

**Table 2** Risk factors for HCC development in all HBV-infected patients by univariate analysis

Factor	95% CI	p value
Age (years) (<50/≥50)	2.15–14.5	<0.001
Sex (male/female)	0.33–1.76	0.520
Initial diagnosis (chronic hepatitis/cirrhosis)	3.75–1.176	<0.001
HBe Ag (positive/negative)	0.31–1.29	0.209
HBV DNA (log copies/ml) (<7.0/>7.0)	0.33–1.35	0.262
AST (IU/l) (<40/≥40)	0.33–2.22	0.742
ALT (IU/l) (<40/≥40)	0.17–1.16	0.188
Total bilirubin (mg/dl) (<1.2/>1.2)	1.43–6.72	0.004
Alb (g/dl) (<3.8/≥3.8)	0.19–0.86	0.019
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> ) (<14/≥14)	0.02–0.31	<0.001
Emergence of LAM-resistant viruses (positive/negative)	0.51–2.03	0.968
IVR (positive/negative)	0.52–3.25	0.575
MVR (positive/negative)	1.04–5.95	0.035

HCC Hepatocellular carcinoma, HBV hepatitis B virus, CI confidence interval, HBe Ag hepatitis B e antigen, HBV hepatitis B virus, AST aspartate aminotransferase, ALT alanine aminotransferase, Alb albumin, IVR initial viral response, MVR maintained viral response, LAM lamivudine

**Table 3** Risk factors for HCC development in all HBV-infected patients by multivariate analysis

Factor	Category	Risk ratio	95% CI	p value
Age (years)	<50	1	1.08–9.53	0.036
	≥50	3.20		
Initial diagnosis	Chronic hepatitis	1	1.75–12.4	0.002
	Cirrhosis	4.64		
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> ) (<14/≥14)	≥14	1	0.05–0.96	0.045
	<14	4.76		
MVR	Negative	1	1.09–6.56	0.032
	Positive	0.37		

HCC Hepatocellular carcinoma, HBV hepatitis B virus, CI confidence interval, MVR maintained viral response

Cumulative incidence of HCC development according to effectiveness of treatment (MVR vs. non-MVR)

Figure 2a shows the Kaplan–Meier curve of cumulative HCC incidence in all HBV-infected patients treated with LAM according to the effectiveness of treatment (MVR vs. non-MVR). The cumulative carcinogenesis rate for MVR-positive patients was 2% at 3 years, 4% at 5 years, and 6% at 7 years. On the other hand, the cumulative carcinogenesis rate for MVR-negative patients was 5% at 3 years, 13% at 5 years, and 16% at 7 years. MVR during LAM significantly suppressed the cumulative HCC incidence

**Table 4** Risk factors for HCC development by univariate analysis (chronic hepatitis/cirrhosis)

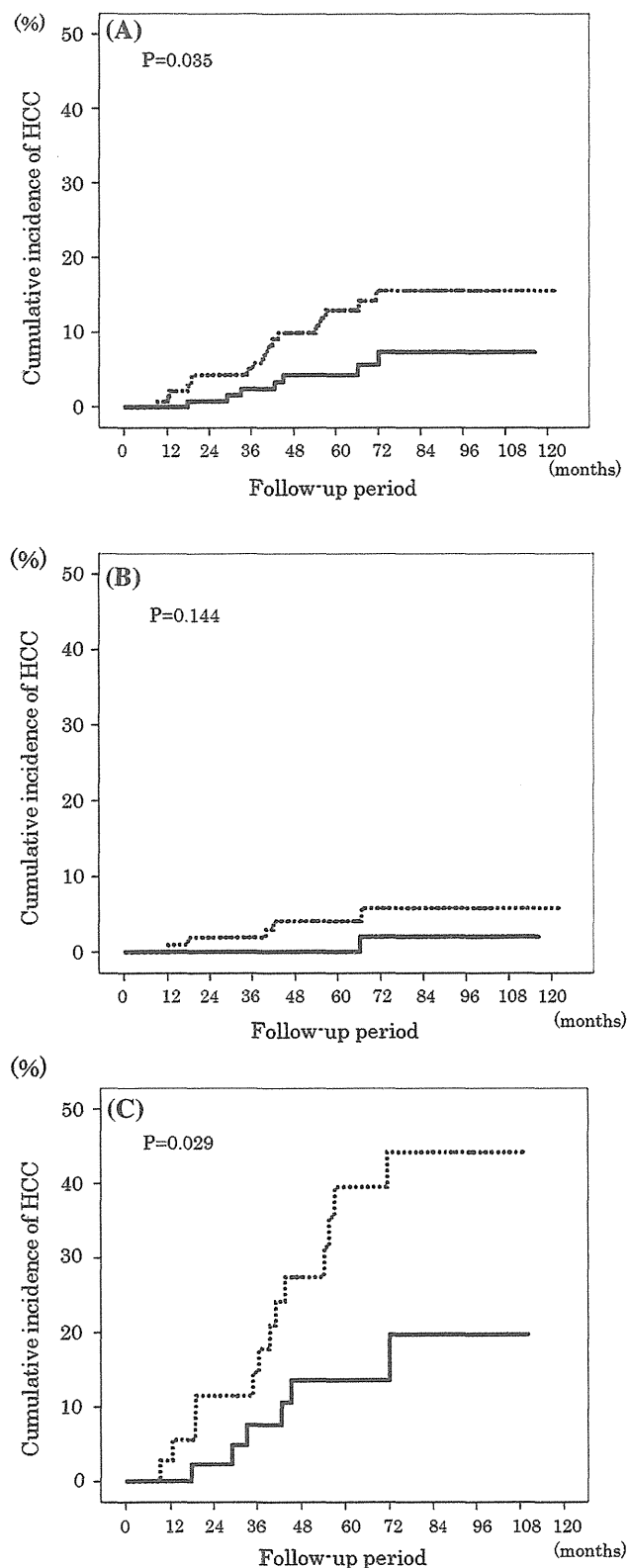
	95% CI	p value
<b>Chronic hepatitis</b>		
Age (years) (<50/≥50)	0.26–8.38	0.002
Sex (male/female)	0.37–6.42	0.556
HBe Ag (positive/negative)	0.01–0.74	0.005
HBV DNA (log copies/ml) (<7.0/>7.0)	0.11–1.99	0.296
AST (IU/l) (<40/≥40)	0.11–2.64	0.482
ALT (IU/l) (<40/≥40)	0.06–1.41	0.101
Total bilirubin (mg/dl) (<1.2/≥1.2)	0.67–6.67	0.574
Alb (g/dl) (<3.8/≥3.8)	0.13–8.58	0.960
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> ) (<14/≥14)	0.01–0.72	0.004
Emergence of LAM-resistant viruses (positive/negative)	0.27–4.28	0.927
IVR (positive/negative)	0.29–8.67	0.590
MVR (positive/negative)	0.51–37.10	0.144
<b>Cirrhosis</b>		
Age (years) (<50/≥50)	0.86–6.17	0.089
Sex (male/female)	0.21–1.82	0.380
HBe Ag (positive/negative)	0.80–4.17	0.149
HBV DNA (log copies/ml) (<7.0/>7.0)	0.40–2.01	0.795
AST (IU/l) (<40/≥40)	0.27–3.07	0.873
ALT (IU/l) (<40/≥40)	0.13–1.47	0.167
Total bilirubin (mg/dl) (<1.2/≥1.2)	0.82–4.80	0.126
Alb (g/dl) (<3.8/≥3.8)	0.28–1.58	0.354
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> ) (<14/≥14)	0.03–1.51	0.084
Emergence of LAM-resistant viruses (positive/negative)	0.44–2.18	0.948
IVR (positive/negative)	0.90–8.32	0.063
MVR (positive/negative)	1.07–0.029	

HCC Hepatocellular carcinoma, HBV hepatitis B virus, CI confidence interval, HBe Ag hepatitis B e antigen, HBV hepatitis B virus, AST aspartate aminotransferase, ALT alanine aminotransferase, Alb albumin, IVR initial viral response, MVR maintained viral response

compared with non-MVR in all HBV-infected patients ( $p = 0.035$ ).

Figure 2b shows the Kaplan–Meier curve of the cumulative HCC incidence in chronic hepatitis patients according to the effectiveness of treatment (MVR vs. non-MVR). The cumulative carcinogenesis rate for MVR-positive patients was 0% at 3 years, 0% at 5 years, and 2% at 7 years. On the other hand, the cumulative carcinogenesis rate for MVR-negative patients was 2% at 3 years, 4% at 5 years, and 6% at 7 years. MVR during LAM did not significantly suppress the cumulative HCC incidence compared with non-MVR in the chronic hepatitis patients ( $p = 0.144$ ).

Figure 2c shows the Kaplan–Meier curve of the cumulative HCC incidence in cirrhosis patients according to the effectiveness of treatment (MVR vs. non-MVR).



**Fig. 2** Cumulative incidence of development of HCC according to the effectiveness of treatment (MVR vs. non-MVR). **a** All cases; **b** chronic hepatitis; **c** cirrhosis. *Solid lines* MVR, *dotted lines* non-MVR. MVR Maintained viral response

The cumulative carcinogenesis rate for MVR-positive patients was 8% at 3 years, 14% at 5 years, and 14% at 7 years. On the other hand, the cumulative carcinogenesis rate for MVR-negative patients was 18% at 3 years, 40% at 5 years, and 44% at 7 years. MVR during LAM significantly suppressed the cumulative HCC incidence compared with non-MVR in the cirrhosis patients ( $p = 0.029$ ).

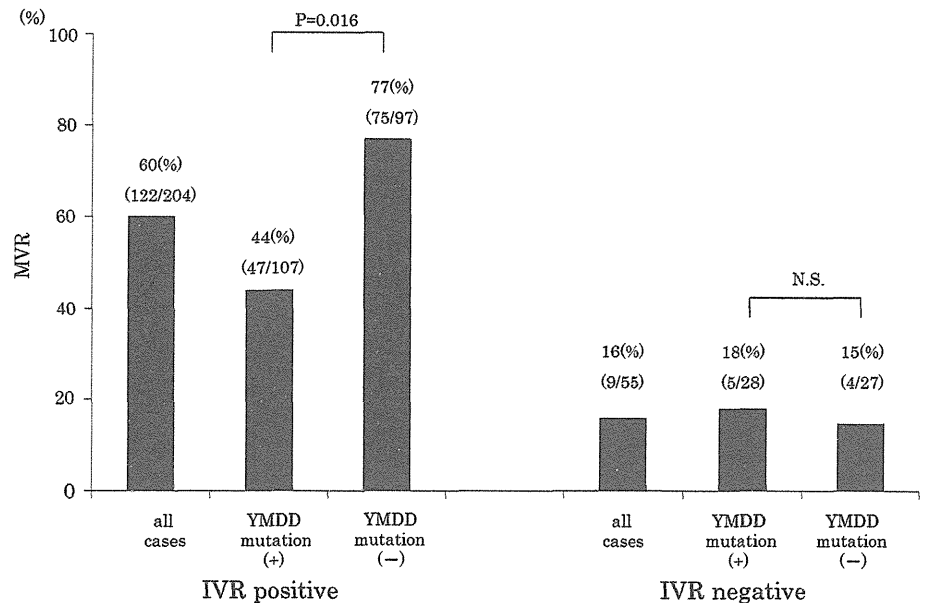
#### Relationship between IVR and MVR

Maintained viral response (MVR) was achieved by 142 (48%) of the 293 patients enrolled in this study. IVR was achieved by 204 (79%) of the 259 patients who were examined for IVR. The relationship between IVR and MVR is shown in Fig. 3; 60% (122/204) of the IVR-positive patients achieved an MVR, while only 16% (9/55) of the IVR-negative patients achieved an MVR ( $p < 0.001$ ). The LAM-resistant YMDD mutant virus was found in 149 (51%) of all patients during follow-up, and in 52% (107/204) of the IVR-positive patients, a finding which was nearly equal to that for the IVR-negative patients (51%, 28/55). Among the IVR-positive patients, the MVR rate was lower in patients with the YMDD mutation, compared with that in those without the YMDD mutation (44%, 47/107 vs. 77%, 75/97,  $p = 0.016$ ), while the MVR rates were low in the IVR-negative patients, irrespective of their YMDD mutation status (with and without the mutation, 15 vs. 18%, respectively). ADV was added to LAM treatment for 73 (68%) of the 107 IVR-positive patients with the YMDD mutation and 20 (36%) of the 55 IVR-negative patients with the YMDD mutation. However, MVR was only achieved at the low rates of 33% (24/73) for the former patients and 20% (4/20) for the latter.

#### Discussion

Lamivudine treatment has been shown to improve the liver histological findings in patients with HBV-infected liver disease by reducing the HBV load and stabilizing inflammatory activity [16–18]. One report has shown that LAM effectively reduced the incidence of HCC in patients with chronic hepatitis B, but the study only compared LAM-treated patients with non-treated patients in a matched case-controlled study [19]. However, there have been few detailed reports about the relationship between virological response and HCC development in HBV-infected patients during LAM treatment. In the present study, we retrospectively examined the incidence of HCC to clarify the indicators of LAM therapy, including median HBV-DNA levels, for reducing the risk of HCC in HBV-infected patients.

**Fig. 3** Relationship between IVR and MVR. *IVR* Initial viral response, *MVR* maintained viral response, *N.S.* not significant



Many investigators have reported that serum HBV DNA levels higher than 4.0–4.5 log copies/ml before HBV treatment serve as a strong risk predictor of HCC [23–25]. Di Marco et al. [26] have reported that the incidence of HCC was higher in patients with serum HBV levels of more than 5.0 log copies/ml, at least once, during LAM therapy than in those in whom serum HBV levels were maintained at 5.0 log copies/ml or less. However, the add-on ADV therapy had not been adopted when the study of Di Marco et al. was reported. When the use of ADV is possible, an evaluation method is needed to measure the antiviral effects of nucleoside/nucleotide analogues against HBV-related liver disease. In the present study, we set the cut-off value for HBV-DNA at 4.0 log copies/ml. The basis of this cut-off value is that a serum HBV DNA level higher than 4.0 log copies/ml before HBV treatment was reported to serve as a strong risk predictor of HCC [23]. MVR, defined as a median HBV-DNA level of less than 4.0 log copies/ml measured every 6 months during therapy, was adopted as an indicator of viral replication, and non-MVR (median HBV-DNA >4.0 log copies/ml) during LAM therapy was shown to be significantly associated with the development of HCC in HBV-infected patients. We also found that a median HBV-DNA level of >4.0 log copies/ml during LAM therapy was a risk factor for HCC development. On the other hand, IVR, defined as HBV-DNA of <4.0 log copies/ml in the first 6 months of the follow-up period after the initiation of therapy, was not associated with the development of HCC in HBV patients in this study. As shown in Fig. 3, 84% of the IVR-negative patients could not achieve an MVR, suggesting that it is crucial to achieve an IVR in order to achieve an MVR. The reason why IVR was not a significant factor for MVR seemed to be the appearance of the YMDD mutation, which reduced the antiviral effect of

LAM for HBV in IVR-positive patients. The LAM-resistant YMDD mutant virus was found in 52% of the IVR-positive patients. Although ADV was added to LAM treatment for 73 patients, only 33% of these patients could achieve an MVR. We speculate that the antiviral effect of ADV is not very strong [27] and it takes time to reduce the YMDD mutant virus, which may explain the low MVR rate (33%) in patients with the add-on ADV therapy. The immediate administration of ADV when the LAM-resistant YMDD mutant virus appears can be important [28]. A switch to ETV, which induces resistant virus less frequently, could also raise MVR rates among IVR-positive patients without the YMDD mutant virus.

As the duration of the add-on ADV therapy was included in this study, we compared the cumulative incidence of HCC in patients receiving LAM monotherapy with that in patients who also received the add-on ADV therapy. Sixteen (10%) of the 164 patients who received the LAM monotherapy developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 10% at 5 years, and 15% at 7 years. On the other hand, 16 (12%) of the 129 patients who received LAM plus ADV developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 12% at 5 years, and 14% at 7 years. No significant difference was found between these two groups ( $p = 0.986$ ). In addition, we examined the cumulative incidence of HCC development according to the effectiveness of treatment (MVR vs. non-MVR) in patients for whom the observation period was terminated when ADV was added, and the same results were obtained (data not shown).

Older age ( $\geq 50$  years), cirrhosis, and low platelet count ( $<14 \times 10^4/\text{mm}^3$ ) were shown to be significantly associated with the development of HCC in patients with HBV infection. These results were consistent with those of

previous reports [29–31], suggesting that patients of older age with advanced fibrosis should be followed up carefully for longer periods in order to detect early stages of HCC even if LAM therapy does effectively suppress HBV. Of note, in the present study we estimated the cumulative HCC incidence according to the initial diagnosis of chronic hepatitis or cirrhosis. In the chronic hepatitis patients, older age ( $\geq 50$  years), HBe Ag negativity, and low platelet count ( $<14 \times 10^4/\text{mm}^3$ ) were significant risk factors for the development of HCC, but this was not the case in the cirrhosis patients. Because liver biopsies had not been performed, the liver fibrosis stage could not be evaluated with respect to the risk factors for HCC in this study. Instead, the factors of age, HBe Ag status, and platelet count may reflect the degree of liver fibrosis in chronic hepatitis patients. In fact, cirrhotic patients, in comparison with chronic hepatitis patients, were of older age (chronic hepatitis vs. cirrhosis:  $46.3 \pm 10.7$  vs.  $51.9 \pm 9.8$  years,  $p < 0.001$ ), had higher rates of HBe Ag negativity (chronic hepatitis vs. cirrhosis: 39 vs. 51%,  $p = 0.065$ ), and had lower platelet counts (chronic hepatitis vs. cirrhosis:  $15.6 \pm 4.9$  vs.  $9.3 \pm 3.8 \times 10^4/\text{mm}^3$ ,  $p < 0.001$ ). This seems to explain why none of these factors were significant risk factors for HCC in cirrhotic patients. On the other hand, in the chronic hepatitis patients, MVR was not a significant factor for HCC development, while MVR was a significant factor for HCC development in the cirrhotic patients. We speculate that HBV suppression induced by LAM therapy could reduce the incidence of HCC in patients infected with HBV, especially those with cirrhosis, who displayed higher malignant potential. Investigation over a longer period is needed to clarify the effect of HBV suppression on the development of HCC in chronic hepatitis patients.

In conclusion, the present study shows that the attainment of an MVR induced by LAM therapy has a significant beneficial effect on the clinical course of HBV-infected patients by decreasing the incidence of HCC. The newer nucleotide analogues, such as ETV and tenofovir, should be able to further reduce the incidence of HCC, given their greater potency.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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## Fibroblast growth factor-2 enhances NK sensitivity of hepatocellular carcinoma cells

Hinako Tsunematsu<sup>1\*</sup>, Tomohide Tatsumi<sup>1\*</sup>, Keisuke Kohga<sup>1</sup>, Masashi Yamamoto<sup>1</sup>, Hiroshi Aketa<sup>1</sup>, Takuya Miyagi<sup>1</sup>, Atsushi Hosui<sup>1</sup>, Naoki Hiramatsu<sup>1</sup>, Tatsuya Kanto<sup>1</sup>, Norio Hayashi<sup>2</sup> and Tetsuo Takehara<sup>1</sup>

<sup>1</sup> Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka, Japan

<sup>2</sup> Kansai-Rosai Hospital, Amagasaki, Hyogo, Japan

The roles of fibroblast growth factor-2 (FGF-2) in the hepatocellular carcinoma (HCC) development are still controversial. In this study, we investigated the expression of FGF-2 in chronic hepatitis (CH) type C patients with or without HCC and the immunoregulation of FGF-2 in NK sensitivity of HCC cells. The FGF-2 expressions were detected in the liver tissues of patients, but not in normal liver. The serum FGF-2 levels of the patients with CH, liver cirrhosis (LC) or HCC were significantly higher than those of healthy volunteers. The serum FGF-2 levels of patients decreased with the progression of chronic liver disease. HCC occurrence of LC patients with high levels of serum FGF-2 was significantly lower than that with low levels of serum FGF-2. Proinflammatory cytokines, such as IL-1 $\beta$  and IL-6, induced FGF-2 expressions in HCC cells and normal hepatocytes. FGF-2 stimulation resulted in increasing the expression of the membrane-bound major histocompatibility complex class I-related chain A (MICA), an NK activating molecule, and decreasing that of human leukocyte antigen (HLA) class I, an NK inhibitory molecule, on HCC cells. This did not occur with normal hepatocytes. Adding anti-FGF receptor-2 neutralizing antibody resulted in inhibiting the change of MICA and HLA class I expressions on FGF-2 stimulated HCC cells. FGF-2 stimulation on HCC cells resulted in increasing NK sensitivity against HCC cells. These findings indicate that FGF-2 produced by HCC cells or normal hepatocytes of chronic liver disease may play critical roles in eliminating HCC cells by innate immunity.

Fibroblast growth factor (FGF)-2 is one of a family of FGFs that includes 22 structurally related members.<sup>1</sup> FGF-2 has been shown to exert a potent angiogenic effect by interacting with tyrosine kinase receptors, FGFR1, FGFR2 and FGFR3, in various cancers including hepatocellular carcinoma (HCC).<sup>2-4</sup> Aside from its angiogenic effect, FGF-2 has also been shown to act as a mitogen for HCC cell proliferation *via* an autocrine mechanism.<sup>5</sup> Uematsu *et al.* reported that the serum FGF-2 of chronic liver disease patients without

HCC tended to be higher than that of those with HCC.<sup>6</sup> Decrease of serum FGF-2 could be observed prior to the emergence of HCC, and this suggests that FGF-2 may play a critical role in the surveillance of HCC. However, the immunological significance of elevating the FGF-2 levels in chronic liver disease patients remains unclear.

HCC is one of the leading causes of cancer deaths worldwide. Chronic liver disease caused by hepatitis virus infection and nonalcoholic steatohepatitis leads to a predisposition for HCC, with liver cirrhosis (LC), in particular, being considered a premalignant condition.<sup>7,8</sup> The liver contains a large compartment of innate immune cells (NK cells and NKT cells) and acquired immune cells (T cells),<sup>9,10</sup> but the activation process of these immune cells in HCC development remains unclear. A recent study has demonstrated that the innate immune system may play a critical role in tumor surveillance *via* an NKG2D signal.<sup>11</sup> Knowing the details of how to activate the abundant NK cells in the liver could lead to the establishment of attractive new strategies for HCC treatment.

In this study, we investigated the expression of FGF-2 in chronic hepatitis (CH) type C patients with or without HCC and the immunoregulation of FGF-2 in NK sensitivity of HCC cells. Of importance are the findings that serum FGF-2 levels in patients with CH and LC without HCC were significantly higher than that in those with HCC and that FGF-2 enhanced the NK sensitivity of HCC cells. The present study

**Key words:** FGF-2, hepatocellular carcinoma, NK cells, MICA, HLA class I

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\*H.T. and T.T. contributed equally to this work and share the first authorship.

**Correspondence to:** Tomohide Tatsumi, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan, Tel.: +81-6-6879-3621, Fax: +81-6-6879-3629, E-mail: tatsumit@gh.med.osaka-u.ac.jp

Table 1. Clinical backgrounds

	Normal	Hepatitis	Cirrhosis	HCC
Number	24	80	84	112
				Stage I/II 51
				Stage III/IV 61
Sex (M/F)	12/12	45/35	44/40	67/45
Age	64 ± 15	56 ± 13	62 ± 13	66 ± 11
Etiology		HCV	HCV	HCV

Abbreviations: Stage: TNM stage; M: male; F: female; HCV: hepatitis C virus.

sheds light on previously unrecognized immunological effects of FGF-2 on HCC cells and thus suggests a role of FGF-2 in HCC development in patients with CH type C.

## Material and Methods

### Liver tissues and immunohistochemistry

Human HCC tissues ( $n = 6$ ) and normal liver tissues ( $n = 2$ ) were obtained at surgical resection. CH tissues ( $n = 4$ ) and LC tissues ( $n = 4$ ) were obtained as liver biopsy samples. Informed consent, under an Institutional Review Board-approved protocol, was obtained from all patients before sample acquisition. Liver sections were subjected to immunohistochemical staining using the ABC procedure (Vector Laboratories, Burlingame, CA). The primary antibody (Ab) was antihuman FGF-2 Ab (Abcam, Cambridge, MA). To confirm the specificity of the staining, the primary antibody was incubated with recombinant human FGF-2 protein (R&D Systems, Minneapolis, MN) for 3 hr and then applied onto liver sections in parallel with staining of the primary antibody as the absorption test.

### HCC cell lines

HepG2 and PLC/PRF/5, human hepatoma cell lines, were purchased from American Type Culture Collection (Rockville, MD) and were cultured with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (GIBCO/Life Technologies, Grand Island, NY) in a humidified incubator at 5% CO<sub>2</sub> and 37°C.

### ELISA

The sera from CH patients ( $n = 80$ ), LC patients ( $n = 84$ ), HCC patients ( $n = 112$ , Stage I/II  $n = 51$  and Stage III/IV  $n = 61$ ) and age-matched healthy volunteers (HVs) ( $n = 24$ ) were subjected to analysis of the FGF-2 level. Clinical backgrounds of patients were summarized in Table 1. Informed consent, under an Institutional Review Board-approved protocol, was obtained from all patients before sample acquisition. The level of FGF-2 and soluble major histocompatibility complex class I-related chain A (MICA) were determined using Quantikine Human FGF basic (R&D Systems) and DuoSet MICA eELISA kit (R&D Systems), respectively.

### HCC cells and normal hepatocytes cultures

Both HepG2 and PLC/PRF/5 cells or normal hepatocytes (ScienCell Research Laboratories, Carlsbad, CA) were cultured for 72 hr in the presence or absence of human interleukin-1 $\beta$  (IL-1 $\beta$ ) (50 ng/ml, Peprotech, Rocky Hill, NJ), human IL-6 (300 ng/ml, Peprotech), human transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) (50 ng/ml, R&D Systems) and human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (100 ng/ml, Peprotech), and the treated cells were harvested and evaluated for expression of FGF-2. In some experiments, HepG2 and PLC/PRF/5 cells were cultured in the presence or absence of recombinant human FGF-2 protein (250 ng/ml, R&D Systems) with or without antihuman FGFR2 neutralizing Ab (10  $\mu$ g/ml, R&D Systems) for 48 hr, and the hepatoma cells were harvested and evaluated for the immunological regulation of the NK cells.

### Flow cytometry

For the detection of membrane-bound MICA, cells were incubated with anti-MICA specific Ab (2C10, Santa Cruz Biotechnology, Santa Cruz, CA) and stained with Goat F(ab')<sub>2</sub> fragment anti-Mouse IgG(H+L)-PE (Beckman Coulter, Fullerton, CA) as a secondary reagent and then subjected to flow cytometric analysis. For the detection of human leukocyte antigen (HLA) class I, cells were incubated with PE-conjugated antihuman HLA-A,B,C Ab (w6/32, BD Biosciences, San Jose, CA). Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

### Western blotting

The total cellular protein was electrophoretically separated using sodium dodecyl sulfate-12% polyacrylamide gels and transferred onto PVDF membranes. The membranes were blocked in Tris-buffered saline-Tween20 containing 5% skim milk for 1 hr and then probed with rabbit polyclonal Ab to FGF-2 (Abcam) at room temperature overnight. Horseradish peroxidase-conjugated anti-rabbit IgG and SuperSignal West Pico System (Pierce, Rockford, IL) were used for the detection of blots.

### Real-time RT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) and was reverse transcribed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA). The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Ready-to-use assay (Applied Biosystems) was used for the quantification of FGF-2 (ID: Hs00960934\_m1), MICA (Hs00792195\_m1) and  $\beta$ -actin (Hs:99999903\_m1) mRNAs according to the manufacturer's instructions.  $\beta$ -actin mRNA from each sample was quantified as endogenous control of internal RNA.

### NK cell analysis

NK cells were isolated from human peripheral blood mononuclear cells by magnetic cell sorting using CD56 MicroBeads (Miltenyi Biotech, Auburn, CA).<sup>12</sup> The cytolytic ability of NK cells against FGF-2-treated HepG2 and PLC/PRF/5 cells was assessed by 4-hr <sup>51</sup>Cr-release assay with or without antihuman MICA/B Ab (BD Biosciences) as previously described.<sup>12</sup> The expressions of NKG2D and NKG2A on NK cells were analyzed by flow cytometry with PE-conjugated antihuman NKG2D Ab (BD Biosciences) and PE-conjugated IgG antihuman NKG2A Ab (R&D Systems).

### Statistics

For human sample data, values were expressed as the median and interquartile range using box plots and the 10th and 90th percentiles as horizontal bars. For comparison of more

than two groups, the Kruskal–Wallis rank sum test was used. If the Kruskal–Wallis test was significant, post hoc multiple comparisons were carried out using the Steel–Dwass procedure. Differences between retreatment and post-treatment values were tested by the paired *t*-test. FGF-2 mRNA values were expressed as the mean and SD, and the statistical significance of differences between the groups was determined by applying Student's *t* test after each group had been tested with equal variance and Fisher's exact probability test. We defined statistical significance as  $p < 0.05$ .

### Results

#### FGF-2 is expressed in the liver and serum of patients with chronic liver diseases

We first examined the FGF-2 expressions in the livers of normal volunteers and the patients with chronic liver diseases. Immunohistochemical analysis revealed that FGF-2 was not expressed in normal liver tissues. In contrast, the expressions of FGF-2 were detected in chronic liver tissues (Fig. 1a). We evaluated the serum FGF-2 levels by specific ELISA. All of the chronic liver disease patients were hepatitis C virus (HCV)-RNA positive. As shown in Figure 1b, the serum FGF-2 levels in CH and LC patients were significantly higher than those of HV, but those in HCC patients were not. Those in CH patients were also significantly higher than those in LC or HCC patients. Those in LC patients tended to be higher than those in HCC patients, although this was not significant. The serum FGF-2 levels in HCC patients were low and significant difference between Stage I/II patients and III/IV patients was not observed (data not shown). We compared the serum FGF-2 levels before and after the

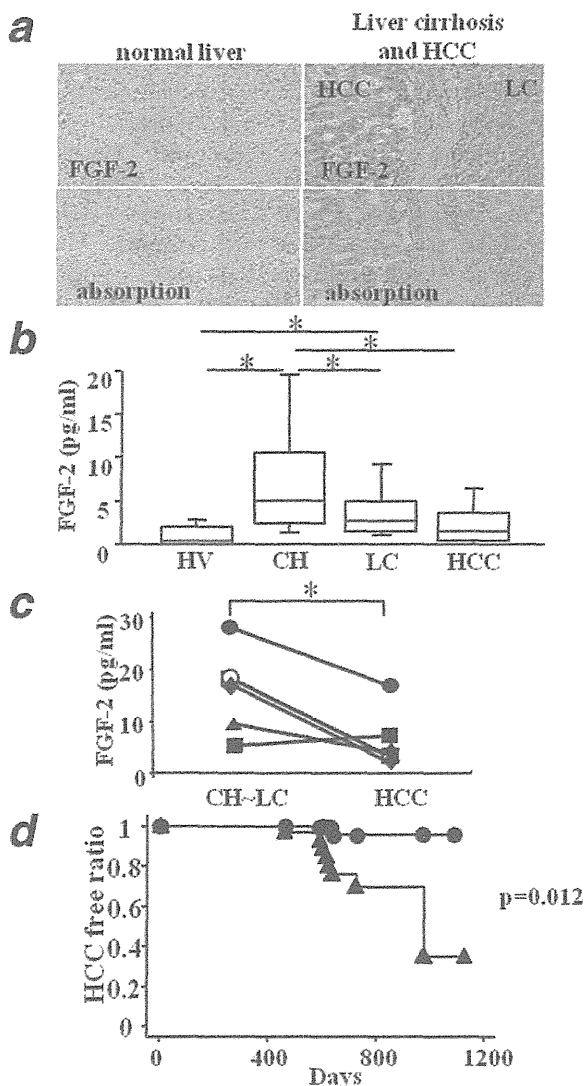


Figure 1. Expressions of FGF-2 in the liver of patients with chronic liver diseases and serum FGF-2 levels in chronic liver disease patients were associated with HCC incidence. (a)

Immunohistochemical analysis of FGF-2 in normal liver tissues ( $N = 2$ ), chronic hepatitis tissues ( $N = 4$ ), liver cirrhosis (LC) tissues ( $N = 4$ ) and hepatocellular carcinoma (HCC) tissues ( $N = 6$ ). Liver sections were stained with the FGF-2 Ab (upper panels). The primary Ab was incubated with recombinant FGF-2 protein and then applied to liver sections in parallel as the absorption test (lower panels). Representative pictures are shown. (b) Serum FGF-2 levels in chronic hepatitis patients (CH,  $N = 80$ ), liver cirrhosis patients (LC,  $N = 84$ ) and HCC patients ( $N = 112$ ) were evaluated by specific ELISA. All patients were HCV-RNA positive. Comparison of serum FGF-2 levels of each group. \*  $p < 0.05$ . (c) Serum FGF-2 levels were compared between before and after HCC development in six chronic liver disease patients. The mean follow-up period was nine years. \*  $p < 0.05$ . (d) The correlation of the FGF-2 level and HCC incidence was evaluated. 84 LC patients were divided into two groups according to serum FGF-2 levels; high (serum FGF-2 concentration  $> 1.8$  pg/ml; 40 patients, ●) and low ( $\leq 1.8$  pg/ml; 44 patients, ▲). We followed these LC patients for three years and compared the rate of HCC-free survival in these groups.



development of HCC in six chronic liver disease patients. The mean follow-up period was nine years. The serum FGF-2 levels of the patients before the occurrence of HCC were significantly higher than those of the same patients after the occurrence of HCC (Fig. 1c). These results demonstrated that the serum FGF-2 levels were highest in CH patients and significantly decreased as the liver disease progressed.

#### **FGF-2 levels were associated with the incidence of HCC in chronic liver disease patients**

The earlier results suggested that increased FGF-2 levels might prevent HCC tumor development. We investigated the correlation of the serum FGF-2 level and HCC incidence. The 84 LC patients were divided into two groups according to serum FGF-2 levels, high (serum FGF-2 concentration > 1.8 pg/ml; 40 patients) and low ( $\leq 1.8$  pg/ml; 44 patients), because the median of FGF-2 levels in these patients was 1.8 pg/ml. We followed these LC patients for three years and compared the rates of HCC-free survival. As shown in Figure 1d, the HCC free ratio of the high FGF-2 patients was significantly higher than that of the low FGF-2 patients. These results suggested that FGF-2 production from chronically diseased liver tissues might be associated with the occurrence of HCC.

#### **Inflammatory cytokines increased FGF-2 expression in HCC cells and normal hepatocytes**

Previous reports demonstrated that FGF-2 expressions were detected in both tumor cells and normal hepatocytes in addition to sinusoidal endothelial cells in HCC tissues.<sup>5</sup> Some inflammatory cytokines, such as IL-1 $\beta$ , IL-6, TGF- $\beta$  and TNF- $\alpha$ , are known to increase in CH patients.<sup>13-15</sup> To examine the effect of such inflammatory cytokines on FGF-2 expression in liver cells, we cultured HepG2 and PLC/PRF/5 HCC cells for 72 hr in the presence or absence of these cytokines. As shown in Figure 2a, IL-1 $\beta$  and IL-6 increased FGF-2 protein levels in both HepG2 and PLC/PRF/5 cells. FGF-2 mRNA levels in HepG2 and PLC/PRF/5 cells treated with IL-1 $\beta$  and IL-6 were significantly higher than those in nontreated control HCC cells (Fig. 2b). We also examined FGF-2 levels in the supernatants of the HCC cells cocultured with inflammatory cytokines. FGF-2 levels of IL-1 $\beta$ - or IL-6-treated HepG2 cells or PLC/PRF/5 cells tended to increase compared with those of nontreated HCC cells (data not shown). FGF-2 mRNA levels in normal hepatocytes treated with IL-1 $\beta$ , but not IL-6, were also significantly higher than those in nontreated control cells (Fig. 2c). These results suggested that both IL-1 $\beta$  and IL-6 were capable of inducing FGF-2 expression in HCC cells and normal hepatocytes. We also examined whether TGF- $\beta$ 1 and TNF- $\alpha$  could induce FGF-2 expressions on HCC cells. We found that FGF-2 expression levels in treated HCC cells did not change in Western blotting or real-time RT-PCR analysis (data not shown).

#### **FGF-2 induced the expression of membrane-bound MICA and suppressed the expression of HLA class I on HCC cells, but FGF-2 did not change the expressions of NKG2D and NKG2A on NK cell**

The above findings suggested that decreasing FGF-2 might affect the HCC development in the patients with chronic liver disease. To investigate whether or not FGF-2 protein directly activates NK cells, we examined whether FGF-2 affected the expression of NKG2D (activating receptor) or NKG2A (inhibitory receptor) on NK cells. We cultured CD56+ NK cells obtained from HVs with FGF-2 for 24 hr and then subjected them to flow cytometric analysis. The expressions of both NKG2D and NKG2A on NK cells did not change by adding FGF-2 protein (Fig. 3a), suggesting that FGF-2 did not have a direct effect on NK cells. We next examined the immunological modification of human HCC cells by adding human FGF-2 protein. We evaluated the expressions of membrane-bound MICA (NK activating molecule) and HLA class I (NK inhibitory molecule) in HepG2 and PLC/PRF/5 cells by flow cytometry. The expressions of MICA on FGF-2-treated cells were higher than those on nontreated cells in both HepG2 and PLC/PRF/5 cells (Fig. 3b). In contrast, those of HLA class I on FGF-2-treated cells were lower than those on nontreated cells in both types of HCC cells (Fig. 3b). FGF-2-treatment could modify the expressions of MICA and HLA class I on HCC cells in a dose-dependent manner (data not shown). The mRNA level of MICA in FGF-2-treated HepG2 cells was also significantly higher than that in nontreated HepG2 cells. The mRNA level of MICA in FGF-2-treated PLC/PRF/5 tended to be higher than that in nontreated cells, although the difference was not statistically significant (Fig. 3b). We examined the expressions of MICA and HLA class I on FGF-2-treated normal hepatocytes. The expressions of both molecules did not change in FGF-2-treated normal hepatocytes (Fig. 3c). We also evaluated FGF-2-dependent MICA regulation on a gastric cancer cell line (KATOIII), colon cancer cell lines (HCT116, HT29) and a cervical cancer cell line (Hela). The MICA expression was induced in FGF-2-treated HCT116 cells and weakly in FGF-2-treated Hela cells, but not in the other two cell lines (data not shown). These results suggested that FGF-2 could modify the MICA expressions in several types of cancers.

#### **The signal via FGF-2/FGF-receptor2 is essential for the induction of MICA and HLA class I expressions on HCC cells**

We examined the FGF receptors (FGFR1, FGFR2, and FGFR3) on both types of HCC cells by flow cytometry. The expressions of FGFR2 were high for both cell types. While FGF-2 has cross-reactivity with FGFR1 and FGFR3, the expressions of FGFR1 and FGFR3 were very low on both types of HCC cells (Fig. 4a). To examine whether the interaction between FGF-2 and FGFR2 could induce the expressions of MICA and HLA class I on both types of HCC cells, we evaluated the expressions of both molecules on FGF-2-treated

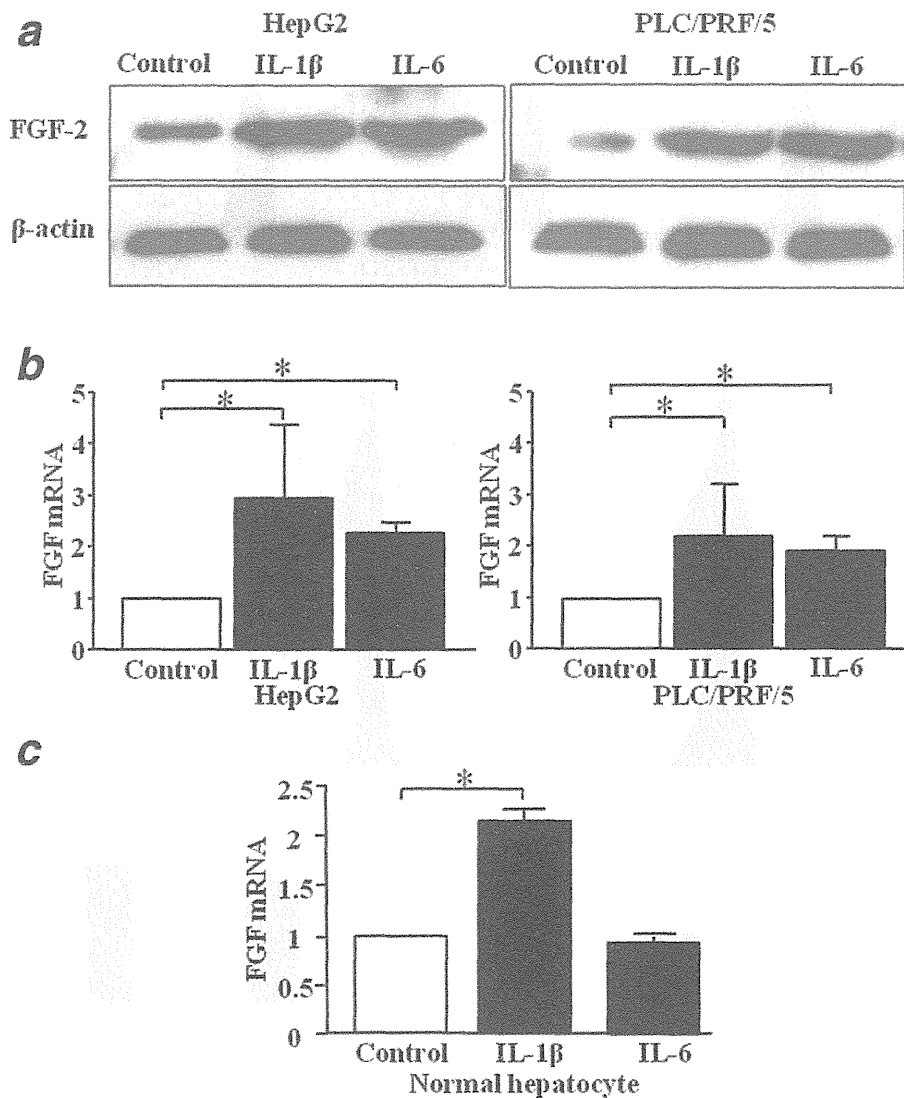


Figure 2. IL-1 $\beta$  and IL-6 increased FGF-2 expressions on human HCC cells and normal hepatocytes. To examine the effect of IL-1 $\beta$  and IL-6 on FGF-2 expression, HepG2 and PLC/PRF/5 cells (a,b) or normal hepatocytes (c) were cultured for 72 hr in the presence or absence of IL-1 $\beta$  (50 ng/ml) and IL-6 (300 ng/ml). FGF-2 expression in these cells was evaluated by Western blotting analysis (a) and real-time RT-PCR analysis (b,c). (a) The proteins were subjected to Western blot assay using each specific Ab. Upper panel is FGF-2 and lower panel is  $\beta$ -actin. (b,c) Total RNA was extracted and reverse transcribed. Relative copy numbers of FGF-2 were determined by real-time PCR analysis and normalized with  $\beta$ -actin expression. Results are expressed as mean  $\pm$ SD. Similar results were obtained in two independent experiments. \*  $p < 0.05$ .

HCC cells with anti-FGFR2 neutralizing Ab. The anti-FGFR2 Ab blocks the ability of FGF-2 to modulate MICA and HLA class I on both HepG2 and PLC/PRF/5 cells (Fig. 4b).

#### FGF-2 enhanced susceptibility to NK cells of HCC cells and the correlation of serum FGF-2 and soluble MICA levels in patients with chronic liver disease

The earlier results suggested that FGF-2 might enhance the susceptibility to NK cells of HCC cells. We next examined

whether FGF-2 could modify the NK sensitivity of human HCC cells. The cytolytic activities of NK cells against FGF-2-treated HepG2 and FGF-2-treated PLC/PRF/5 cells were higher than those against nontreated HCC cells (Fig. 5a). The cytolytic activity against FGF-2-treated HCC cells decreased to the control levels on addition of anti-MICA/B blocking antibody (Fig. 5a) but not on addition of isotype IgG antibody (Fig. 5b). These results demonstrated that adding FGF-2 enhanced the NK sensitivity of HCC cells *via*

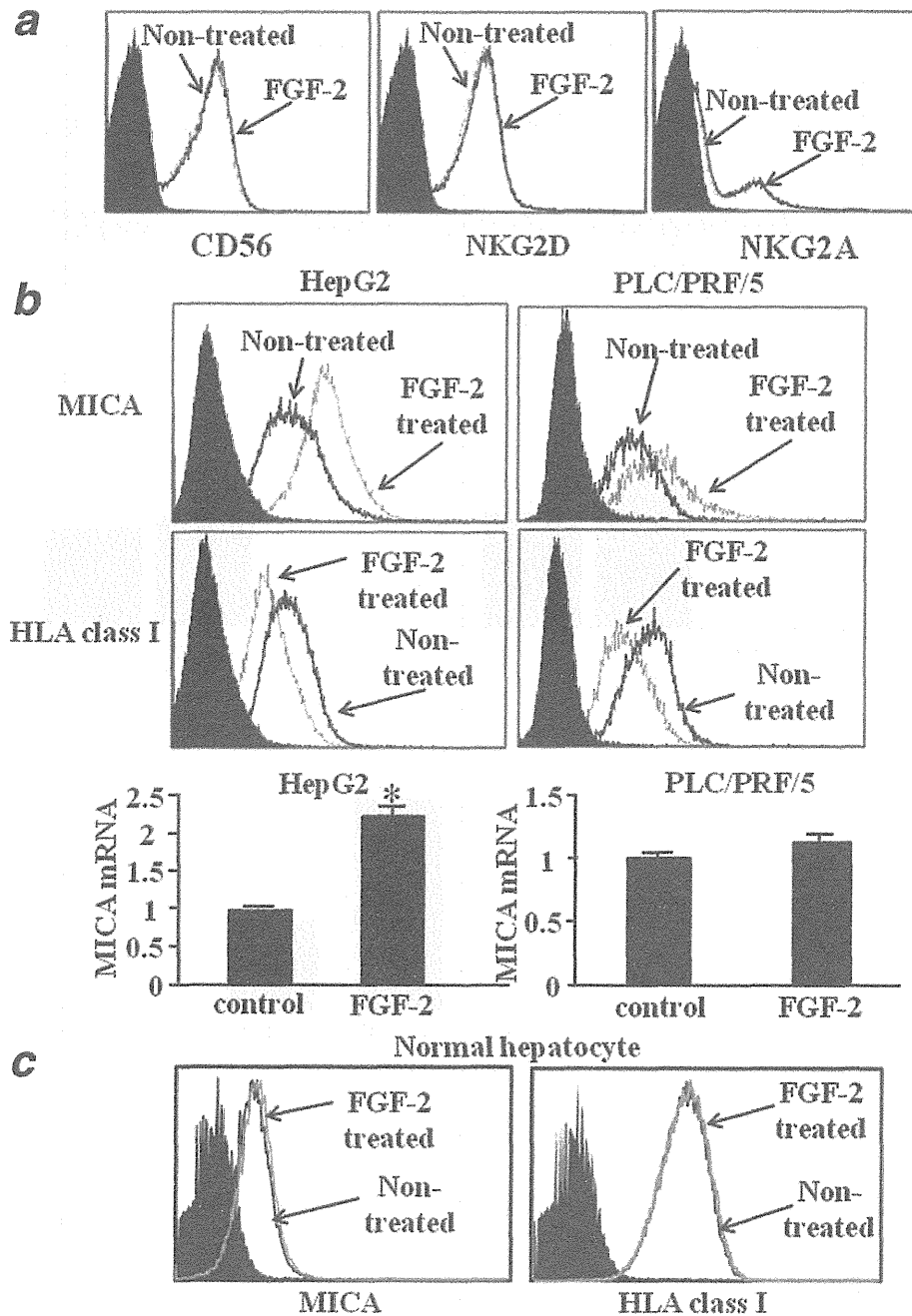
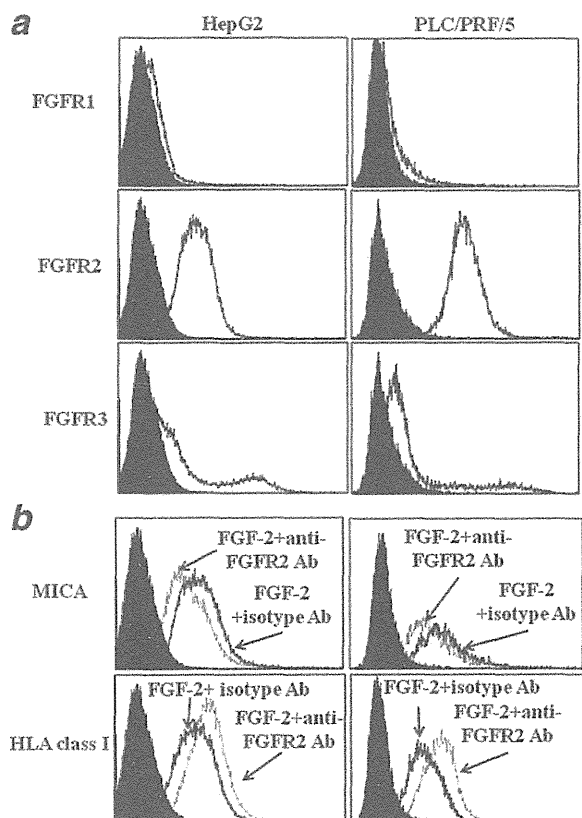
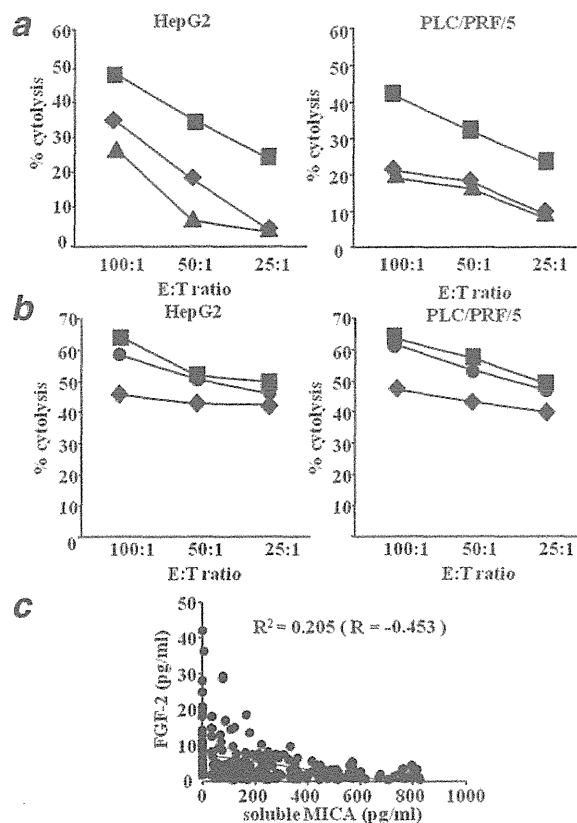


Figure 3. The expressions of NKG2D and NKG2A on FGF-2-treated NK cells and the expressions of MICA and HLA class I on FGF-2-treated hepatoma cells. (a) The expressions of NKG2D or NKG2A on FGF-2-treated or nontreated NK cells were evaluated. NK cells obtained from healthy volunteers ( $2 \times 10^6$  cells/well) were cultured with or without FGF-2 protein (250 ng/ml) for 24 hr, and the expressions of NKG2D and NKG2A on NK cells were evaluated by flow cytometry. Representative results were shown. (b,c) HCC cells (B: HepG2 and PLC/PRF/5) or normal hepatocytes (c) were treated with 250 ng/ml FGF-2 or control medium for 48 hr and subjected to flow cytometric analysis of MICA and HLA class I surface expression. Black line histograms: MICA or HLA class I staining of nontreated cells; gray line histograms: MICA or HLA class I staining of FGF-2-treated cells; shaded/black histograms: control IgG isotype Ab staining of each molecule. (b) Lower panel, mRNA levels of MICA in FGF-2-treated or nontreated HCC cells were examined by real-time PCR. Representative data are shown. Similar results were obtained from two independent experiments. \*  $p < 0.05$ .



**Figure 4.** The expressions of FGF receptors on hepatoma cells. (a) The expressions of FGF receptors (FGFR1, FGFR2, and FGFR3) on both HepG2 and PLC/PRF/5 cells were evaluated by flow cytometry. Black line histograms: staining of each FGF receptors (FGFR1, FGFR2, FGFR3), shaded/black histograms: control isotype Ab staining of each molecule. (b) To confirm that adding of FGF-2 protein resulted in modifying the expressions of MICA and HLA class I on both HCC cells, the expressions of both molecules on FGF-2- (250 ng/ml) treated HCC cells with anti-FGFR2 neutralizing Ab (10  $\mu$ g/ml) or isotype control Ab (murine isotype control IgG 10  $\mu$ g/ml) were evaluated by flow cytometry. FGF-2+anti-FGFR2 Ab, the expression of MICA or HLA class I on FGF-2-treated HCC cells with anti-FGFR2 neutralizing Ab. FGF-2+isotype Ab, the expression of MICA or HLA class I on FGF-2-treated HCC cells with isotype control Ab. shaded/black histograms: control isotype Ab staining of each molecule. Representative results were shown. Similar results were obtained in three independent experiments.

increased expression of membrane-bound MICA. We next examined the correlation of serum FGF-2 and soluble MICA in patients with chronic liver disease. Serum FGF-2 levels in patients with chronic liver disease correlated with soluble MICA levels (Fig. 5c). These results suggested that high FGF-2 levels in patients with chronic liver disease may prevent the shedding of MICA in liver tissues.



**Figure 5.** The cytolytic activity against FGF-2-treated HCC cells and the correlation between serum FGF-2 and soluble MICA in patients with chronic liver disease. (a, b) Both HepG2 and PLC/PRF/5 cells were cultured with or without FGF-2 protein (250 ng/ml) for 48 hr, and the cytolytic activities of NK cells against FGF-2-treated HepG2 and PLC/PRF/5 cells or nontreated HCC cells were evaluated by  $^{51}$ Cr-release assay. Nontreated HCC cells ( $\blacklozenge$ ) or FGF-2-treated HCC cells without ( $\blacksquare$ ) or with blocking Ab of MICA/B (6D4) (a,  $\blacktriangle$ ) or isotype IgG Ab (b,  $\bullet$ ). Representative results are shown. Similar results were obtained from three independent experiments. (c) Correlation of serum FGF-2 levels and soluble MICA levels in patients with chronic liver disease (chronic hepatitis patients,  $N = 80$ , liver cirrhosis patients,  $N = 84$  and HCC patients,  $N = 112$ ). The serum FGF-2 and soluble MICA were evaluated by specific ELISA respectively.

## Discussion

The FGF-2 levels in chronic liver disease, a premalignant condition, have not been well studied. Uematsu *et al.* reported that the serum FGF-2 levels of patients with LC or HCC were significantly higher than those of HVs, and serum FGF-2 levels of HCC patients tended to be lower than those of LC patients without HCC.<sup>6</sup> In contrast, Jinno *et al.* reported that the circulating FGF-2 levels in HCC patients were significantly higher than those in CH and LC patients.<sup>16</sup> In the present study, we analyzed the serum FGF-2 levels on

a larger scale for patients with chronic liver disease. Consistent with Uematsu's report, the serum FGF-2 levels significantly decreased along the progression of chronic liver disease and those in HCC patients were significantly lower than those in CH or LC patients. These results suggested that decreasing FGF-2 levels might be associated with the occurrence of HCC during the progression of chronic liver disease. FGF-2 has been shown to act as a potent angiogenic factor in a number of cell lines and solid tumors.<sup>1,2</sup> As for HCC development, FGF-2 has been reported to augment vascular endothelial growth factor (VEGF)-mediated angiogenesis in HCC development.<sup>17</sup> However, at present, in contrast to the clear roles of VEGF in the angiogenesis of HCC, the roles of FGF-2 in the HCC development are still controversial and should be elucidated.

Immunohistochemical analysis revealed that hepatocytes in patients with chronic liver diseases seemed to produce FGF-2, but those in healthy donors did not. This suggested that inflammatory responses in liver tissues might have roles in the production of FGF-2. Some inflammatory cytokines, such as IL-1 $\beta$  and IL-6, increased in CH or LC patients.<sup>13-15</sup> Aside from liver cells, IL-6 could induce FGF-2 expressions in basal cell carcinoma cell line<sup>18</sup> or Kaposi's sarcoma cell and human umbilical vein endothelial cells.<sup>19</sup> On the basis of these reports, we examined the effect of such inflammatory cytokines on FGF-2 expression in HCC cells and normal hepatocytes. The FGF-2 expression could be, at least in part, induced by IL-1 $\beta$  and IL-6. Both IL-1 $\beta$  and IL-6 are produced mainly by local immune cells, including activated Kupffer cells.<sup>20</sup> Although the detail mechanism of the induction of FGF-2 expression in HCC cells and normal hepatocytes is little known, the production of these cytokines might contribute to preventing HCC development *via* promoting FGF-2 expression in the liver.

Guerra *et al.* reported that NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy,<sup>11</sup> suggesting that NK-dependent immune-surveillance might play a critical role in tumor development. However, the mechanism of tumor surveillance of NK cells remains unclear in HCC development. We previously demonstrated that membrane-bound MICA on HCC cells plays essential roles in the NK sensitivity of HCC cells.<sup>21</sup> We therefore evaluated the MICA (activating molecule of NK cells) and HLA class I (inhibitory molecule of NK cells) on HCC cells treated with FGF-2. This treatment resulted in increasing MICA expression and decreasing HLA class I on HCC cells. Consistent with these results, the cytolytic activity of NK cells against FGF-2-treated HCC cells was higher than that against nontreated HCC cells. These results suggested that FGF-2 enhanced the NK sensitivity of HCC cells by upregulating MICA expression and downregulating HLA class I on the cellular surface. Interestingly, adding FGF-2 did not change the expressions of MICA and HLA class I on normal hepatocytes. These demonstrated that FGF-2 could enhance the NK sensitivity of HCC cells but not that of normal hepatocytes.

We also evaluated the expressions of MICA and HLA class I on other growth factors (such as VEGF or PDGF)-treated HCC cells. The expressions of MICA and HLA class I on VEGF- or PDGF-treated HCC cells were similar to those on nontreated HCC cells (Tsunematsu H, unpublished data). In this study, we demonstrated that FGF-2 production from liver tissues decreased along the progression of chronic liver disease. FGF-2 production from liver tissues might prevent the occurrence of HCC by eliminating HCC cell by enhancing NK sensitivity. If the innate immunity of the liver can be efficiently activated, preventing the occurrence of HCC could be expected. We previously demonstrated that anti-HCC chemotherapy and molecular targeted therapy using sorafenib resulted in enhancing NK sensitivity of HCC cells *via* upregulation of membrane-bound MICA on HCC cells.<sup>12,22</sup> These results suggested the possibility of new routes for chemoprevention of HCC, which could improve the prognosis of chronic liver disease patients. Also, on the basis of our results, FGF-2 supplementation therapy may be a rational approach for eliminating HCC cells in the chronic liver disease.

The concentration of FGF-2 in our *in vitro* study was high compared with the serum FGF-2 concentration level. Previous reports demonstrated that FGF-2 produced in the liver tissues acts in an autocrine or paracrine fashion.<sup>2,5</sup> We demonstrated that serum FGF-2 levels in chronic liver disease were significantly higher than those in HVs and that serum FGF-2 levels decrease with the progression of liver disease. These results suggested that FGF-2 production from liver tissues might also decrease with the progression of liver disease. Although the local FGF-2 concentration in the liver tissues still remains unknown and may differ from the serum FGF-2 concentration, our results have at least demonstrated that FGF-2 could enhance NK sensitivity of HCC cells *via* modification of the activating and inhibitory molecules on HCC cells.

The expression of NKG2D has been reported in all NK cells. However, this has also been reported in most NKT cells, subsets of  $\gamma\delta$  T cells and all human CD8+ T cells and a subset of CD4+ T cells.<sup>23</sup> In addition to NK cells, the MICA-NKG2D pathway plays roles in the costimulation or recognition of each cell. Our results demonstrated that FGF-2 might increase the membrane-bound MICA on HCC cells. It might be possible that the increased expression of MICA may also activate other lymphocytes expressing NKG2D and that these cells may also contribute to the elimination of HCC cells.

The earlier results suggested that FGF-2 levels might contribute to the eradication of HCC cells in liver tissues, which would prevent the incidence of HCC in chronic liver disease. Our patients' data demonstrated that HCC occurrence of the patients with high levels of FGF-2 was significantly lower than that with low levels of FGF-2, which is consistent with the results of NK sensitivity of FGF-2-treated HCC cells. Moreover, the FGF-2 levels in patients before HCC occurrence were significantly higher than those in the same

patients after HCC occurrence. The decreasing levels of serum FGF-2 may be a prediction factor for the occurrence of HCC in chronic liver disease.

Despite recent progress in understanding HCC development, unknown mechanisms remain. We have shown here that FGF-2 levels in chronic liver disease were significantly

higher than those in HVs, and serum FGF-2 levels decreases along the progression of liver disease. Importantly, FGF-2 enhances NK sensitivity of HCC cells *via* modification of the activating and inhibitory molecules on HCC cells. These findings suggested that FGF-2 might play roles in eliminating occurring HCC cells by innate immunity.

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## Association of enhanced activity of indoleamine 2,3-dioxygenase in dendritic cells with the induction of regulatory T cells in chronic hepatitis C infection

Koyo Higashitani · Tatsuya Kanto · Shoko Kuroda · Sachiyo Yoshio · Tokuhiko Matsubara · Naruyasu Kakita · Tsugiko Oze · Masanori Miyazaki · Mitsuru Sakakibara · Naoki Hiramatsu · Eiji Mita · Yasuharu Imai · Akinori Kasahara · Alato Okuno · Osamu Takikawa · Norio Hayashi · Tetsuo Takehara

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### Abstract

**Background** Altered functions of dendritic cells (DCs) and/or increases of regulatory T cells (Tregs) are involved in the pathogenesis of chronic hepatitis C virus (HCV) infection. A tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO), is reported to be an inducer of immune tolerance. Our aim was to clarify whether or not

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K. Higashitani · T. Kanto (✉) · S. Kuroda · S. Yoshio · T. Matsubara · N. Kakita · T. Oze · M. Miyazaki · N. Hiramatsu · T. Takehara  
Department of Gastroenterology and Hepatology,  
Osaka University Graduate School of Medicine,  
2-2 Yamadaoka, Suita, Osaka, Japan  
e-mail: kantot@gh.med.osaka-u.ac.jp

M. Sakakibara  
Osaka Medical Center for Cancer and Cardiovascular Diseases,  
1-3-3 Nakamichi, Higashinari, Osaka, Japan

E. Mita  
National Hospital Organization Osaka National Hospital,  
2-1-14 Hoenzaka, Chuo, Osaka, Japan

Y. Imai  
Ikeda Municipal Hospital, 3-1-18 Jonan, Ikeda, Osaka, Japan

A. Kasahara  
Department of General Medicine, Osaka University Hospital,  
2-15 Yamadaoka, Suita, Osaka, Japan

A. Okuno · O. Takikawa  
Laboratory of Radiation Safety, National Center for Geriatrics  
and Gerontology, 35 Gengo, Morioka, Obu, Aichi, Japan

N. Hayashi  
Kansai Rosai Hospital, 3-1-69 Amagasaki, Hyogo, Japan

IDO is activated in chronic hepatitis C patients and its role in immune responses.

**Methods** This study enrolled 176 patients with chronic HCV infection and 37 healthy volunteers. Serum kynurenine concentration was evaluated by high-performance liquid chromatography, and its correlation with clinical parameters was examined. Monocyte-derived DCs were prepared from the subjects and subsequently stimulated with a combination of lipopolysaccharide and interferon-gamma to induce functional IDO (defined as IDO-DCs). The phenotypes, kynurenine or cytokine production, and T-cell responses with IDO-DCs were compared between the patients and healthy volunteers.

**Results** The serum kynurenine level in the patients was significantly higher than that in the healthy volunteers, and the level of serum kynurenine was positively correlated with the histological activity or fibrosis score. IDO activity in IDO-DCs from the patients was significantly higher than that in IDO-DCs from the volunteers. Furthermore, IDO-DCs from the patients induced more Tregs in vitro compared with those from the volunteers, and the frequency of induced Tregs by IDO-DCs was decreased with an IDO-specific inhibitor.

**Conclusions** Systemic IDO activity is enhanced in chronic hepatitis C patients in correlation with the degree of liver inflammation and fibrosis. In response to inflammatory stimuli, DCs from the patients tend to induce Tregs, with some of this action being dependent on IDO.

**Keywords** Hepatitis C virus · Dendritic cell · Regulatory T cell · Indoleamine 2,3-dioxygenase

### Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. It is estimated that 170 million people



are chronically infected with HCV and are at risk of developing liver cirrhosis and/or hepatocellular carcinoma [1]. Approximately 70 % of those exposed to HCV progress to a chronically infected state [2]. The mechanisms of HCV leading to persistent infection have been ascribed to escape mutations of the HCV genome and insufficient immune responses to HCV in hosts, but the precise mechanisms are still largely unknown.

Dendritic cells (DCs) are key regulators of the immune system and are capable of promoting or suppressing T-cell responses depending on their environment [3, 4]. One of the crucial machineries of HCV-induced immune dysfunction is impaired abilities of DCs. Several research groups, including ours [5, 6] have demonstrated that DCs from chronically HCV-infected patients have lower ability to stimulate T cells and to drive T-helper 1 (Th1) polarization than those from healthy controls [7, 8]. Regulatory T cells (Tregs) are specialized suppressor cells that maintain immune tolerance against auto-reactive T cells or against pathogens [9]. In patients with chronic HCV infection, the frequency of Tregs in peripheral blood mononuclear cells (PBMCs) is higher than that in healthy individuals, suggesting the active roles of Tregs in immune alteration or alleviation of inflammation [10, 11]. However, the mechanisms of DC dysfunction or Treg expansion in chronic HCV infection have not been completely elucidated.

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyzes the initial and rate-limiting steps in the catabolism of the essential amino acid tryptophan (Trp), resulting in the generation of kynurenine (Kyn). IDO is widely expressed in human tissues [12] and cell subsets [13] and is induced during inflammation by interferon-gamma (IFN- $\gamma$ ) and/or other inflammatory cytokines [14–16]. Recent studies have demonstrated a crucial role of IDO in the induction of immune tolerance during infection, pregnancy, transplantation, autoimmunity, and cancers [17–21]. IDO expressed by DCs promotes immune tolerance by inhibiting T-cell activation and proliferation or by inducing Tregs through Trp starvation and/or the accumulation of Trp catabolites, such as Kyn, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid [22–25]. With respect to chronic HCV infection, a small-sized study showed that IDO expression was up-regulated in the liver and was associated with increased serum IDO activity [26]. However, the functions of IDO in immune cells in HCV infection still remain obscure.

In this study, we aimed to clarify whether or not IDO in DCs has a role in chronic HCV infection. We found that systemic IDO activity was enhanced in chronic hepatitis C patients. By comprehensively comparing the function of IDO-expressing DCs between the patients and healthy volunteers, we showed that IDO in DCs may be related to the induction of Tregs.

## Subjects, materials, and methods

### Subjects

This study enrolled 176 patients chronically infected with HCV serotype 1 (CHC group) who had been followed at Osaka University Hospital (Suita, Japan), National Hospital Organization Osaka National Hospital (Osaka, Japan), or Ikeda Municipal Hospital (Ikeda, Japan). All of them were confirmed to be positive for both serum anti-HCV antibody and HCV-RNA but were negative for other viral infections, including hepatitis B virus (HBV) and human immunodeficiency virus. The presence of other liver diseases, such as alcoholic, metabolic, or autoimmune hepatitis was ruled out, and the presence of liver cirrhosis and hepatocellular carcinoma was excluded by the use of laboratory and imaging analyses. As controls, we examined 37 healthy volunteers (HV group), working as medical staff at Osaka University Hospital, who were negative for HCV and HBV markers. As disease controls, 13 patients with chronic HBV infection followed at National Hospital Organization Osaka National Hospital were also enrolled. They were positive for hepatitis B surface (HBs) antigen and had abnormal levels of alanine aminotransferase (ALT). The characteristics of the group were: male/female 10/3, hepatitis B envelope (HBe) antigen-positive/HBe antigen-negative 6/7, mean age  $43.9 \pm 15.0$  years, mean serum ALT level  $218.7 \pm 282.5$  IU/L, and mean HBV-DNA level [assayed by the COBAS AmpliPrep<sup>TM</sup>/COBAS TaqMan<sup>TM</sup> HBV test (Roche, Branchburg, NJ, USA)]  $6.1 \pm 2.3$  Log copies/mL. At enrollment, written informed consent was obtained from each subject. The study protocol was approved by the ethics committee of each institution.

In this study, because of the limitations of sampling from multiple centers, the conditions for blood collection and preservation differed among the facilities. Thus, for the precise comparison of IDO activity between the patients and healthy volunteers, firstly, we examined the samples collected and preserved under the same conditions at Osaka University Hospital (Cohort I, Table 1). Secondly, because liver biopsy was not carried out in Cohort I patients, we used another cohort (Cohort II, Table 1) for our analysis of the correlation between IDO activity and clinical parameters. Cohort II consisted of the remaining 127 patients, whose samples were collected at National Hospital Organization Osaka National Hospital or Ikeda Municipal Hospital. Histological examination was performed according to the METAVIR scoring system. The clinical backgrounds of the patients in Cohorts I and II, except for HCV-RNA quantity, were not different.



**Table 1** Clinical backgrounds of subjects

	HV (Cohort I)	CHC (Cohort I)	CHC (Cohort II)
<i>N</i>	37	49	127
Male/female	20/17	24/25	58/69
Age (years) <sup>a</sup>	44.3 ± 14.6 <sup>b</sup>	57.8 ± 12.6	56.5 ± 10.9
ALT (IU/L) <sup>a</sup>	ND	55.8 ± 39.9	64.6 ± 47.9
Plts (×10 <sup>4</sup> /μL) <sup>a</sup>	ND	16.8 ± 6.4	17.3 ± 6.1
HCV-RNA <sup>c</sup> (Log copies/mL) <sup>a</sup>	ND	6.1 ± 1.0	6.6 ± 0.6 <sup>b</sup>
METAVIR activity (A0/1/2/3)	ND	ND	10/78/35/4
METAVIR fibrosis (F0/1/2/3/4)	ND	ND	0/70/29/21/7

CHC chronic hepatitis C patients, HV healthy volunteers, ALT alanine aminotransferase, Plts platelets, ND not determined

<sup>a</sup> Values are expressed as means ± SD

<sup>b</sup> Statistical significance was analyzed by the Mann–Whitney *U*-test (*P* < 0.05), compared with CHC group (Cohort I)

<sup>c</sup> Serum HCV-RNA titer was quantitated using the COBAS AmpliPrep<sup>TM</sup>/COBAS TaqMan<sup>TM</sup> HCV test (Roche)

### Reagents and antibodies

Recombinant human interleukin-4 (IL-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF) were purchased from PeproTech (Rocky Hill, NJ, USA). Recombinant human IFN- $\gamma$  was purchased from R&D Systems (Minneapolis, MN, USA). Lipopolysaccharide (LPS) from *Escherichia coli*, L-tryptophan, L-kynurenine, and 1-methyl-L-tryptophan (1-MT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein monoclonal antibodies (mAbs) against human CD4 (clone, SK3), CD11c (B-ly6), CD25 (M-A251), CD40 (5C3), CD80 (L307.4), CD86 (IT2.2), CD127 (HIL-7R-M21), CD274/PD-L1 (MIH1), HLA-DR (L243), Foxp3 (259D/C7), and isotype control Abs were purchased from BD Biosciences (San Jose, CA, USA).

### Generation of CD14+ monocyte-derived dendritic cells

Monocyte-derived DCs (MoDCs) were generated from CD14+ cells as reported previously [27]. In brief, CD14+ cells were cultured for 7 days at 37 °C and 5 % CO<sub>2</sub> in DC culture medium [Iscove's modified Dulbecco's medium (IMDM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 10 % fetal calf serum, 50 IU/mL of penicillin, 50 mg/mL of streptomycin, 2 mM of L-glutamine, 10 mM of Hepes buffer, and 10 mM of nonessential amino acids] in the presence of 20 ng/mL of IL-4 and 50 ng/mL of GM-CSF. On day 5 of the culture, cells were stimulated with 50 ng/mL of LPS and/or 50 ng/mL of IFN- $\gamma$  to induce functional IDO, and cultured for 48 h. On day 7, cells were harvested and subjected to phenotypic and functional analysis. At the same time, the supernatant of the culture was also collected and subjected to cytokine assays. As controls, unstimulated MoDCs were also prepared.

### Flow cytometric analysis

For the analysis of cell surface markers, cells were stained as reported previously [27]. In this study, Tregs were defined as CD4+CD25+CD127-Foxp3+ cells, the frequency of which in PBMCs was analyzed as reported previously [11]. Flow cytometric analyses were performed with the use of a FACSCantoII flow cytometer (BD Biosciences). Analyses of data were done with FACSDiva 6.1 software (BD Biosciences).

### Analysis of IDO activity by high-performance liquid chromatography (HPLC)

For the measurement of Kyn and Trp, the HPLC analysis was performed according to the procedure developed by Takikawa et al. [28]. As an index of IDO activity in vivo, the serum kynurenine-to-tryptophan ratio (KTR) was determined by HPLC [26, 29], after deproteinization by the addition of one-tenth volume 2.4 M perchloric acid and centrifugation at 20000×*g* for 10 min. To assay the functional IDO in MoDCs in vitro, the cells were harvested on day 7 of the culture, washed, and resuspended in Hanks' balanced salt solution (HBSS; Gibco Laboratories) containing 100 μM L-Trp. The cells were incubated for an additional 24 h, and Kyn in the culture supernatants was determined by HPLC. IDO activity in vitro was expressed as the concentration of Kyn (μM) in the supernatant, converted from 100 μM L-Trp by IDO.

### T-cell stimulation and cytokine analyses

Naive CD4+ T cells were isolated from the allogeneic healthy volunteer using a Naive CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. After 7 days of the culture, the

graded numbers of IDO-DCs (MoDCs stimulated with LPS and IFN- $\gamma$  for 48 h) were co-cultured with  $1 \times 10^5$  naive CD4<sup>+</sup> T cells in DC culture medium for 4 days. An IDO-specific inhibitor, 1-MT, was used to confirm the specificity of the IDO activity in the T-cell responses. On day 0 of the co-culture, 1-MT was added to IDO-DCs and T-cell cultures at a final concentration of 1 mM. On day 4, half of the supernatants were collected to assess the Th1/Th2 polarization, which was done by measuring the various cytokines. Next, WST-8 reagent in the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) was added to the cultures, followed by incubation for 4 h. The T-cell proliferation index was measured at the absorbance 450 nm of reduced WST-8 using the plate reader. Assays were performed in triplicate wells.

#### Cytokine bead assay

To analyze the cytokine secretion of IDO-DCs and of naive CD4<sup>+</sup> T cells primed with IDO-DCs, the concentrations of IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IFN- $\gamma$ , or tumor necrosis factor-alpha (TNF- $\alpha$ ) in the supernatants were assayed using the Cytometric Bead Array System (BD Biosciences) according to the manufacturer's instructions.

#### Treg induction

To assess the potential effects of IDO on Treg induction from naive CD4<sup>+</sup> T cells, the cells were primed with allogeneic IDO-DCs at a 10:1 ratio in HBSS containing 100  $\mu$ M L-Trp. After 7 days, the primed T cells were harvested and assessed for their surface phenotype and intracellular Foxp3 expression. Phenotyping of the cells after the co-culture was performed using anti-CD4-PerCP, anti-CD25-APC, and anti-CD127-PE. To exclude dead lymphocytes after the co-culture, Near-IR LIVE/DEAD Fixable Dead Cell Stain (Invitrogen, Carlsbad, CA, USA) was used, according to the manufacturer's instructions. Next, the cells were fixed, permeabilized, and stained with anti-Foxp3-Alexa Fluor 488, using the Human FoxP3 Buffer Set (BD Biosciences) according to the manufacturer's instructions. The frequency of CD4<sup>+</sup>CD25<sup>+</sup>CD127-Foxp3<sup>+</sup> Tregs generated from each priming culture condition was determined by flow cytometry. As described above, 1 mM of 1-MT was added on day 0 to test for IDO-dependent effects.

#### Statistical analysis

The values were analyzed by nonparametric tests—the Mann-Whitney *U*-test, the Wilcoxon signed rank test, or Spearman's rank correlation test—or by linear regression analysis, using GraphPad Prism software, version 5.04

(Graph Pad Software, San Diego, CA, USA). A *P* value of <0.05 was considered to be statistically significant.

## Results

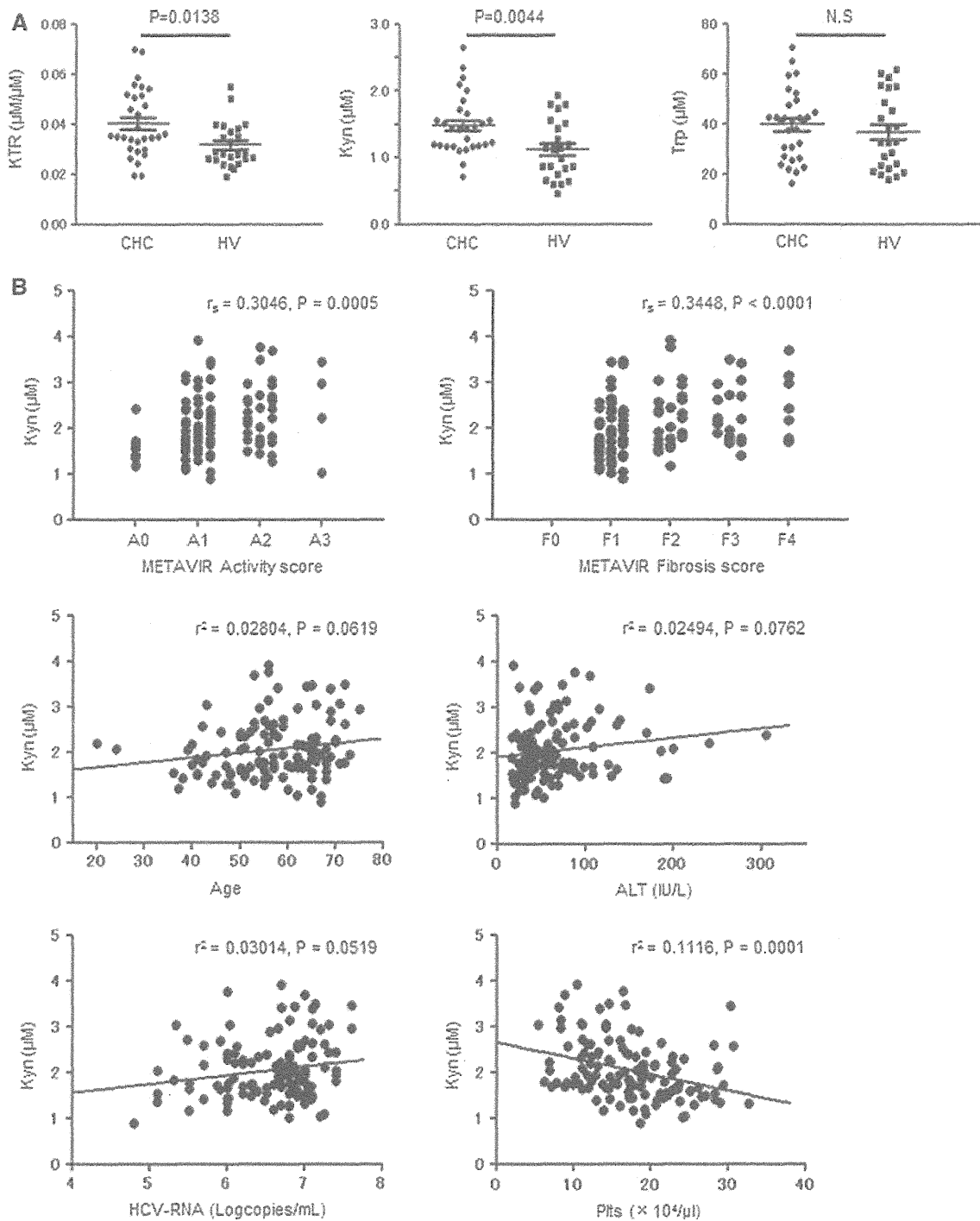
### Systemic IDO activity is enhanced in chronic hepatitis C patients

To examine whether or not IDO activity is up-regulated in chronically HCV-infected patients, we compared the serum Kyn and Trp levels between the groups in Cohort I. The serum KTR was significantly higher in the CHC group than that in the HV group (Fig. 1a). Furthermore, we found that the concentration of Kyn in the CHC group was significantly higher than that in the HV group, whereas the levels of Trp were comparable in the two groups (Fig. 1a). These results show that the KTR level in serum, as a surrogate for systemic IDO activity, was higher in chronic hepatitis C patients than in uninfected controls. Furthermore, as the KTR and Kyn levels were correlated (data not shown), the serum Kyn level can be regarded as a surrogate marker for systemic IDO activity.

Next, in order to examine whether or not the enhanced systemic IDO activity was specific for chronically HCV-infected patients, we compared serum Kyn concentrations among chronic hepatitis B patients, chronic hepatitis C patients (Cohort II), and healthy subjects. The serum Kyn concentration in chronic hepatitis B patients was significantly higher than those in the healthy subjects and the patients with chronic hepatitis C (chronic hepatitis B patients:  $2.42 \pm 0.11 \mu$ M, healthy subjects,  $1.12 \pm 0.09 \mu$ M, chronic hepatitis C patients in Cohort II:  $2.04 \pm 0.06 \mu$ M), suggesting that systemic IDO activity is enhanced in chronic HBV infection as well.

### Systemic IDO activity correlates with activity grade and fibrosis stage in the liver

Next, to investigate the underlying mechanisms of enhanced IDO activity in chronically HCV-infected patients, we assessed whether or not serum Kyn levels in Cohort II were correlated with various clinical parameters and the METAVIR scores. A significant positive correlation was observed between serum Kyn levels and the histological activity or fibrosis scores (Fig. 1b). However, there was no correlation between the Kyn level and age, ALT level, or HCV-RNA quantity (Fig. 1b). These results show that the more advanced the inflammation and fibrosis of the liver, the higher the serum Kyn, and vice versa. The inverse correlation between serum Kyn and platelet counts was consistent with the correlation between Kyn and the fibrosis score (Fig. 1b).



**Fig. 1** Systemic indoleamine 2,3-dioxygenase (IDO) activity is enhanced in chronic hepatitis C patients. **a** Serum kynurenine (*Kyn*) and tryptophan (*Trp*) were assayed by HPLC as described in “Subjects, materials, and methods”, and the kynurenine-to-tryptophan ratio (*KTR*) was calculated from their concentrations. Scatter plots of 30 chronic hepatitis C patients (*CHC*) and 24 healthy volunteers (*HV*) are shown. Horizontal bars depict mean  $\pm$  SEM. Statistical analyses were performed using the nonparametric Mann–

Whitney *U*-test. **b** Correlation analyses were performed between the serum *Kyn* concentration and histological scores in the liver, and clinical parameters (age, alanine aminotransferase [*ALT*], hepatitis C virus [*HCV*]-RNA titers, and platelet counts [*Plts*]) in 127 chronic hepatitis C patients. Spearman’s correlation or simple linear regression analyses were performed.  $r_s$  Spearman’s correlation coefficient,  $r^2$  linear regression coefficient. *N.S* not significant

### Lipopolysaccharide and IFN- $\gamma$ induce functional IDO in DCs

DCs have been reported to be the most prominent IDO inducer in blood cells in response to inflammatory stimuli [13]. We first assayed the IDO activity (i.e., production of Kyn) of unstimulated MoDCs from chronic hepatitis C patients and found that they did not induce functional IDO (Fig. 2a). In order to simulate the inflammatory condition of DCs in vivo, we examined whether or not IDO was inducible in MoDCs with different combinations of cytokines for various incubation times. In this context, we examined the IDO activity of MoDCs stimulated with LPS alone, IFN- $\gamma$  alone, or LPS plus IFN- $\gamma$  for 48 h. The Kyn concentration in media from MoDCs stimulated with LPS alone did not differ from that in unstimulated MoDCs, whereas Kyn concentrations in media from MoDCs stimulated with IFN- $\gamma$  alone or LPS plus IFN- $\gamma$  were elevated (Fig. 2a). These results show that the combination of LPS and IFN- $\gamma$  for 48 h significantly induces functional IDO in MoDCs. Therefore, in the following experiments, we used a combination of LPS and IFN- $\gamma$  to induce functional IDO.

### DCs from chronic hepatitis C patients induce more IDO in response to LPS and IFN- $\gamma$ than those from healthy volunteers

First, we compared the phenotype of IDO-DCs and unstimulated MoDCs from each group. The expressions of CD40, CD80, CD86, HLA-DR, and CD274/PD-L1 on IDO-DCs were significantly up-regulated compared with those on unstimulated MoDCs, and their expression levels were not different between the CHC and HV groups (Fig. 2b).

Next, we examined the concentration of Kyn in the culture supernatants. In the CHC group, Kyn levels from MoDC culture were significantly enhanced by the stimulation with LPS and IFN- $\gamma$  (Fig. 2c). Moreover, the Kyn levels in the IDO-DC culture from the CHC group were significantly higher than those in the HV group, whereas those in unstimulated MoDCs did not differ between the groups (Fig. 2c). This increase of Kyn was blocked by the addition of 1-MT, showing that the production of Kyn is specifically dependent on IDO activity (Fig. 2c). These results show that IDO activity is enhanced more in DCs from chronic hepatitis C patients than in DCs from healthy subjects.

Finally, we compared the ability of IDO-DCs to produce various cytokines. The levels of IL-6, IL-10, IL-12p70, and TNF- $\alpha$  from IDO-DCs were not different between the hepatitis C patients and healthy controls (Fig. 2d).

**Fig. 2** Enhanced induction of IDO in dendritic cells (DCs) from chronic hepatitis C patients in response to a combination of lipopolysaccharide (LPS) and interferon- $\gamma$  (IFN- $\gamma$ ). **a** The levels of Kyn in the culture supernatants of monocyte-derived DCs (MoDCs) in the presence of LPS (50 ng/mL) and/or IFN- $\gamma$  (50 ng/mL) were determined by HPLC, as described in "Subjects, materials, and methods". The results are expressed as the mean  $\pm$  SEM from 4 chronic hepatitis C patients. \* $P$  < 0.05 by nonparametric Wilcoxon signed rank test. **Controls**, unstimulated MoDCs. **b** Phenotype analysis of IDO-DCs was performed as described in "Subjects, materials, and methods". The values are expressed as mean fluorescence intensity (MFI). The MFI of each marker is represented as the mean  $\pm$  SEM from 9 patients and 7 healthy volunteers. \* $P$  < 0.05 by nonparametric Wilcoxon signed rank test. **IDO-DCs**, MoDCs stimulated with LPS and IFN- $\gamma$  for 48 h. **c** The levels of Kyn in the culture supernatants were assayed by HPLC as described in "Subjects, materials, and methods". The samples were obtained from MoDCs in the presence (IDO-DCs) or absence (controls) of a combination of LPS and IFN- $\gamma$ . In parallel, the same experiments were performed in the presence or the absence of 1 mM of 1-methyl-L-tryptophan (1-MT). The results are expressed as the mean  $\pm$  SEM from 12 chronic hepatitis C patients and 10 healthy controls. \* $P$  < 0.05 by Wilcoxon signed rank test, \*\* $P$  < 0.05 by Mann-Whitney  $U$ -test. **d** The levels of cytokines in the culture supernatants from IDO-DCs were assayed with the Cytometric Bead Array System, as described in "Subjects, materials, and methods". **Bars** depict the mean concentration of each cytokine  $\pm$  SEM from 10 healthy volunteers and 10 chronic hepatitis C patients. **IL** interleukin, **TNF- $\alpha$**  tumor necrosis factor- $\alpha$

### IDO is not involved in allogeneic T-cell proliferation and Th1/Th2 differentiation with DCs from chronic hepatitis C patients

With regard to the allogeneic CD4+ T-cell response, IDO-DCs from the CHC group tended to have a lower stimulatory capacity than those from the HV group (Fig. 3a). To examine whether this phenomenon was dependent on IDO activity, we compared T-cell proliferation with IDO-DCs in the presence and absence of 1-MT. The CD4+ T-cell responses with IDO-DCs were not restored by the addition of 1-MT, regardless of HCV infection (Fig. 3a).

In order to examine whether functional IDO in DCs is involved in Th1/Th2 differentiation, we quantified cytokines in the supernatants obtained from the co-culture of IDO-DCs and CD4+ T cells. In samples from chronic hepatitis C patients, the levels of Th1 cytokines (IL-2, IFN- $\gamma$ ) and Th2 cytokines (IL-4, IL-10, IL-13) tended to be higher than the levels in samples from healthy volunteers, though the difference was not significant. The levels of all cytokines, except for IL-4, tended to decrease with the addition of 1-MT (Fig. 3b). Thus, IDO in DCs is not actively involved in Th1/Th2 differentiation.

### IDO is involved in the induction of regulatory T cells

We examined whether or not IDO in DCs was involved in the generation of Tregs. With IDO-DCs from the CHC