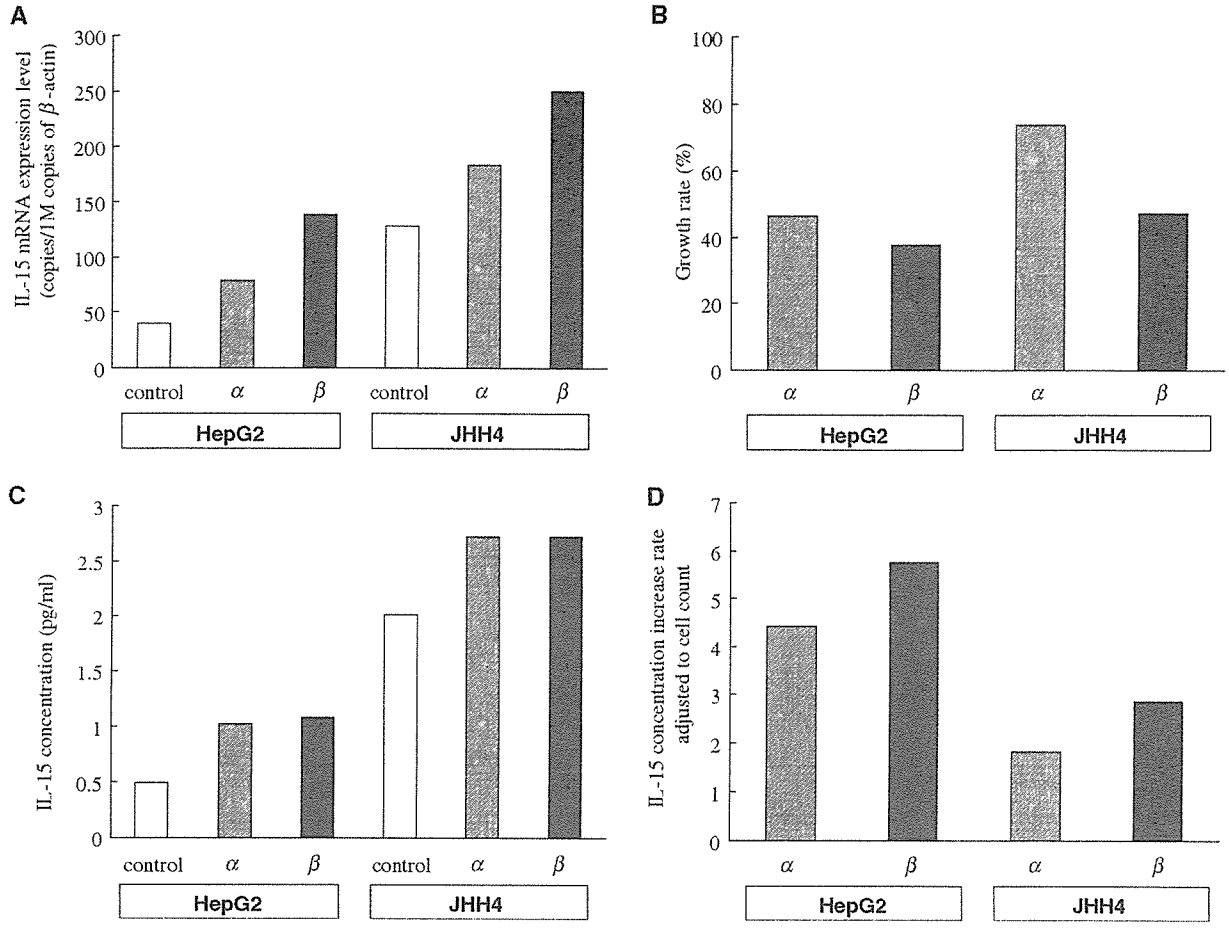


**Fig. 3** The effects of IFN-α and -β on IL-15 expression in HepG2 and JHH4 cells. HepG2 cells and JHH4 cells were cultured with or without 1,000 U/ml IFN-α/β for 72 h. **a** Expression levels of IL-15 and β-actin gene were quantified by reverse-transcription real-time PCR. The IL-15 mRNA expression level was adjusted to the β-actin mRNA expression level. **b** Cell numbers were counted by flow cytometry after detachment with trypsinization, and the cell growth rate of IFN-treated cells in comparison with the controls was calculated. **c** The IL-15 concentration in culture supernatant was quantified by ELISA. **d** The supernatant IL-15 concentration was adjusted to the cell number, and the IL-15 production increase rate of IFN-treated cells in comparison with the controls was calculated. All experiments were repeated three times and representative results are depicted

production, not only in Huh7 but also in other HCC cell lines such as HepG2 and JHH4.

## IL-15 mRNA expression in liver biopsy samples from chronic hepatitis C patients

To investigate if IL-15 is expressed in human liver, we examined IL-15 mRNA expression in liver biopsy samples obtained from six chronic hepatitis C patients. The amplification of IL-15 transcript by PCR showed that IL-15 mRNA was expressed in all the liver biopsy samples examined (Fig. 4a). We also quantified IL-15 transcripts in liver samples, and the data adjusted by β-actin mRNA expression levels is shown in Fig. 4b. The IL-15 mRNA expression level in the liver showed little valiation between the samples, but generally, it was similar to that of Huh7 cells. These results suggest that IL-15 is expressed in the human liver.

## Increased serum IL-15 level in chronic hepatitis C patients during Type I IFN treatment

To investigate the induction of IL-15 production by type I IFNs in vivo, we examined alterations of the serum IL-15 level in 21 chronic hepatitis C patients during treatment with IFN-α or -β. Table 1 shows the clinical characteristics of 11 patients treated with IFN-α, 10 patients treated with IFN-β, and 29 control subjects. No significant difference in sex or age was found between the control subjects and chronic hepatitis C patients. No significant pretreatment difference in IL-15 level (Fig. 5) was found between the samples from controls and the chronic hepatitis C patients. In the chronic hepatitis C patients, the IL-15 level significantly increased during the IFN treatment period in comparison with pretreatment (week 2; $P<0.0001$, week 4; $P<0.0001$, end of treatment; $P<0.0001$). At 6 months after cessation of

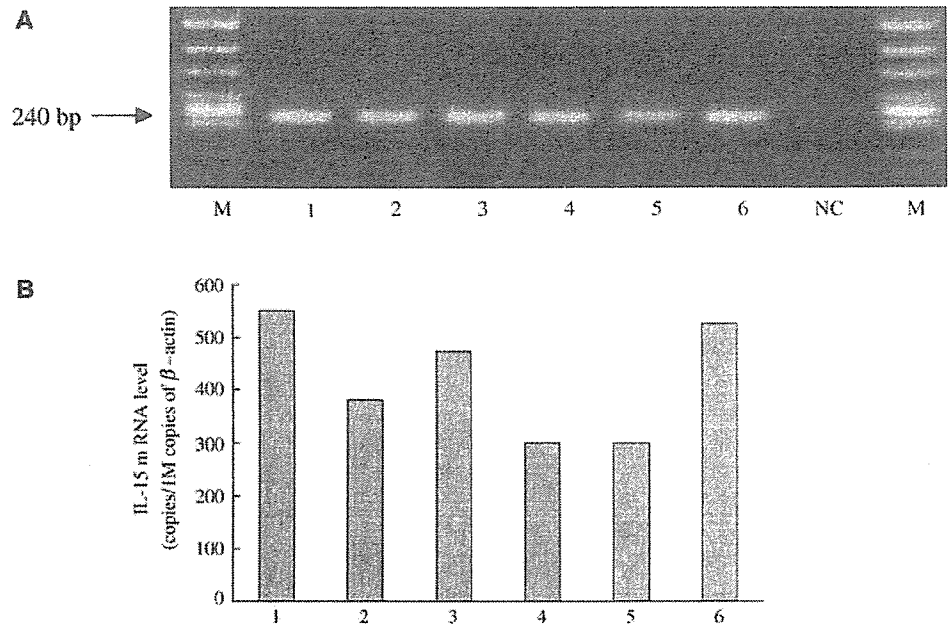


**Fig. 4** Interleukin-15 mRNA expression in liver biopsy samples of chronic hepatitis C patients. **a** The IL-15 transcripts were amplified from the liver biopsy samples of six patients by reverse-transcription PCR. DEPC-treated water was used as the negative control. lanes 1–6; liver biopsy samples, lane 7; negative control, M; DNA marker. **b** Expression levels of IL-15 and the $\beta$-actin gene were quantified by reverse-transcription real-time PCR. The IL-15 mRNA expression level was adjusted to the $\beta$-actin mRNA expression level

treatment, the serum IL-15 had returned to a level similar to the pretreatment. No significant differences in the serum IL-15 level of patients by type of IFN received or by virological or biochemical response were found during the observation period (data not shown). These results suggest that both IFN-$\alpha$ and -$\beta$ increased the serum IL-15 level of the chronic hepatitis C patients during the administration period.

## Discussion

Interleukin-15 is considered a key factor for both innate and adaptive immune response. IL-15 expression has been implicated in clinical studies to be associated with graft rejection in transplantations[34–36] and with synovial T cell activation in rheumatoid arthritis[37, 38], suggesting that IL-15 plays an important role in local immune reaction. It has also been reported that IL-15 enhances antitumor immune response by NK cells and CTLs [39–42], and it is expected that IL-15 can be applied to antitumor immunotherapy in the future [43, 44]. The present study revealed that IFN-$\alpha$ and -$\beta$ upregulate IL-15 production from HCC cell lines in vitro. The finding suggests a possibility that type I IFNs may induce IL-15 production in HCC cells in vivo leading to proliferation and activation of surrounding immune cells, such as NK and CTL. It has been demonstrated that some cancer cells can regulate immune response and lead to inhibition of antitumor immunity [45]. Type I IFNs may elicit the opposite immune regulatory effect, i.e. activation of antitumor immunity through IL-15 production from cancer cells. This may be one of the mechanisms contributing to the antitumor effect of IFN-$\alpha$ and -$\beta$.

The serum IL-15 levels of the chronic hepatitis C patients in this study significantly increased during the IFN administration period, indicating that IFN-$\alpha$ and -$\beta$ stimulate IL-15 production in humans in vivo. In the present study, IL-15 production was increased in human HCC cell lines by IFN-$\alpha/\beta$, and a comparable level of IL-15 mRNA expression was observed in liver biopsy samples. It has been reported that multiple STAT signals are activated by IFN-$\alpha$ in hepatocytes [46]. The IL-15 promotor includes two enhancer elements, the binding sites of NF-$\kappa$B and IRF-1 which are transcription factors in the downstream of type I IFN signals [47]. These findings led us to believe that IFN-$\alpha$ and -$\beta$ upregulate IL-15 production in hepatocytes in vivo, which in turn contributes to the elevation of serum IL-15 in chronic hepatitis C patients during IFN administration. It is also possible that IL-15 production is increased in immune cells, although a recent report suggested that type I IFN-mediated production of IL-15 was impaired in monocyte-derived dendritic cells of chronic hepatitis C patients[48]. Which cells or tissues were involved in type I IFN-mediated production of IL-15 in vivo remains unknown. The present study, however, demonstrated that type I IFNs upregulate IL-15 production in vivo, and the results suggest that IL-15 is associated with type I IFN-induced immune response.

In the present study, in vitro IFN-$\beta$ showed a stronger activity than IFN-$\alpha$ in IL-15 upregulation of HCC cell lines. IFN-$\beta$ also inhibited the growth of HCC cell lines more efficiently than IFN-$\alpha$, as reported previously [49]. It is unclear what causes these differences between IFN-$\alpha$ and -$\beta$, since IFN-$\alpha$ and -$\beta$ bind to the same receptor. The receptor, type I IFN-R, is composed of at least two subunits; $\alpha$ chain, IFNAR1 and $\beta$ subunit, IFNAR2 which has short ($\beta_S$) and long ($\beta_L$) forms. In a previous report,

**Table 1** Clinical characteristics of 21 patients treated with IFN-α or -β and 29 control subjects

| characteristics | controls (n = 29) | HCV-infected patients (n = 21) | *p* value |
|---|---|---|---|
| sex, n (%) | | | > 0.999 † |
| male | 16 (55.2) | 13 (61.9) | |
| female | 13 (44.8) | 8 (38.1) | |
| age, years§ | 58.1 ± 10.8 | 52.2 ± 10.7 | 0.0632 ‡ |
| HCV viral load¶, n (%) | | | |
| $10^3$ | - | 2 (6.9) | |
| $10^4$ | - | 2 (6.9) | |
| $10^5$ | - | 4 (13.8) | |
| $10^6$ | - | 10 (34.5) | |
| $10^7$ | - | 3 (10.3) | |
| HCV genotype, n (%) | | | |
| 1b | - | 13 (44.8) | |
| 2a | - | 7 (24.1) | |
| 2b | - | 1 (3.4) | |
| histology, n (%) | | | |
| CPH | - | 5 (17.2) | |
| CAH | - | 8 (27.6) | |
| LC | - | 5 (17.2) | |
| NT | 29 (100) | 3 (10.3) | |
| virological responder, n (%) | - | 8 (27.6) | |
| biochemical responder, n (%) | - | 9 (31.0) | |

§ Mean value ± SD
¶ The unit of HCV viral load is "copies per 50 μl of serum"
† Comparison by $\chi^2$ test with Yate's compensation
‡ Comparison by unpaired student's *t*-test
CPH: chronic persistent hepatitis
CAH: chronic active hepatitis
LC: liver cirrhosis
NT: not tested

it was shown that IFN-α2 and IFN-β require a distinct intracytoplasmic region of the $\beta_L$ subunit for their antiviral response, suggesting activation of a distinct signaling pathway by IFN-β [50]. In another report, it was shown that expression of CXCL11 (alias β-R1, I-TAC), a CXC chemokine ligand for CXCR3, was selectively induced by IFN-β, but not by IFN-α in a fibrosarcoma cell line [51, 52]. These findings suggested differences in biological activity between IFN-α and -β, and that it may be associated with the differences in the level of activity in IL-15 upregulation. We have also reported differences in clinical outcome between patients treated with IFN-α and -β. Sustained virological response was predicted by disappearance of HCV viremia early in the course of IFN administration in patients treated with IFN-α [53], but not in patients treated with IFN-β [11]. Moreover, by IFN-α treatment, the risk of HCC was significantly reduced in chronic hepatitis C patients with a biochemical response. In contrast, in patients treated with IFN-β, there was no difference in the incidence of HCC between patients with and without biochemical response, and there was a tendency for the risk to be reduced irrespective of the biochemical response of patients treated with IFN-β [14]. Thus, differences in biological activity between IFN-α and -β have been suggested by both basic and clinical studies, and these differences may be associated with the differences in the level of activity in IL-15 upregulation.

There was no significant difference in the serum IL-15 level of the patients treated with IFN-α and -β during the administration period. One possible explanation is that the administration routes are different for IFN-α and -β, i.e. intramuscular and intravenous injection, respectively. This difference would affect the drug concentration in tissues including liver. Another possibility is varied response to type I IFN among different cell types,

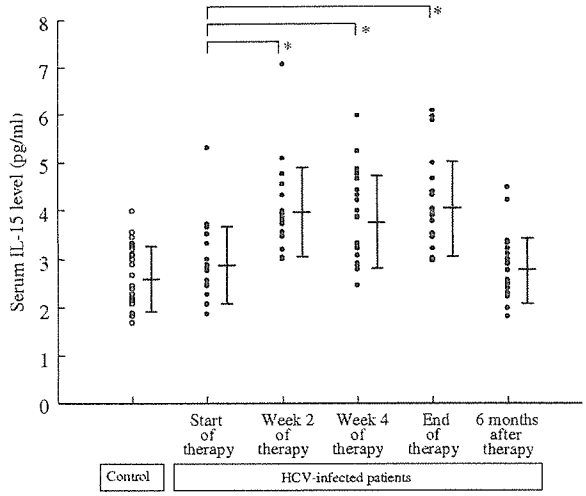


**Fig. 5** The serum IL-15 level of IFN-treated patients with type C chronic liver disease and controls. The IL-15 level was quantified by ELISA. Unpaired Student's *t*-test was used to assess the statistical significance of differences in serum IL-15 levels between sera from controls and HCV-infected patients before treatment. Paired Student's *t*-test was used to compare serially assayed serum IL-15 of the HCV-infected patients. *Circles* indicate the individual serum IL-15 levels of the controls (*open circle*) and HCV-infected patients (*closed circle*). *Horizontal bars* show mean ± SD in each sample group. $*P < 0.0001$

or that only some cells or organs show differences in IL-15 expression response as between IFN-α and -β, resulting in the differences being overshadowed by other aspects of systemic response in vivo.

The present study provided the first evidence that type I IFN induces IL-15 production in vitro in HCC cell lines and also suggests a mechanism by which IL-15 production from non-immune cells mediates IFN-α/β-induced immune response. Our study also demonstrated that IFN-α and -β induce IL-15 production in human in vivo. These findings may help us better understand the immune regulatory mechanism of IFN-α/β and its implication in IL-15 expression. However, the precise roles of IFN-α/β are still unclear and further study is needed to clarify these issues. Such study will be useful for the development of selective and effective therapies for viral infection and cancer.

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# Long-term lamivudine treatment for chronic hepatitis B in Japanese patients: A project of Kyushu University Liver Disease Study

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## Abstract

**AIM:** To determine the efficacy of long-term lamivudine treatment of a large number of Japanese patients with chronic hepatitis B.

**METHODS:** In this retrospective, multi-center trial, 318 Japanese patients with chronic hepatitis B received 100 mg of lamivudine daily for up to 36 (median 21) mo. Virological response was a decline to a serum HBV DNA level less than 3.7 log copies/mL. Virological breakthrough was defined as the reappearance of a serum HBV DNA level to more than 10-fold the minimum during treatment.

**RESULTS:** Lamivudine produced virological response in 86.8% of the 318 patients at 6 mo, in 80.2% of 252 patients at 12 mo, in 69.2% of 133 patients at 24 mo, and in 53.6% of 28 patients at 36 mo. Forward stepwise logistic regression analysis showed an HBV DNA level less than 6.8 log copies/mL ($P<0.0001$), HBeAg negativity ($P<0.0001$), a platelet count of $100\times10^9$/L or more ($P=0.0162$) at baseline, and a decline of the HBV DNA level of more than 3.2 log copies/mL as compared with the baseline level at 3 mo after the start of treatment ($P=0.0003$) to be significantly associated with virological response. Among patients with a virological response, virological breakthrough was seen in 5.3% of 19 patients who responded virologically at 1 mo, in 20.7% of 203 patients at 3 mo, in 27.5% of 51 patients at 6 mo, in 33.3% of 12 patients at 9 mo, and in 100% of 3 patients at ≥15 mo. A virological breakthrough was found significantly more often in patients with delayed virological response.

**CONCLUSION:** Lamivudine treatment could suppress serum HBV DNA in most of the tested Japanese patients. Long-term efficacy might be seen in patients without HBeAg at baseline, in the absence of cirrhosis, and in patients with a decline in HBV DNA level soon after the start of treatment.



**Key words:** Hepatitis B virus; Lamivudine; HBeAg; Cirrhosis

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## INTRODUCTION

Chronic hepatitis B virus (HBV) infection affects 400 million people worldwide, three-quarters of whom reside in Asia[1]. The morbidity and mortality of chronic HBV infection are a major public health concern. Vertical transmission of HBV is the main cause of chronic HBV infection in the endemic areas of Asia. In Japan which is endemic for HBV infection, transmission can be easily prevented by vaccination of at risk-infants. The prevalence of hepatitis B surface antigen (HBsAg) carriage in the general population was reported to be less than 2%[2]. However, in areas of high HBV endemicity, persons with HBV-related cirrhosis have an approximately threefold higher risk of hepatocellular carcinoma (HCC) than those with chronic hepatitis but without cirrhosis and a 16-fold higher risk of HCC than the carriers in whom the virus is inactive[3]. Interferon-alpha (IFN-α) has been approved for the treatment of chronic hepatitis B[4], but it is poorly tolerated and effective in only 20%-30% of patients. Since the late 1990s, several studies have demonstrated the effectiveness of the initially available antiviral medicine, lamivudine, for patients with chronic hepatitis B: HBV DNA suppression, normalization of alanine aminotransferase (ALT), loss of hepatitis B e antigen (HBeAg). Moreover, studies have demonstrated the improvement of hepatic histology by the administration of lamivudine compared to placebo[5-8].

Lamivudine, an oral cytosine nucleoside analog, is the (-) - beta-enantiomer of 2',3'-dideoxy-3'-thiacytidine. HBV replicates through a pregenomic RNA intermediate. Lamivudine interferes with HBV reverse transcriptase (DNA polymerase) activity and causes chain termination of nascent viral DNA, leading to the inhibition of HBV replication[9]. Long-term treatment with lamivudine is not an option because it leads to drug resistance in most cases[10,11]. Lamivudine treatment, especially for chronic HBV-infected patients with cirrhosis, may also act as a bridge to more definitive treatments, such as liver transplantation. However, in several countries, including Japan, liver transplantation is not easily available because of insufficiency of donors, and even in other countries, many patients have to wait for long periods for transplantation. Although several non-Asian studies, from North America and Europe, have shown the efficacy of long-term use of lamivudine[12,13], few studies have assessed the efficacy of long-term lamivudine treatment of a large number of Japanese patients with chronic hepatitis B.

To acquire more data on these issues, 37 Japanese liver units involved in the management of HBV-related chronic liver diseases in Kyushu, Japan cooperated in this study. The objective of the present study was to analyze the results of long-term lamivudine administration for the suppression of HBV replication and the clinical outcomes of a large number of Japanese patients with chronic hepatitis B.

## MATERIALS AND METHODS

### Patients

This retrospective analysis encompassed 318 Japanese chronic hepatitis B patients (231 males and 87 females, mean age 47.8 years) on lamivudine monotherapy for up to 36 (median 21, range 9-36) mo. Clinical features from 403 HBsAg-positive patients with chronic liver diseases, who started lamivudine treatment between December 2000 and March 2004 in 37 Japanese liver units in Kyushu, were recorded in a centralized database. All patients were determined to be serum HBV DNA-positive via polymerase chain reaction (PCR) assay prior to treatment. The diagnosis of chronic hepatitis and cirrhosis was based on a liver biopsy in most patients, if unavailable, on clinical laboratory, and ultrasound data. Eighty five patients were excluded from the present analysis because of one or more of the following reasons: age below 18 years; positive for antibody to hepatitis C virus or human immunodeficiency virus type 1; diagnosis of HCC within 3 mo after enrolment; time of lamivudine treatment within 9 mo; or treatment with anticancer drugs or corticosteroid drugs for other malignancies, such as leukemia, lymphoma or autoimmune diseases. Because this was a retrospective analysis of treated patients, there were no predefined criteria for treatment withdrawal or combination treatments. Criteria for withdrawal and combination treatments after the start of the treatment were dependent upon the strategy used by the physician at each center. In the present study, follow-up was stopped for patients who discontinued lamivudine treatment or started receiving a combination treatment with IFN and lamivudine, or with adefovir dipivoxil and lamivudine.

### Therapeutic protocol

The patients received lamivudine (Zeffix®, Glaxo Smith Kline, UK) orally in a single daily dose of 100 mg. Data concerning age, sex, history of prior IFN treatment, Child-Turcotte-Pugh (CTP) score, series of serum laboratory testing of ALT, total bilirubin, albumin, HBeAg, and HBV DNA level were collected. Also, we analyzed virological (time of virological response and virological breakthrough) and biological events (time of ALT normalization, ALT breakthrough, and hepatitis flare) during the observation period. The clinical events recorded were hepatic decompensation (ascites, portal hypertensive bleeding, and hepatic encephalopathy) and liver-related death during the study period.

### Biochemical and virological measurement

Quantification of serum HBV DNA was performed at each center using one of the following commercial assays according to local availability: quantitative PCR assay (Amplicor HBV Monitor, Roche Diagnostics, Mannheim, Germany), over a detection range from 2.6 (corresponding to 400 copies/mL) to 7.5 log copies/mL; or transcription-mediated amplification and hybridization protect assay (TMA-HPA, Chugai Diagnostics, Tokyo, Japan), over a detection range from 3.7 log genome equivalents (LGE)/mL (corresponding to 5000 copies/mL) to 8.7 LGE/mL. A decline of the serum HBV DNA level to less than 3.7 log copies/mL during treatment was considered as a virological response. Virological breakthrough was defined as the reappearance of a serum HBV DNA level to more

than 10-fold the minimum during treatment. We analyzed whether or not an early decline of the HBV DNA level at 3 and 6 mo after the start of the treatment was related to virological response and breakthrough.

The serum ALT, bilirubin, and albumin levels were serially determined using the standard method every month before treatment and during the treatment. The upper normal limits for the ALT level were slightly different in each facility, ranging between 30 and 40 IU/mL. Normalization with an ALT level 667 or below during the treatment was considered as a biological response. A deterioration of ALT to an abnormal level after normalization during the treatment was considered as an ALT breakthrough. A deterioration of the ALT level more than 10 times the upper limit of normal (ULN) was considered as a hepatitis flare.

### Statistical analysis

Categorical variables were analyzed using $\chi^2$ test or Fisher's exact test. The Mann-Whitney *U*-test was also used to compare responders and non-responders with regard to various characteristics, when appropriate. The Cochran-Armitage's trend test was used to determine the relationship between the increases or decreases in the virological breakthrough rates of patients with virological response. Independent factors associated with responders were studied using forward stepwise logistic regression analysis of the following variables: age at the start of treatment, sex, history of prior IFN treatment, histological staging and grading, pretreatment laboratory data, serum pretreatment HBV DNA level, and the median declines of HBV DNA level at 3 and 6 mo after the start of the treatment. Forward stepwise logistic regression analysis was performed using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA, USA) for the IBM 3090 system computer. The BMDP program LR was used to evaluate the relationship between the clinical features and SVR. Using this method, the most significant associated variable was entered into the model. After adjusting for that variable, the next most significant variable was added to the model. Two-tailed *P* values less than 0.05 were considered statistically significant.

## RESULTS

### Baseline assessment

The mean age and percentage over 35 years were significantly higher in patients with cirrhosis than in those without cirrhosis, while the mean ALT level, albumin, and platelet counts were significantly higher in patients without cirrhosis than in those with cirrhosis. No significant differences in sex distribution, total bilirubin, positivity of HBeAg, or HBV DNA level were observed between these groups (Table 1). This study consisted of 173 HBeAg-positive and 145 HBeAg-negative patients with a mean pretreatment HBV DNA level of 6.8±1.2 (median 7.0) log copies/mL. Concerning the relationship between HBeAg and HBV DNA level, the mean HBV DNA level was significantly higher in HBeAg-positive patients with (7.3±1.1 log copies/mL) and without cirrhosis (7.2±1.0 log copies/mL) as compared with HBeAg-negative patients with (6.3±1.2 log copies/mL) and without cirrhosis (6.2±1.1 log copies/mL) (both $P<0.0001$). No significant difference in HBV DNA level, even classified by HBeAg status, was observed between patients with and without cirrhosis.

**Table 1 Baseline characteristics of 318 patients with chronic HBV infection treated with lamivudine (mean± SD)**

| Characteristics | Cirrhosis | | *P* |
|---|---|---|---|
| | No *n* = 216 | Yes *n* = 102 | |
| Number of men (%) | 154 (71.3) | 77 (75.5) | 0.5168 |
| Age (yr) | 45.0±11.1 | 53.7±9.7 | <0.0001 |
| Number with 35 yr old and over (%) | 173 (80.1) | 98 (96.1) | 0.0003 |
| ALT (IU/L) | 320.9±503.3 | 101.5±95.4 | <0.0001 |
| Total bilirubin (mg/dL) | 1.2±1.3 | 1.4±1.3 | 0.1880 |
| Albumin (g/dL) | 4.0±0.4 | 3.5±0.6 | <0.0001 |
| Platelet count (mean ± SD) ($\times 10^4/\mu L$) | 16.2±5.3 | 9.8±4.4 | <0.0001 |
| Number of HBeAg positivity (%) | 119 (55.1) | 54 (52.9) | 0.8112 |
| HBV-DNA[1] | 6.8±1.3 | 6.6±1.2 | 0.1344 |
| Lamivudine treatment (mo) | 20.2±8.9 | 21.8±9.7 | 0.1429 |

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; SD, standard deviation [1]Logarithmic transformed copies/mL.

### Virological and biological efficacy during lamivudine treatment period

In analyses of an early decline of HBV DNA level by lamivudine, the mean declines of all the studied patients were 3.2±1.2 (median 3.2) log copies/mL at 3 mo and 3.6±1.1 (median 3.8)log copies/mL at 6 mo after the start of treatment. During the treatment period of up to 36 (median 21) mo, a virological response was found in 90.6% (288/318) patients and ALT normalization was found in 86.2% (274/318) patients. Of the 288 with virological response, 255 (88.5%) had ALT normalization. Of the remaining 30 without virological response, 19 (63.3%), who had achieved virological suppression with a low HBV DNA level of 3.7-4.0 log copies/mL by lamivudine, had ALT normalization, but 11 (36.7%) had no ALT normalization and an HBV DNA level of more than 4.0 log copies/mL.

The mean pretreatment HBV DNA level was significantly lower in patients with virological response (6.6±1.2 log copies/mL) than those without virological response (7.7±0.7 log copies/mL) ($P<0.0001$). The frequency of pretreatment HBeAg positivity was significantly lower in patients with virological response (51.0%, 147/288) than those without virological response (86.7%, 26/30) ($P=0.0004$). No significant differences in sex distribution, age, ALT level, platelet count, presence of cirrhosis, or CTP score were found between the patients with and without virological response (Table 2).

Lamivudine suppressed serum HBV DNA to less than 3.7 log copies/mL in 69.2% patients at 3 mo, in 86.8% patients at 6 mo, in 80.2% patients at 12 mo, in 69.2% patients at 24 mo, and in 53.6% patients at 36 mo. The efficacy rate of virological response decreased with the length of the treatment period of patients who received lamivudine for over 6 mo (Figure 1).

Of the 288 patients with virological response, 224

**Table 2 Virological response of 318 patients with chronic HBV infection treated with lamivudine (mean± SD)**

| Characteristics | Virological response | | P |
|---|---|---|---|
| | No n = 288 | Yes n = 30 | |
| Number of men (%) | 207 (71.9) | 24 (80.0) | 0.4624 |
| Age (yr) | 47.9±11.4 | 46.9 (22-73) | 0.6453 |
| Number of cirrhosis (%) | 90 (31.2) | 12 (40.0) | 0.4403 |
| Baseline laboratory data | | | |
| Total bilirubin (mg/dL) | 1.3±1.5 | 1.1±0.6 | 0.6895 |
| Albumin (g/dL) | 3.9±0.6 | 3.8±0.6 | 0.2381 |
| Platelet count ($\times 10^4/\mu L$) | 14.3±5.9 | 13.2±5.8 | 0.2624 |
| Number of HBeAg positivity (%) | 147 (51.0) | 26 (86.7) | 0.0004 |
| HBV-DNA[1] | 6.6±1.2 | 7.7±0.7 | <0.0001 |

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; SD, standard deviation [1]Logarithmic transformed copies/mL

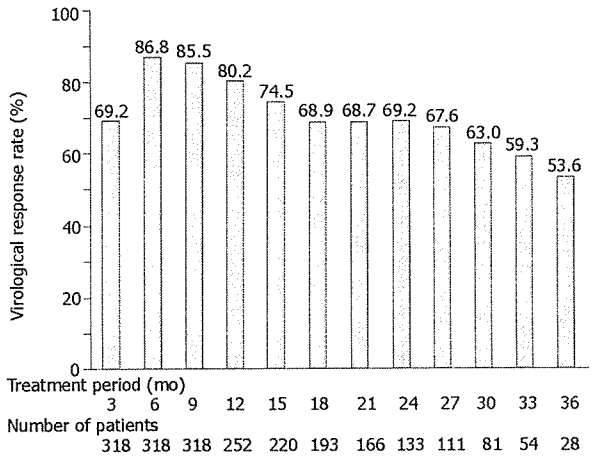


**Figure 1** Treatment period and virological response rates to lamivudine treatment of Japanese patients with chronic hepatitis B virus infection.

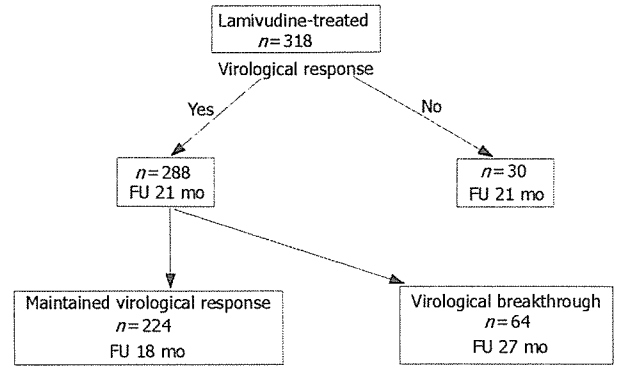


**Figure 2** Virological events in all patients during lamivudine treatment period. FU: Follow-up period.

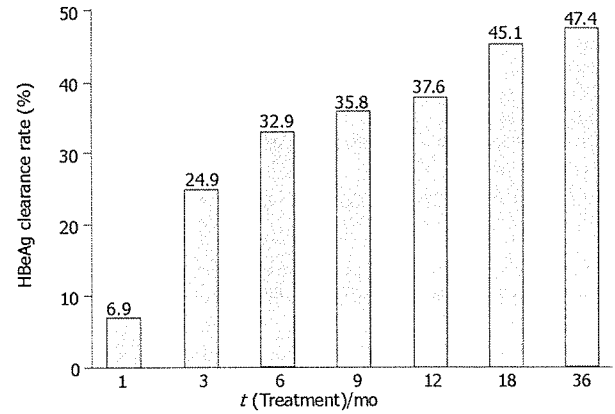


**Figure 3** Relationship between treatment period and HBeAg clearance rate during lamivudine treatment of Japanese patients with chronic HBV infection.

(77.8%) had a sustained virological response, and 64 (22.2%) had a virological breakthrough. The median follow-up time was significantly shorter for patients with sustained virological response (18 mo) than those with virological breakthrough (27 mo). The frequencies of pretreatment HBeAg positivity [65.6% (42/64) *vs* 46.8% (105/224); $P=0.0123$] and cirrhosis [43.8% (28/64) *vs* 27.7% (62/224); $P=0.0218$] were significantly higher for patients with virological breakthrough than those without a breakthrough (Figure 2). No significant differences in sex distribution, age, or pretreatment HBV DNA level were observed between these groups.

### HBeAg status during lamivudine treatment

Of the 318 patients, 173 (54.4%) were detected to have HBeAg in their sera at baseline. Of the 173 HBeAg-positive patients, 82 (47.4%) had clearance of HBeAg and 91 (52.6%) continued to have HBeAg during treatment. Lamivudine led to HBeAg clearance by 6.9% of the patients at 1 mo, by 24.9% of the patients at 3 mo, by 32.9% at 6 mo, by 35.8% at 9 mo, by 37.6% at 12 mo, by 45.1% at 18 mo, and by 47.4% at 36 mo, suggesting that HBeAg clearance rates increased with the duration of lamivudine treatment (Figure 3). HBeAg clearance always occurred after virological response in all the 82 who cleared HBeAg. No significant differences in sex distribution, age, ALT level, platelet count, presence of cirrhosis, or CTP score were found between the patients with and without HBeAg clearance. Of the 145 patients with HBeAg negative at baseline, no patient reversed to HBeAg positive. We observed that the patients who cleared HBeAg (79/82, 96.3%) and the patients with HBeAg negative at baseline (141/145, 97.2%) had a significantly higher virological response rate than those without HBeAg clearance (68/91, 74.8%, $P=0.0002$, $P<0.0001$, respectively).

### ALT breakthrough and hepatitis flare during lamivudine treatment

Of the 274 patients with ALT normalization by lamivudine, 231 (84.3%) had sustained ALT normalization, and 43 (15.7%) had an ALT breakthrough. Of the 43 patients with an ALT breakthrough, 4 (9.3%) had a hepatitis flare: 4 males, 3 with cirrhosis and 1 without cirrhosis, and 4 with HBeAg. However, no patient with hepatic decompensation, who had marked hyperbilirubinemia, or had a liver-related death, was observed in this study. The

**Table 3 Forward stepwise logistic regression analysis for all independent factors contributing to virological response**

| Factors | Odds ratio | 95% CI | *P* |
|---|---|---|---|
| (At baseline) | | | |
| HBV DNA less than 6.8[1] | 434.7 | 104.1-2000 | < 0.0001 |
| HBeAg negativity | 7.142 | 2.136-238.0 | <0.0001 |
| Platelet count more than $100\times10^9$/L | 4.625 | 1.242-17.22 | 0.0224 |
| (During treatment) | | | |
| Decline of HBV-DNA more than 3.2[1] within 3 mo of the start of treatment | 51.13 | 11.21-233.0 | <0.0001 |

HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; CI, confidence interval of odds ratios [1]Logarithmic transformed copies/mL.

**Table 4 Forward stepwise logistic regression analysis for all independent factors contributing to virological breakthrough**

| Factors | Odds ratio | 95% CI | *P* |
|---|---|---|---|
| (At baseline) | | | |
| Cirrhosis | 3.527 | 1.687-7.371 | 0.0008 |
| HBeAg positivity | 2.512 | 1.265-4.989 | 0.0085 |
| Platelet counts less than $100\times10^9$/L | 2.386 | 1.003-5.676 | 0.0491 |
| (During treatment) | | | |
| Decline of HBV-DNA less than 3.9[1] within 6 mo of the start of treatment | 2.358 | 1.246-4.464 | 0.0084 |

HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; CI, confidence interval of odds ratios [1]Logarithmic transformed copies/mL.

time of ALT changes always depended on the time of virological change: ALT deterioration after normalization followed an increase in the HBV DNA level in all cases. The frequency of HBeAg positivity at baseline was significantly higher in patients with a breakthrough than those without a breakthrough [72.1% (37/43) *vs* 51.5% (119/231); $P<0.0001$]. No significant differences in sex distribution, age, pretreatment HBV DNA level, presence of cirrhosis or CTP score were observed between these groups.

### *Relationship between early virological response and virological breakthrough*

Among the 288 with virological response, virological breakthrough was seen in 1 (5.3%) of 19 who had virological response at one month, in 42 (20.7%) of 203 at 3 mo, in 14 (27.5%) of 51 at 6 mo, in 4 (33.3%) of 12 at 9 mo, and in 3 (100%) of 3 at ≥15 mo. Cochran-Armitage's trend test revealed that virological breakthrough was significantly more prevalent in patients with delayed virological response ($P<0.0001$) (Figure 4).

### *Factors contributing to virological response and breakthrough*

At baseline, an HBV DNA level less than 6.8 log copies/mL ($P<0.0001$), HBeAg negativity ($P<0.0001$), and platelets count of $100\times10^9$/L or more ($P=0.0224$) were significantly associated with virological response in the 318 studied patients (Table 3). Of the treatment factors, an early decline of 3.2 or more log copies/mL of HBV DNA at 3 mo after the start of the treatment was significantly associated with the response ($P<0.0001$).

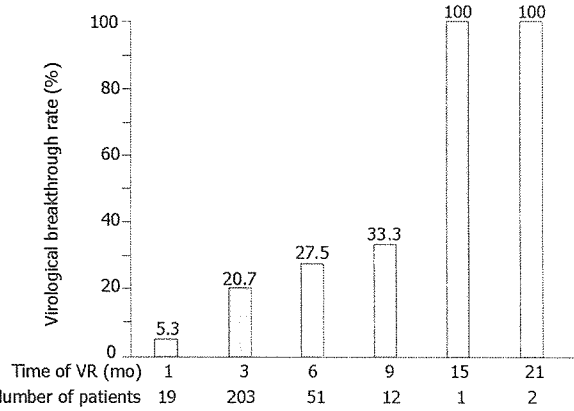


**Figure 4** Relationship between time of virological response and virological breakthrough rate during lamivudine treatment of Japanese patients with chronic HBV infection. VR: Virological response.

At baseline, cirrhosis ($P=0.008$), HBeAg positivity ($P=0.0085$), and platelets count less than $100\times10^9$/L ($P=0.0491$) were significantly associated with a virological breakthrough in the 288 patients with virological response (Table 4). Of the treatment factors, an early decline of 3.8 or less log copies/mL of HBV DNA at 6 mo after the start of the treatment was significantly associated with the breakthrough ($P=0.0084$).

## DISCUSSION

To our best knowledge, no such large-scale studies as this of lamivudine have been carried out for Japanese chronic hepatitis B patients. In this retrospective study, good virological and biological efficacy for up to 36 mo of lamivudine treatment was seen in Japanese patients with chronic hepatitis B, with no relation to sex, age, or ALT level at baseline. The effect was sustained for the patients with HBeAg-negative before treatment, absence of cirrhosis, and with an early decline of the HBV DNA level after the start of the treatment. During the treatment, very few patients with a hepatitis flare were seen and none with hepatic decompensation, marked hyperbilirubinemia, or liver-related death were seen in this study. The aims of treatment for chronic hepatitis B are to achieve sustained suppression of HBV replication and remission of inflammation in the liver. The antiviral responses for chronic hepatitis B are categorized as biochemical (ALT normalization), virological (decrease of HBV DNA to less than 5 log copies/mL and loss of HBeAg), and histological, and as on-therapy or sustained off-therapy[14]. Treatment for chronic hepatitis B patients seems to be necessary when the HBV DNA level exceeds 5 log copies/mL, independent of ALT activity[11]. Lamivudine well inhibited HBV DNA replication in Japanese chronic hepatitis B patients.

HBeAg clearance usually predicts long-lasting suppression of HBV, reduced infectivity and an improved clinical prognosis[15]. In this study, 47.4% of patients with HBeAg at baseline had HBeAg eliminated from their sera. Follow-up reports of the multicenter Asian study for Chinese patients showed that HBeAg clearance rates increased with the duration of lamivudine treatment, from 17% to 22% at

12 mo, 27 to 29% at 24 mo, and 33 to 40% at 36 mo[12,16,17]. The results of our study were consistent with those of these non-Japanese patient, although the HBeAg clearance rates within 24 mo were relatively high in our study. Lamivudine was effective in terms of HBeAg clearance in Japanese chronic hepatitis B patients. Patients successfully treated for chronic hepatitis B are less likely to develop cirrhosis, liver failure, and HCC in comparison with those who do not respond to treatment[18]. A randomized controlled trial of lamivudine for chronic hepatitis B patients demonstrated that HCC incidence was reduced by lamivudine antiviral therapy, showing an incidence of 3.9% in lamivudine-treated patients and 7.4% in a placebo control group, with a hazard ratio of 0.49 (95%CI = 0.25-0.99)[19]. For chronic hepatitis B patients, antiviral therapy with lamivudine that results in sustained suppression of HBV DNA replication and hepatic necroinflammation may reduce the incidence of HCC.

It has been reported that resistance to lamivudine often develops after 6 mo of treatment[10]. The present study was limited in its value because we detected viruses resistant to lamivudine. However, in our study, the emergence of resistant viruses could be defined by the virological breakthrough (reappearance of serum HBV DNA levels more than 10-fold increase from the minimum). A serious drawback of long-term lamivudine treatment is the development of resistant HBV mutants, i.e., the mutations in a tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV polymerase gene, associated with increase in serum HBV DNA and the ALT level[19]. The present study showed that the HBV DNA suppression rates by lamivudine decreased with the duration of treatment, but that a relapse of biochemical response, ALT breakthrough was found only in 15.7% of patients during these treatment periods. Lamivudine treatment withdrawal can cause HBV DNA to revert to pretreatment levels, with the relapse of clinical hepatitis[20]. With the excellent safety and tolerability of lamivudine, continuous therapy is suggested as beneficial[4]. After the start of phylogenetic analyses, based on inter group divergence of 8% or more over the complete HBV nucleotide sequence, seven different genotypes, arbitrarily designed A-G, have been recognized[21,22]. Several reports have shown geographical distribution of the genotypes, with genotypes A and D predominant in Western Europe, B and C in South Asia and the Far East, and F in South America[21-26]. Due to the geographical distribution pattern, HBV genotypes B and C are commonly observed in Japan[24-27]. Moreover, Japan is apparently at a geographical boundary for genotypes B and C, forming a south to north gradient in which genotype C is more frequent in the south of Kyushu, and genotype B is more frequent in the north of Tohoku. Interestingly, however, genotype B is more frequent in Okinawa, the southern-most area of Japan[27]. Our previous epidemiological study of the Japanese HBV genotype distribution showed that 95% of the patients studied had genotype C[24]. Genotype C has been reported to cause more severe liver damage and to have lower rates of HBeAg clearance, which usually indicates cessation of HBV replication and represents a later stage of chronic HBV infection, than genotype B in Japanese patients[24-26]. Accordingly, our results were equivalent in the response to lamivudine to Japanese HBV genotype C patients, although we did not determine the genotyping of our patients.

Another noteworthy finding of our study was that predictive marker of the efficacy to lamivudine and its durability were HBeAg negativity and a low HBV DNA level at baseline. HBV DNA reappears in serum after cessation of lamivudine treatment because HBV replication within the HBV-infected hepatocytes originates primarily from the covalently closed circular DNA (cccDNA) of HBV in the liver. Lamivudine appears to have no effect on the level of cccDNA[28]. Liver injury seems to be particularly severe and rapidly progressive in HBeAg-negative patients, but clinically significant HBV replication persists in them[24]. Most HBeAg-negative chronic hepatitis B patients who are HBV DNA-positive harbor HBV variants with mutations in the precore or core promotor region, which can suppress synthesis of HBeAg[11,26]. The clearance of HBeAg is perhaps a reflection of a loss of the cccDNA pool of HBV in the liver[29]. The great concern of clinicians is that HBeAg negativity and a low HBV DNA level at baseline are significant predictive markers for lamivudine treatment in Japanese patients.

A previous report on Japanese patients showed that the emergence rate of lamivudine-resistant viruses in patients with cirrhosis was higher than those without cirrhosis[26], suggesting that a virological breakthrough appears more frequently in patients with cirrhosis than those without cirrhosis. The present study showed that lamivudine treatment was not so effective or durable in patients with cirrhosis and low platelet counts. Clinicians should always do close monitoring or use other antiviral drugs because hepatitis flare was occasionally severe, especially in patients with cirrhosis. The present study also showed that an early virological response to lamivudine was predictive of both efficacy and durability, but a lack of an early virological response was found to predict a virological breakthrough. A high HBV DNA level reflects a greater pool of virus and a higher rate of virus replication, thereby increasing the likelihood that drug-resistant mutations will be selected. Such an early decrease of viral load after the start of lamivudine might be associated with the lack of viral resistance.

In conclusion, the present study suggests a long-term lamivudine treatment to be safe and to result in the reduction of serum HBV DNA in most Japanese patients with chronic hepatitis B. The efficacy is sustained in patients with HBeAg-negative at baseline, absence of cirrhosis, and a reduction of the HBV DNA level soon after the start of the treatment.

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# A comparison of the antitumor effects of interferon-α and β on human hepatocellular carcinoma cell lines

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**Abstract**

The antiviral, antiproliferative and immunomodulatory effects of type I interferons (IFNs) are well documented, however, few studies have been published concerning differences in the antitumor effects of IFN-α and β. In the present study, differences in antitumor effect, including the antiproliferative effect, cell cycle change, apoptosis, and the IFN-stimulated gene (ISG) were examined by flow cytometry between IFN-α and β on three human hepatocellular carcinoma (HCC) cell lines (HepG2, Huh7 and JHH4). The antiproliferative effect of both IFNs on the HCC cell lines was time- and dose-dependent, and IFN-β was significantly stronger than IFN-α. The cell cycle effect by both IFNs was an S-phase accumulation, with IFN-β having a tendency to increase the S-phase ratio more strongly than IFN-α, especially in Huh7. Apoptosis marker expression, Fas antigen and intracellular active caspase-3, was increased after the addition of IFNs, especially of IFN-β. The expression of human leukocyte antigen-class I molecules, ISG-encoded protein, was increased after the addition of IFNs, especially of IFN-β. These data suggest that IFN-β has a greater antitumor effect than IFN-α on HCC of a very early stage in patients with chronic hepatitis C.


*Keywords:* Antitumor effects; Hepatocellular carcinoma; Interferon-α; Interferon-β

## 1. Introduction

Hepatocellular carcinoma (HCC) is a common cancer worldwide. The hepatitis C (HCV), and hepatitis B viruses (HBV) have been directly linked to the development of HCC, especially in patients who have chronic active hepatitis with cirrhosis [1,2]. In Japan, the HCC incidence has increased, resulting in it becoming the third leading cause of death due to cancer.

Interferons (IFNs) are a family of cytokines that elicit a pleiotropic biological effect. IFNs have antiviral, antiproliferative and immunomodulatory effects, and are classified as type I (IFN-α, β and ω) and type II (IFN-γ) [3,4]. IFNs mediate their effects by binding to cell surface receptors (IFN receptors) and activating Janus kinases (JAK), resulting in the phosphorylation of the signal transducers and activators of transcription (STAT). STAT proteins homo- or heterodimerize and form complexes with other transcription factors to activate transcription of IFN-stimulated genes (ISGs) [3]. IFN actions are largely mediated by the proteins encoded by ISGs [5–7]. A number of IFN-related proteins, such as dsRNA-dependent protein kinase (PKR), the 2-5A system, human leukocyte antigen (HLA)-class I molecules and Mx proteins, mediate the antiviral actions of IFNs [3], and IFN-α and β are effective for the treatment of chronic hepatitis C [8,9].

In oncology, IFN-α and β are used for the treatment of a number of solid tumors and hematological malignancies,

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such as malignant melanoma, renal cell carcinoma, and chronic myelogeneous leukemia [4]. Recent reports showed that IFN-α treatment reduced the risk for HCC in patients with chronic hepatitis C [10,11]. We previously reported that IFN-β treatment also reduced the risk of HCC in such patients [12]. Moreover, this study revealed that a reduction in HCC occurrence was independent of virological or biochemical responses of IFN-β [12]. Although the antitumor effect of IFN-α on HCC cell lines has been reported in vitro [13,14], few studies have been published concerning differences in the antitumor effects of IFN-α and β on HCC cell lines [15,16], as was done in this study.

## 2. Materials and methods

### 2.1. Cell lines and reagents

The three human HCC cell lines, HepG2, Huh7 and JHH4, were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan). HepG2 was established by Aden et al. [17] from a liver tumor biopsy obtained from a 15-yr-old Caucasian male. The morphological characteristics and epithelial cell shape were compatible with that of liver parenchymal cells. Histology of the liver biopsy revealed well differentiated hepatocellular carcinoma with a trabecular pattern. Huh7 was established by Nakabayashi et al. [18] from a hepatoma tissue of a 57-yr-old Japanese male with well differentiated hepatocellular carcinoma. JHH4 was established by Homma [19] from a liver tumor biopsy obtained from a 51-yr-old Japanese male with hepatocellular carcinoma. These cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 25 mM HEPES, 50 units (U)/mL penicillin, 50 μg/mL streptomycin, and 10% heat-inactivated fetal calf serum (FCS) at 37 °C in a humidified incubator with 5% $CO_2$ in air.

Human natural lymphoblast IFN-α (Sumiferon), with a specific activity of $2.25 \times 10^8$ IU/mg, was kindly supplied by Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan). Human natural fibroblast IFN-β (FERON), with a specific activity of $3.08 \times 10^8$ IU/mg, was kindly provided by Daiichi Pharmaceuticals Co., Ltd. (Tokyo, Japan). Since these natural IFN-α and β were of high purity, they did not contain other cytokines, that might have modulating effects such as tumor necrosis factor.

Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human Fas antigen monoclonal antibodies (mAbs) and HLA-class I molecule mAbs were purchased from Beckman Coulter (Miami, FL). Phycoerythrin (PE)-conjugated polyclonal rabbit anti-active caspase-3 antibodies were purchased from BD Biosciences (San Jose, CA). DMEN, FCS, trypsin/EDTA, and penicillin/streptomycin were purchased from Gibco BRL (Life Technologies, Inc., Gaithersburg, MD).

### 2.2. Antiproliferative effect of IFN-α and β

The antiproliferative effect of the IFNs was analyzed for three HCC cell lines, HepG2, Huh7 and JHH4. Cells ($1 \times 10^5$/well) were added in triplicate to a 6-well culture plate (Becton Dickinson). The medium was replaced 24 h later by 1.5 mL of fresh medium containing IFN-α and β. Concentrations of IFN-α and β were $1 \times 10^3$ IU/mL and cell lines cultured in medium alone were used as a control. Proliferation of HCC cell lines was determined over a period of 96 h after IFN addition. After the culture, the adhering cells were washed with PBS and detached using 0.25% trypsin/EDTA. The resulting single-cell suspension was washed in washing buffer (PBS containing bovine serum albumin and sodium azide), and the number of viable cells was counted by flow cytometer, CYTORON ABSOLUTE with ImmunoCount 2 software (Ortho Diagnostic Systems). In some experiments, the concentrations of IFN-α and β ranged from $10^2$ to $10^4$ IU/mL. Cell viability was determined using the trypan blue dye exclusion method and exceeded 95% in all experiments. All assays were analyzed in at least three independent experiments.

### 2.3. Cell cycle

The effect of the IFNs on the cell cycle phase distribution of the HCC cell lines was analyzed by flow cytometry using the CycleTEST™ PLUS DNA reagent kit (Becton Dickinson Immunocytometry Systems, San Jose, CA) according to the manufacturer's instructions. Briefly, cells ($1 \times 10^5$/well) were added in triplicate to a 6-well culture plate, and the medium was replaced 24 h later by 1.5 mL of fresh medium containing $10^3$ IU/mL IFN-α or $10^3$ IU/mL IFN-β. Cell lines cultured in medium alone were used as a control. The cultured cells were detached 24 h later using 0.25% trypsin/EDTA after washing with PBS. Cells were washed twice with PBS, 250 μL of Solution A (trypsin buffer) was added and the cells were incubated for 10 min at room temperature, followed by the addition of 200 μL of Solution B (trypsin inhibitor and RNase buffer) and incubation for a further period of 10 min at room temperature. Finally, 200 μL of cold Solution C (propidium iodide stain solution) was added and the cells were incubated on ice for 10 min in the dark. The samples were filtered through a 44-μm nylon mesh, and analyzed by flow cytometer, EPICS XL with EXPO32 software (Beckman Coulter).

### 2.4. Apoptosis-related markers

The expression of surface Fas antigen on the HCC cell lines was analyzed by flow cytometry. Cells ($1 \times 10^5$/well) were cultured with medium alone as a control, $10^3$ IU/mL IFN-α, or $10^3$ IU/mL IFN-β. Twenty-four hours after the addition of IFNs, the cells were washed with PBS and detached using 0.25% trypsin/EDTA. Washed cells were incubated at 4 °C for 30 min in 10 μL of FITC-conjugated mouse anti-human Fas antigen mAbs. The samples were then washed with the washing buffer and analyzed by flow cytometer, EPICS XL with EXPO32 software.

Active caspase-3, a marker for cells undergoing apoptosis, consists of a heterodimer of 17 and 12 kDa subunits which are derived from the 32 kDa proenzyme. Caspase-3 is a key

protease that is activated during the early stages of apoptosis [20]. In this study, intracellular active caspase-3 in the HCC cell lines was analyzed. Cells ($1 \times 10^5$/well) were cultured with medium alone as a control, $10^3$ IU/mL IFN-α, or $10^3$ IU/mL IFN-β. The cells were washed with PBS 24 h after the addition of IFNs and detached using 0.25% trypsin/EDTA. Washed cells were fixed and permeabilized using Cytofix/Cytoperm kit (PharMingen) according to the manufacturer's instructions, and were incubated in the dark at 4 °C for 30 min in 10 μL of PE-conjugated anti-active caspase-3 antibodies. The samples were then washed with a washing buffer and analyzed by flow cytometer, EPICS XL with EXPO32 software.

### 2.5. Expression of HLA-class I molecules on HCC cell lines

Cells ($1 \times 10^5$/well) were cultured with medium alone as a control, $10^3$ IU/mL IFN-α, or $10^3$ IU/mL IFN-β. The cells were washed with PBS 24 h after the addition of IFNs and detached using 0.25% trypsin/EDTA. Washed cells were incubated in the dark at 4 °C for 30 min in 10 μL of FITC-conjugated mouse anti-human HLA-class I molecule mAbs. The samples were then washed with the washing buffer and analyzed by flow cytometer, CYTORON ABSOLUTE with ImmunoCount 2 software.

### 2.6. Statistical analyses

Statistical analysis was by the Stat View J-5.0 program (SAS Institute Inc., Cary, NC). Statistical differences between the control and IFN treatment groups were calculated by unpaired student's *t*-test and considered significant at $P < 0.05$.

## 3. Results

### 3.1. Antiproliferative effect of IFN-α and β

As shown in Fig. 1, panels (a)–(c), the IFNs showed a significant time-dependent antiproliferative effect on HepG2 (control, IFN-α, IFN-β; $9.8 \times 10^5 \pm 0.5 \times 10^5$ cells, $8.4 \times 10^5 \pm 0.3 \times 10^5$ cells, $4.4 \times 10^5 \pm 0.2 \times 10^5$ cells, respectively), Huh7 (control, IFN-α, IFN-β; $8.8 \times 10^5 \pm 0.4 \times 10^5$ cells, $6.6 \times 10^5 \pm 0.7 \times 10^5$ cells, $3.3 \times 10^5 \pm 0.3 \times 10^5$ cells, respectively) and JHH4 (control, IFN-α, IFN-β; $17 \times 10^5 \pm 2.2 \times 10^5$ cells, $12 \times 10^5 \pm 0.4 \times 10^5$ cells, $9 \times 10^5 \pm 0.6 \times 10^5$ cells, respectively) compared with the control at 96 h after the addition of the IFNs ($P < 0.05$). Furthermore, IFN-β was significantly stronger than IFN-α in time-dependent antiproliferative effect, with the first significant effect observed at 48 h in both HepG2 (IFN-α; $3.9 \times 10^5 \pm 0.3 \times 10^5$ cells, IFN-β; $3.0 \times 10^5 \pm 0.3 \times 10^5$ cells, $P < 0.05$) and Huh7 (IFN-α; $3.1 \times 10^5 \pm 0.1 \times 10^5$ cells, IFN-β; $2.2 \times 10^5 \pm 0.2 \times 10^5$ cells, $P < 0.05$)

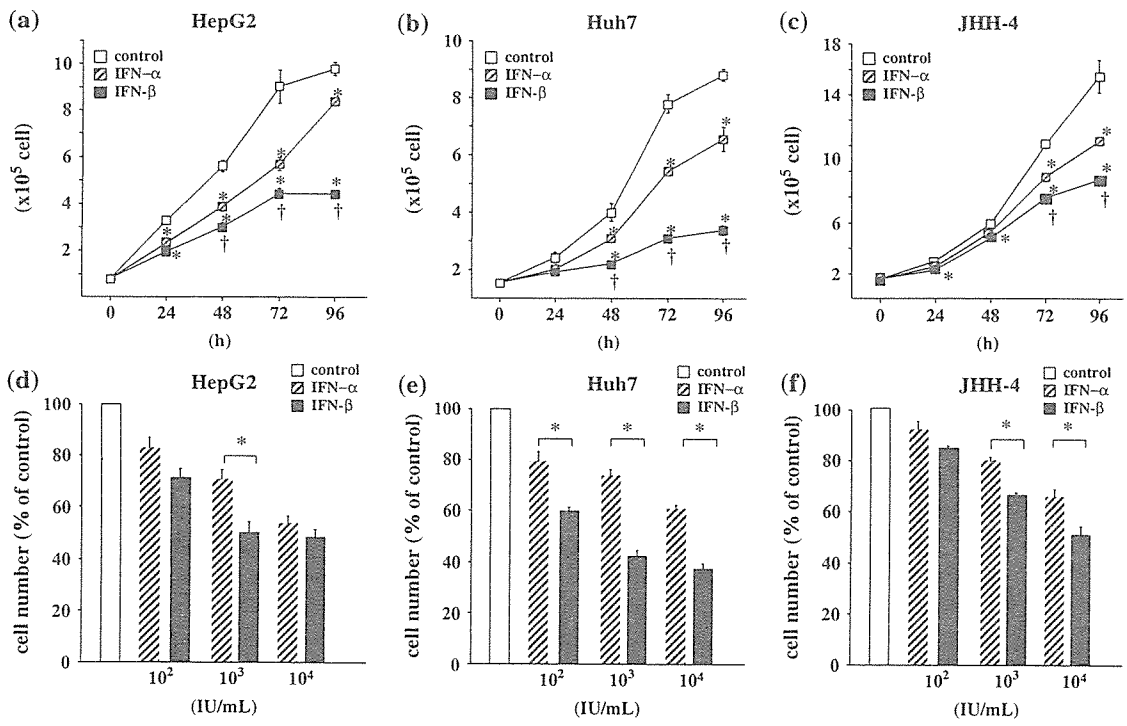


Fig. 1. Effect of IFN-α and β on the cell proliferation of HCC cell lines. Time course of HepG2 (a), Huh7 (b) and JHH4 (c) cell numbers. Dose effect of HepG2 (d), Huh7 (e) and JHH4 (f) cell numbers. Three HCC cell lines were cultured with medium alone as a control, $10^3$ IU/mL IFN-α and $10^3$ IU/mL IFN-β. Cell numbers were measured by flow cytometry after harvest up to 96 h as described in the "Section 2". Alternatively, three HCC cell lines were cultured with medium alone as, IFN-α ($10^2$, $10^3$ and $10^4$ IU/mL) and IFN-β ($10^2$, $10^3$ and $10^4$ IU/mL) for 72 h, and cell numbers were measured by flow cytometry after harvest. Values are the mean ± SD. Representative results from three independent experiments, each carried out in triplicate are shown. (a)–(c): * And † indicate statistically significant differences ($P < 0.05$) between the indicated experimental groups (* vs. control, † vs. IFN-α). (d)–(f): The proportion of viable cells cultured with medium alone as a control was considered 100%. * Indicates a statistically significant difference between the indicated experimental groups ($P < 0.05$).

and at 72 h in JHH4 (IFN-α; $9.6 \times 10^5 \pm 0.3 \times 10^5$ cells, IFN-β; $7.9 \times 10^5 \pm 0.1 \times 10^5$ cells, $P < 0.05$).

As shown in Fig. 1, panels (d)–(f), IFNs showed a dose-dependent antiproliferative effect on HepG2 (control, $10^2$ U/mL IFN-α and β, $10^3$ U/mL IFN-α and β, $10^4$ U/mL IFN-α and β; 100%, 83 ± 6.5% and 71 ± 6.0% of control, 70 ± 6.5% and 50 ± 6.9% of control, 54 ± 4.7% and 48 ± 2.1% of control, respectively), Huh7 (control, $10^2$ U/mL IFN-α and β, $10^3$ U/mL IFN-α and β, $10^4$ U/mL IFN-α and β; 100%, 79 ± 7.2% and 60 ± 2.1% of control, 74 ± 4.0% and 42 ± 3.5% of control, 61 ± 2.1% and 37 ± 3.2% of control, respectively) and JHH4 (control, $10^2$ U/mL IFN-α and β, $10^3$ U/mL IFN-α and β, $10^4$ U/mL IFN-α and β; 100%, 91 ± 5.8% and 84 ± 1.7% of control, 79 ± 2.6% and 66 ± 1.5% of control, 65 ± 5.0% and 51 ± 5.9% of control, respectively) at 72 h after the addition of the IFNs. The antiproliferative effect of IFN-β was especially notable in Huh7, since the cell number in the culture with $10^2$ IU/mL IFN-β was almost equal to $10^4$ IU/mL of IFN-α.

### *3.2. Effect of IFN-α and β on the cell cycle distribution of HCC cell lines*

We next analyzed the mechanism of the antiproliferative effect on HCC cell lines after the addition of IFNs. As shown in Fig. 2 and Table 1, at 24 h, the addition of IFNs significantly increased the S-phase ratio and slightly decreased the $G_2$/M phase ratio compared with the controls. Furthermore, the increase of the S-phase ratio induced by IFN-β was significantly stronger than that induced by IFN-α in three HCC cell lines. These results suggest that the difference in effect on the cell cycle distribution is a mechanism contributing to the IFN-related antiproliferative effect.

### *3.3. Effect of IFN-α and β on the expression pattern of apoptosis-related markers of HCC cell lines*

Apoptosis is thought to be related to another mechanism of IFN-related antiproliferative effect [3]. To examine the effect of IFN-α and β on apoptosis in HCC cell lines, the expression of surface Fas antigen, a protein encoded by ISGs, and intracellular active caspase-3 were analyzed by flow cytometry. As shown in Table 2, IFNs increased the mean fluorescence intensity (MFI) of Fas antigen on the three HCC lines. IFN-β significantly increased the cell surface expression of Fas antigen on HepG2 and Huh7 in comparison with IFN-α. Furthermore, both IFNs increased the MFI of intracellular active caspase-3 in the three HCC cell lines, and all had a tendency to be more strongly induced by IFN-β than IFN-α. These results suggest that apoptosis is another mechanism contributing to the antiproliferative effect of IFN-β as well as IFN-α.

### *3.4. Effect of IFN-α and β on the expression pattern of HLA-class I molecules of HCC cell lines*

Ligation of IFNs with IFN receptors results in the upregulation of ISGs [5]. We compared the capacity of IFN-α and β to induce HLA-class I molecules, a protein also encoded by ISGs. As shown in Table 2, the expression of HLA-class I molecules on the three HCC cell lines was significantly increased by both IFNs compared with controls. The increase

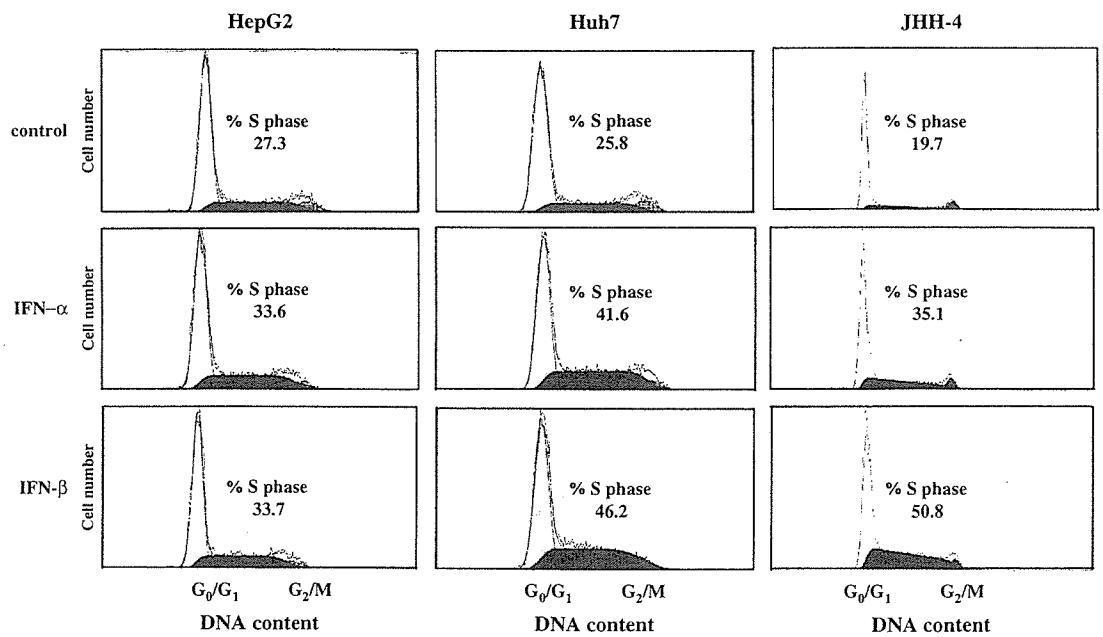


Fig. 2. Effect of IFN-α and β on the cell cycle distribution of HCC cell lines. HepG2, Huh7 and JHH4 were cultured with medium alone as a control, $10^3$ IU/mL IFN-α and $10^3$ IU/mL IFN-β for 24 h. The cell cycle phase distribution of both HCC cell lines were analyzed after harvest by flow cytometry using CycleTEST™ PLUS DNA Reagent Kit according to the manufacturer's instructions as described in the "Section 2". The x axis indicates DNA content, and the y axis indicates cell number. Results show representative data of three independent experiments.

Table 1
Effect of IFN-α and β on the cell cycle distribution of HCC cell lines

| | HepG2 | | | Huh7 | | | JHH-4 | | |
|---|---|---|---|---|---|---|---|---|---|
| | $G_0/G_1$ phase (%) | S phase (%) | $G_2/M$ phase (%) | $G_0/G_1$ phase (%) | S phase (%) | $G_2/M$ phase (%) | $G_0/G_1$ phase (%) | S phase (%) | $G_2/M$ phase (%) |
| Control | 65 (64 ± 1.5) | 29 (29 ± 0.8) | 7.3 (7.5 ± 1.1) | 66 (68 ± 1.8) | 25 (25 ± 0.6) | 8.5 (7.6 ± 1.9) | 70 (72 ± 1.7) | 22 (21 ± 0.8) | 7.8 (7.0 ± 0.9) |
| IFN-α ($10^3$ IU/mL) | 62 (61 ± 0.6) | 33* (33 ± 0.9) | 4.6 (6.1 ± 1.5) | 53* (56 ± 2.3) | 42* (41 ± 2.0) | 2.5 (3.4 ± 0.9) | 58* (58 ± 0.5) | 35* (36 ± 0.5) | 6.6 (6.5 ± 0.1) |
| IFN-β ($10^3$ IU/mL) | 57* (56 ± 2.9) | 34*,† (38 ± 4.3) | 6.3 (5.9 ± 1.8) | 53* (49 ± 4.4) | 47*,† (51 ± 4.4) | 0*,† (0) | 46*,† (47 ± 0.6) | 49*,† (49 ± 0.8) | 4.5† (3.9 ± 1.4) |

Variables were expressed as median (mean ± S.E.) in three independent experiments.
* Significant difference ($P < 0.05$) compared with control.
† Significant difference ($P < 0.05$) compared with IFN-α.

Table 2
Effect of IFN-α and β on the expression pattern of apoptosis-related markers and HLA-class I molecules of HCC cell lines

| | HepG2 | | | Huh7 | | | JHH-4 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Fas | Caspase-3 | HLA-class I | Fas | Caspase-3 | HLA-class I | Fas | Caspase-3 | HLA-class I |
| Control | 220 (216 ± 5.10) | 170 (176 ± 4.00) | 140 (137 ± 4.87) | 170 (168 ± 6.29) | 165 (163 ± 4.97) | 110 (111 ± 3.60) | 270 (270 ± 20.8) | 130 (137 ± 6.67) | 60 (58.8 ± 3.15) |
| IFN-α ($10^3$ IU/mL) | 283* (283 ± 19.1) | 210* (233 ± 24.6) | 280* (255 ± 36.9) | 195 (188 ± 9.47) | 210* (268 ± 64.9) | 564* (508 ± 120) | 370 (355 ± 34.8) | 190* (193 ± 8.82) | 150* (147 ± 13.6) |
| IFN-β ($10^3$ IU/mL) | 374*,† (367 ± 27.3) | 280* (295 ± 24.6) | 500* (445 ± 73.1) | 225*,† (230 ± 12.2) | 325* (373 ± 52.7) | 1678* (1589 ± 512) | 455* (438 ± 42.5) | 190* (200 ± 10.0) | 190* (185 ± 12.3) |

Variables indicated mean fluorescence intensity, and were expressed as median (mean ± S.E.) in three independent experiments.
* Significant difference ($P < 0.05$) compared with control.
† Significant difference ($P < 0.05$) compared with IFN-α.

of the MFI of three HCC cell lines tended to be more strongly induced by IFN-β than IFN-α.

## 4. Discussion

There is accumulating evidence that IFN-β has a preferential antiproliferative effect on glioma, melanoma, and breast carcinoma cells, and that this effect is stronger than that by IFN-α [21–24]. The present study showed that IFN-β also had a superior antiproliferative effect on HCC cell lines than IFN-α. Type I IFNs exert their effects through the type I IFN receptor, which is composed of two major subunits, IFN-α receptor 1 (IFNAR-1) and 2c (IFNAR-2c) [3], which are potentially expressed in HCC cells [13]. IFN-α and β probably utilize a common receptor complex. Natural IFN-α (Sumiferon) was composed of approximately 20 subtypes, in which that contained α 2 subtype of 25%. IFN-α 2 is the subtype that is used as an antitumor and antiviral agent in the clinical setting, including chronic myelogenous leukemia, hairy cell leukemia, renal cell carcinoma and hepatitis C virus (HCV). Natural IFN-β (FERON) was composed of only one subtype, and has found clinical applications in several malignancies and viral diseases such as glioblastoma, melanoma, medulloblastoma and HCV. It was possible that the difference of component between natural IFN-α and β affected the antitumor effects on HCC cell lines in the present study. Previous reports showed that IFN-β had a greater antitumor effects on several cancer cell lines, such as melanoma cell, squamous cervical carcinoma cell, breast cancer cell compared with recombinant IFN-α 2 [21–25]. Because the α 2 subtype is the major subtype of which natural IFN-α is composed, the difference in the antitumor effects between both IFNs used may be involved with that of biological activity, rather than component, in the present study. Although it is still unknown why IFN-α and β have different biological effects, it is possible that IFN-α and β have different signaling events at the receptor level. IFN-β, but not IFN-α, formed a strong complex with IFNAR-1 and IFNAR-2c [26] and, alternatively, an IFN-β specific signaling domain within the cytoplasmic regions of the IFNAR chain was found in IFNAR-2c [27]. These reports suggest that the specific assembly of type I IFNAR leads to the differing biological responses to IFN-α and β. The present study showed that ISGs were more induced strongly by IFN-β than IFN-α since Fas antigen and HLA-class I molecules, proteins also encoded by ISGs, were more effectively upregulated by IFN-β. These results suggest that there are some differences in receptor interaction between IFN-α and β in HCC.

The present study showed that the antiproliferative effect of both IFNs on the HCC cell lines was time- and dose-dependent, and that IFN-β was significantly stronger than IFN-α. IFN-β showed a significantly stronger antiproliferative effect on Huh7 at any concentration examined than IFN-α, after 72 h of incubation, as shown in Fig. 1, panel (e). On other two cell lines, the antiproliferative effect at low ($10^2$ units/mL) as well as high ($10^4$ units/mL) concentrations had a tendency to be more strongly induced by IFN-β than IFN-α, although that was not statistically significant, as shown in Fig. 1, panels (d) and (f). It is possible that HCC cell lines differed in their sensitivity to IFNs, but we suppose that IFN-β has a stronger antiproliferative effect on HCC cell lines compared with IFN-α.

Type I IFNs are known to modify the cell cycle [3]. Although previous studies demonstrated that IFNs induced an inhibitory effect on $G_1$–S phase transition [28,29], it was recently demonstrated that the S phase of HCC cell lines was delayed by IFN-α [13,14]. We showed a greater increase in the S phase population of HCC cell lines treated with IFN-β than with IFN-α. Qin et al. [30] has reported that IFN-β preferentially induced S phase accumulation in human transformed cells by losing or inactivating the normal $G_1$ checkpoint conferred by the retinoblastoma protein, which acts as a cell cycle inhibitor. It is possible that IFN-β influences the normal $G_1$ checkpoint of HCC cell lines.

Induction of apoptosis is a highly attractive mechanism of the antitumor effect of IFNs. Apoptosis plays a critical role in the elimination of cells that sustain DNA damage or undergo uncontrolled cellular proliferation [7,31], and probably occurs as an independent cell cycle arrest [32]. The mechanism of apoptosis has been shown to occur through the ligation of death receptors on the cell surface, such as Fas or tumor necrosis factor-related apoptosis inducing ligand (TRAIL). This leads to the activation of an adaptor protein, Fas associated death domain (FADD) and to the subsequent activation of caspase-8. Activated caspase-8 cleaves additional downstream caspases, including caspase-3, a major effector caspase, and elicits the morphological hallmarks of apoptosis [7,32]. While IFN-α has been shown to induce apoptosis in HCC cell lines [13,14], the present study demonstrated that IFN-β does the same. Previous studies reported that IFN-β preferentially induced apoptosis in non-HCC cell lines, which was correlated with a stronger induction of TRAIL by IFN-β [25,33,34]. The difference in the induction of apoptosis by IFN-β seen in the present study may be related to the more effective induction of ISGs with an apoptotic function, such as Fas and TRAIL.

Tatsumi et al. [35] reported that IFN-α increased the expression of HLA-class I molecules on HCC cell lines. We also showed that HLA-class I molecules were more effectively upregulated by IFN-β. The immunomodulatory effects of type I IFNs occurred by enhancing the expression of HLA-class I molecules, activating $CD8^+$ cytotoxic T lymphocytes, natural killer cells and dendritic cells [3]. These data suggest a more effective antitumor immune response against HCC by IFN-β than by IFN-α.

It is still disputable if the prevention of HCC in patients with chronic hepatitis C treated with IFN-α and β is due to the direct antitumor effect on cancer cells. Several studies showed that the prevention of HCC would be associated with the virological or biochemical responses of IFNs [10,11]. Furthermore, our previous study [12] revealed that a reduction in the HCC development was independent of the biochemical response in natural IFN-β treated patients with chronic hepatitis C, but not in natural IFN-α treated patients, although similar rates of the HCC development were found

in patients with chronic HCV viremia treated with either IFN-α or β. Thus, IFN-β, rather than IFN-α, may directly inhibit HCC growth at a very early stage in patients with chronic hepatitis C, as suggested in the present study, although the results obtained from this study have been done by in vitro model.

In conclusion, IFN-β had a stronger antiproliferative effect than IFN-α by inducing cell cycle change and apoptosis, and upregulated HLA-class I molecules more strongly than IFN-α in three HCC cell lines, indicating that ISGs would be more strongly induced by IFN-β than by IFN-α. These data suggest that IFN-β has a greater antitumor effect than IFN-α in the early stage of HCC in patients with chronic hepatitis C.

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