entecavir.

## Patients and methods

### Patients

This study included 44 consecutive CH-B patients from 10 institutions in the Osaka area of Japan (Otemae Hospital, Sumitomo Hospital, Osaka Police Hospital, Suita Municipal Hospital, Yao Municipal Hospital, Osaka Rousai Hospital, Ikeda Municipal Hospital, National Hospital Organization Osaka National Hospital, Itami City Hospital and Osaka University Hospital) who underwent continuous lamivudine treatment (100 mg/day) for more than 6 months and showed no apparent evidence of lamivudine resistance. Before starting the preceding lamivudine treatment, all patients had abnormal ALT, positive hepatitis B surface antigen (HBsAg) and a detectable level of HBV DNA according to PCR-based assay (Amplicor HB Monitor, Roche Diagnostics) or branched DNA assay (Quantiplex HBV DNA, Chiron). None of them showed evidence of dual infection with hepatitis C virus or human immunodeficiency virus, or other forms of liver diseases such as alcoholic liver disorder, autoimmune hepatitis and drug-induced liver injury. The total duration of the preceding lamivudine treatment ranged from 6 to 73 (median, 14)