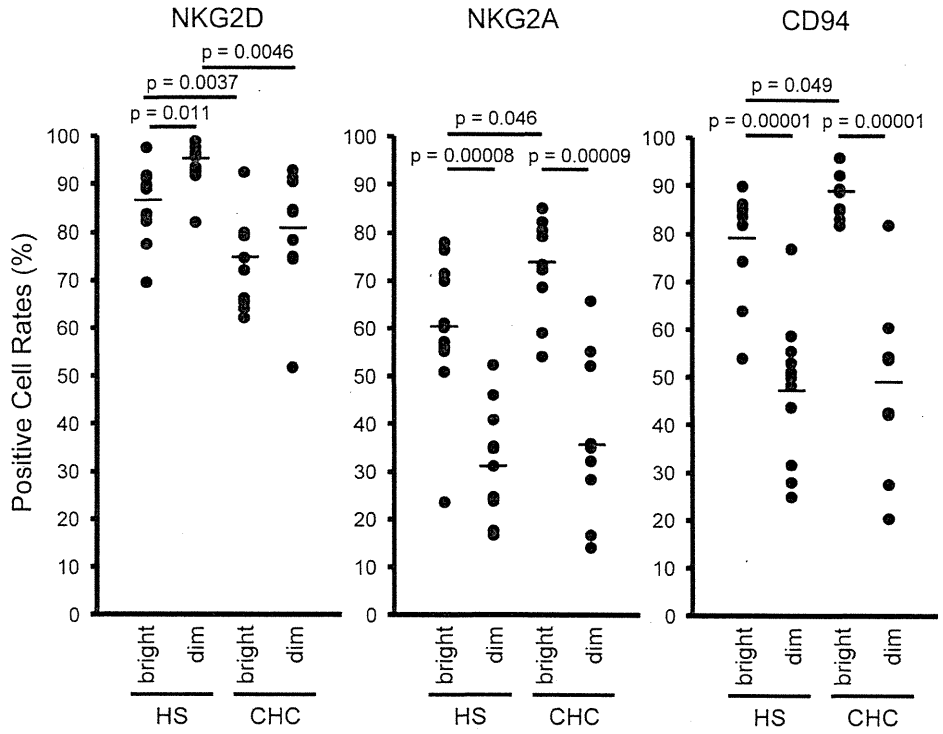

Fig. 2 NK receptor expression on NK cell subsets. The expression of NK activating or inhibitory receptors, NKG2D or NKG2A and CD94, respectively, on $CD56^{bright}$ NK cell subset (bright) and $CD56^{dim}$ NK cell subset (dim) was evaluated by flow cytometry with isotype control staining, electronically gating on CD56 bright $CD3^-$ cells and CD56 dim $CD3^-$ cells. PBMCs were derived from patients with chronic HCV infection (CHC) ($n = 9$) and healthy subjects (HS) ($n = 11$). Positive cells (positive cell rate) were determined based on isotype control staining. Comparisons of those NK receptor expression levels between bright and dim subsets in a subject group or between CHC and HS in a subset are shown as positive cell rates with the statistically significant p values. Each *circle* represents data for an individual. *Horizontal bars* represent means. Statistical significance was analyzed using the unpaired Student's t-test

the phosphorylation level of STAT4 occurring in response to IFN-α in the CHC patients was significantly lower than that in the HS in both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells (Fig. 4b). On the other hand, the phosphorylation level of STAT1 occurring in response to IFN-α in the CHC patients was significantly greater than that in the HS in both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells.

We next examined the relationship between STAT1 phosphorylation and STAT4 phosphorylation occurring in response to IFN-α in the NK cell subsets. Upon stimulation with IFN-α, the whole population of $CD56^{bright}$ NK cells phosphorylated both STAT1 and STAT4, while some of the $CD56^{dim}$ NK cells more strongly phosphorylated STAT1 but more weakly phosphorylated STAT4, compared with the remaining $CD56^{dim}$ NK cells, which more weakly phosphorylated STAT1 but more strongly phosphorylated STAT4 (Fig. 5a). Moreover, the frequency of the 'high-pSTAT1 population' in response to IFN-α in $CD56^{dim}$ NK cells in the CHC patient group was significantly greater than that in the HS group (Fig. 5a, b).

Regulation of NK receptor expression level on $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells occurring in response to IFN-α-based therapy in vivo

To examine whether $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells would respond differently to IFN-α treatment in vivo, we evaluated the frequency, the expression level of NK receptors, and the STAT1 expression level in $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells before and after the initiation of IFN-α-based therapy. The frequency of $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells did not show any significant change between before and 1 day after initiation of the therapy (data not shown). The expression levels of NKG2A/CD94 on both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells were significantly decreased in response to the therapy 1 day after its initiation (Fig. 6). On the other hand, the expression level of NKG2D on $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells did not show any significant change between before and 1 day after initiation of the therapy. The STAT1 expression levels in both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells were significantly increased in response to the therapy (data not shown).

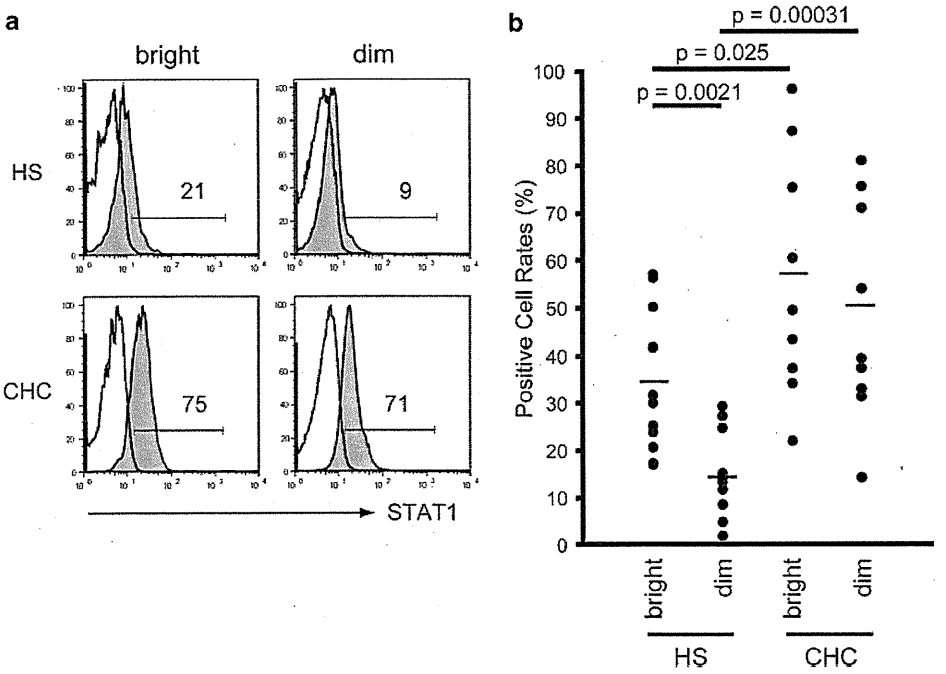

Fig. 3 Signal transducer and activator of transcription 1 (*STAT1*) expression in NK cell subsets. Intracellular STAT1 expression levels in $CD56^{bright}$ NK cell subset (bright) and $CD56^{dim}$ NK cell subset (dim) were evaluated by flow cytometry with isotype control staining, electronically gating on CD56 bright $CD3^-$ cells and CD56 dim $CD3^-$ cells. PBMCs were derived from patients with chronic HCV infection (CHC) ($n = 9$) and healthy subjects (HS) ($n = 11$). **a** Representative histograms from a patient and a healthy subject (*HS*) are shown. *Dotted lines* show staining with the isotype control. *Thick lines* with *shaded areas* show staining with the antibody. Numbers are percentages of positive cells (positive cell rate) determined based on isotype control staining. **b** Comparisons of STAT1 expression level between bright and dim subsets in a subject group or between CHC and HS in a subset are shown as positive cell rates with the statistically significant p values. Each *circle* represents data for an individual. *Horizontal bars* represent means. Statistical significance was analyzed using the unpaired Student's t-test

Discussion

In the present study, we found clear differences between $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells in their responses to cytokines, as well as the cell frequency and the surface expression level of the NK receptors. We also found some differences between these subsets in the alteration caused by chronic HCV infection. Of interest and novelty are the findings that the NK cell subsets displayed different intracellular STAT1 expression levels (Fig. 3) and responded differently to cytokine stimulation to lead to differences in the phosphorylation of STAT1/4 (Figs. 4, 5) and that some of the differences were altered in the CHC patients. Furthermore, both subsets showed alterations of IFN-α signaling in the CHC patients, compared with the HS (Fig. 4).

We have recently shown that NK cells from patients with CHC display a higher level of STAT1 expression than those from HS [24] and suggested that the up-regulation of STAT1 expression might result from a host response to HCV infection with IFN-α and/or IFN-γ production, because STAT1 itself is one of the IFN-stimulated genes (ISGs) whose expression is up-regulated by IFN-α or IFN-γ [27, 28], which has been reported to be detected in the sera of patients with CHC [29, 30]. The present study has shown that both NK cell subsets from the patients with CHC displayed a higher level of STAT1 expression than those from the HS (Fig. 3b); this might also have been induced similarly in both subsets by a host response to HCV infection. Since a host response to HCV infection would be associated with the liver inflammation and subsequent fibrosis, we examined whether the STAT1 expression level in these NK cell subsets could be correlated with the level of liver inflammation or fibrosis which had been histologically evaluated using liver biopsy samples. Although no significant correlation was observed between the STAT1 expression level in the $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells and the level of liver inflammation or fibrosis, there was a tendency of a higher level of inflammation or fibrosis being correlated with a higher level of STAT1 expression in NK cells, including $CD56^{bright}$ and $CD56^{dim}$ subsets, in our limited number of patients (T. Miyagi et al. unpublished data); further investigation should be done with a larger number of subjects. Another question that emerged was whether our findings in peripheral blood could be applied to the liver in CHC patients. Chen et al. [31]

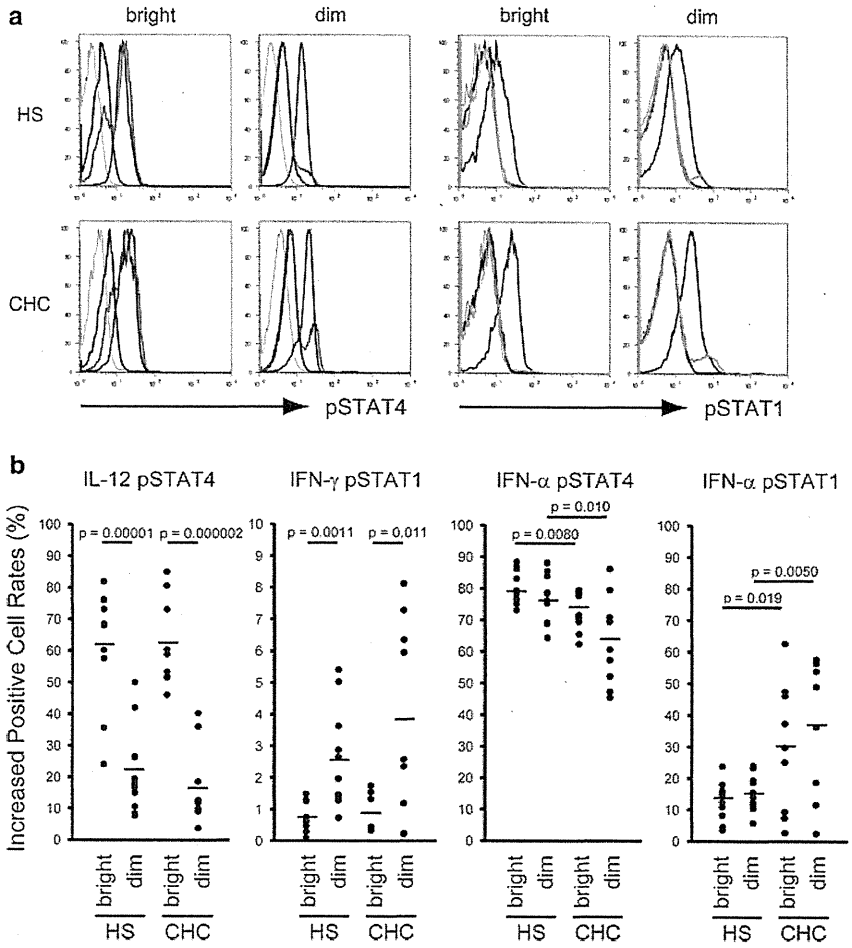

Fig. 4 Activation of STAT1/4 occurring in response to interleukin-12 (*IL-12*), interferon-γ (*IFN-γ*), or IFN-α in NK cell subsets. Phosphorylated STAT1 (*pSTAT1*) and pSTAT4 protein levels were evaluated by flow cytometry with isotype control staining. PBMCs were derived from patients with chronic HCV infection (CHC) ($n = 9$) and healthy subjects (HS) ($n = 11$). Prepared PBMCs were unstimulated or stimulated with natural IFN-α, IFN-γ, or IL-12 for 90 min in vitro, and then collected. pSTAT1 and pSTAT4 protein levels in $CD56^{bright}$ NK (bright) and $CD56^{dim}$ NK (dim) cell subsets were evaluated by flow cytometry, electronically gating on CD56 bright $CD3^-$ cells and CD56 dim $CD3^-$ cells. **a** Representative histograms of a patient and a healthy subject (*HS*) are shown. *Green lines* show staining of IFN-α-stimulated cells with isotype control. *Purple lines* show staining of unstimulated cells with the antibody. *Red, orange,* and *blue lines* show staining of IL-12-, IFN-γ- and IFN-α-stimulated cells, respectively, with the antibody. **b** Positive cell rates were determined based on staining with isotype controls. Increased positive cell rates were determined by subtracting the positive cell rate of unstimulated cells from those of stimulated cells. Comparisons of pSTAT1/4 level in response to IFN-α, IFN-γ, or IL-12 between bright and dim subsets in a subject group or between CHC and HS in a subset are shown as increased pSTAT1/4 positive cell rate with the statistically significant p values. Each *circle* represents individual data. *Horizontal bars* represent means. Statistical significance was analyzed using the unpaired Student's t-test

reported that the hepatic gene expression level in a subset of ISGs, including STAT1, was greater in CHC patients than in normal subjects. Sarasin-Filipowicz et al. [32] showed that the gene expression level in a subset of ISGs in CHC patients was greater in whole liver, including hepatocytes and nonparenchymal cells such as lymphocytes, than in PBMC, and suggested that chronic HCV infection had stronger local effects on the IFN system in the liver than in PBMC. Also, Tateno et al. [33] showed that the gene expression level of STAT1 in liver-infiltrating lymphocytes was about twofold greater than that in hepatocytes in CHC patients. Considering these reports, we speculate that the NK cell subsets in the liver as well as in the peripheral blood of CHC patients might display a high level of STAT1 expression. Whether our findings in peripheral blood could be applied to the liver in CHC patients requires further investigation. We also examined whether our findings with CHC patients would be observed in CHB patients. Unlike in the CHC patients, the CHB patient expression levels of STAT1 in either $CD56^{bright}$ or $CD56^{dim}$ subsets was not significantly higher than that in the HS, which would be consistent with the report of the

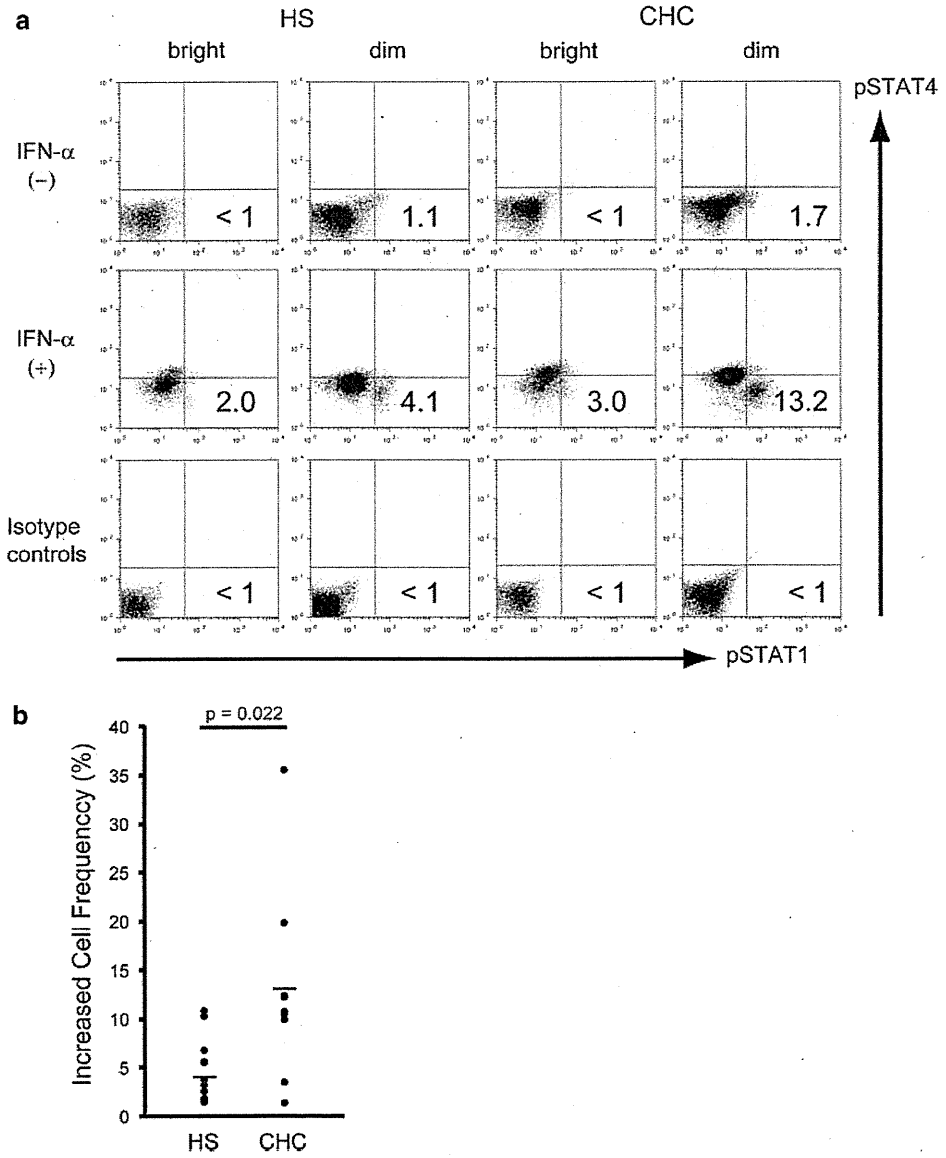

Fig. 5 Relationship between STAT1/4 phosphorylation occurring in response to IFN-α in NK cell subsets. pSTAT1 and pSTAT4 protein levels were simultaneously evaluated by flow cytometry with isotype control staining. PBMCs were derived from patients with chronic HCV infection (CHC) ($n = 9$) and healthy subjects (HS) ($n = 11$). Prepared PBMCs were unstimulated or stimulated with natural IFN-α for 90 min in vitro, and then collected. pSTAT1 and pSTAT4 protein levels in $CD56^{bright}$ NK (bright) and $CD56^{dim}$ NK (dim) cell subsets were evaluated by flow cytometry, electronically gating on CD56 bright $CD3^-$ cells and CD56 dim $CD3^-$ cells. **a** Representative dot plots of untreated or IFN-α treated cells stained with antibody or isotype controls from a patient and a healthy subject (*HS*) are shown. *Numbers* are frequencies of gated cells that strongly phosphorylated STAT1 but weakly phosphorylated STAT4 in the corresponding subsets. **b** Increased cell frequency was determined by subtracting the gated cell frequency of unstimulated cells from those of stimulated cells. Comparisons of the increased cell frequency of the high-pSTAT1 population in response to IFN-α stimulation in the $CD56^{dim}$ NK cell subset between CHC and HS are shown with the statistically significant p value. Each *circle* represents individual data. *Horizontal bars* represent means. Statistical significance was analyzed using the unpaired Student's t-test

STAT1 signaling pathway being less activated in CHB than in CHC [34].

Lines of evidence have shown that $CD56^{dim}$ NK cells, but not $CD56^{bright}$ NK cells, decrease in number in peripheral blood in patients with CHC [12, 14, 15, 35]. In agreement with these reports, we observed a lower frequency of $CD56^{dim}$ NK cells, but not of $CD56^{bright}$ NK cells, in the CHC patients than in the HS (Fig. 1b). Although we observed significant up-regulation of STAT1 expression in both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells, the magnitude of the up-regulation of STAT1 expression in the CHC patients, compared with that in the

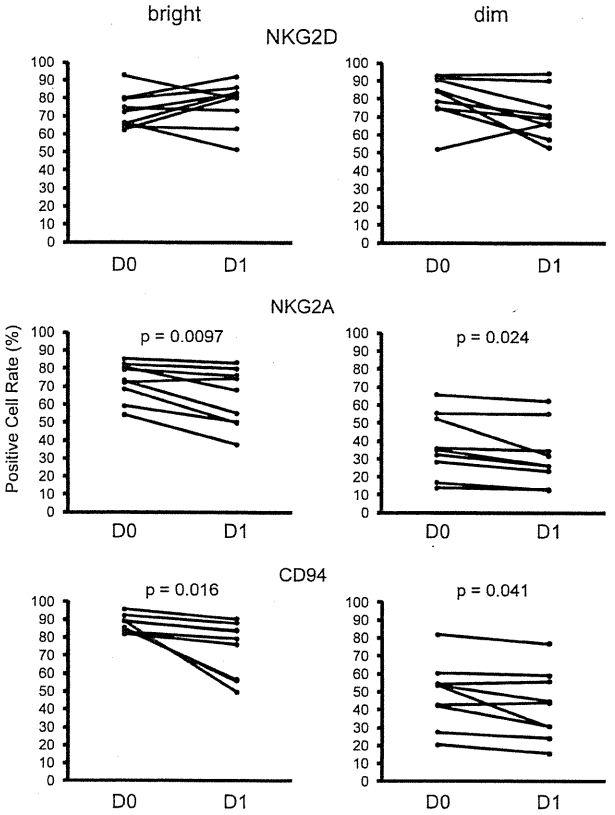

Fig. 6 Regulation of NK receptor expression in response to IFN-α-based therapy. The expression of NK activating or inhibitory receptors, NKG2D or NKG2A and CD94, respectively, on $CD56^{bright}$ NK (bright) and $CD56^{dim}$ NK (dim) cell subsets was evaluated by flow cytometry with isotype control staining, electronically gating on CD56 bright $CD3^-$ cells and CD56 dim $CD3^-$ cells. PBMCs were derived from patients treated with IFN-α-based therapy ($n = 9$) before (*D0*) and 1 day after (*D1*) the initiation of the therapy. Positive cells (positive cell rate) were determined based on isotype control staining. The changes in the NK receptor expression levels between D0 and D1 are shown as positive cell rates with the statistically significant p values. Each *circle* represents individual data. Statistical significance was analyzed using the paired Student's t-test

HS, was clearly greater in $CD56^{dim}$ NK cells than in $CD56^{bright}$ NK cells (Fig. 3b). Considering that STAT1 transmits the anti-proliferative effects induced by IFN-α [36–38], the greater up-regulation of STAT1 in $CD56^{dim}$ NK cells, compared with that of $CD56^{bright}$ NK cells, might have resulted in the significantly reduced frequency in $CD56^{dim}$ NK cells but not in $CD56^{bright}$ NK cells. Further study is required to examine this.

The most prolific producer of IFN-γ is the $CD56^{bright}$ NK cell rather than the $CD56^{dim}$ NK cell [4, 5]. In the present study, we found that $CD56^{bright}$ NK cells responded to IL-12 to phosphorylate STAT4 much more than $CD56^{dim}$ NK cells (Fig. 4a, b). IL-12 is one of the strongest stimulators of IFN-γ production from NK cells, which is transmitted by STAT4 phosphorylation [1, 3, 39, 40]. The preferential activation of STAT4 in $CD56^{bright}$ NK cells, compared with $CD56^{dim}$ NK cells, might be one of the underlying mechanisms by which $CD56^{bright}$ NK cells, compared with $CD56^{dim}$ NK cells, are armed to produce IFN-γ. On the other hand, we found that $CD56^{dim}$ NK cells responded to IFN-γ to phosphorylate STAT1, while $CD56^{bright}$ NK cells hardly did so (Fig. 4a, b). Moreover, some of the $CD56^{dim}$ NK cells responded to IFN-α to more strongly phosphorylate STAT1 than $CD56^{bright}$ NK cells (Fig. 5a). The $CD56^{dim}$ NK cells are strongly cytolytic armed effector cells [4, 5]. IFN-α or IFN-γ is one of the strongest inducers of the cytolytic function of NK cells, which is transmitted by STAT1 phosphorylation [1, 3, 38, 41]. Thus, the predominant activation of STAT1 in $CD56^{dim}$ NK cells, compared with $CD56^{bright}$ NK cells, might be one of the underlying mechanisms by which $CD56^{dim}$ NK cells become armed with a strong cytotoxic function. The differences in cytokine response to activate STAT molecules between these NK cell subsets might lead to the differences in their armed functions, such as cytolysis and cytokine production.

Ahlenstiel et al. [42] have recently reported that chronic exposure to HCV-induced IFN-α rendered NK cells with a functional polarization toward a cytotoxic phenotype, but without an increase in IFN-γ production. Moreover, Oliviero et al. [16] showed that NK cells from CHC patients were of a predominantly activating phenotype and that these phenotypic changes were associated with enhanced cytotoxic activity and defective IFN-γ production. These reports may be associated with our finding that NK cells, including $CD56^{bright}$ and $CD56^{dim}$ subsets, from the CHC patients displayed a high level of STAT1 expression (Fig. 3). Cytotoxic molecules such as perforin and granzyme, as well as STAT1, are among the ISGs [28, 41]. A high level of STAT1 in NK cells, particularly in $CD56^{dim}$ NK cells that are armed with a cytolytic function, in CHC patients might correspond to a high level of cytotoxic molecules in NK cells, resulting in enhanced cytotoxic activity. Indeed, the frequency of the population that strongly phosphorylated STAT1 upon IFN-α stimulation in $CD56^{dim}$ NK cells was significantly higher in the CHC patients than in the HS (Fig. 5b). This population might be highly armed cells with a cytolytic function. On the other hand, it has been reported that the STAT1 expression level in NK cells was correlated negatively with the activation of STAT4 to produce IFN-γ in response to IFN-α in NK cells [22, 24]. A high level of STAT1 in NK cells, particularly in $CD56^{bright}$ NK cells that are armed to produce IFN-γ, might cause defective IFN-γ production in the NK cells of patients with CHC.

Recent studies have demonstrated that a higher level of ISGs in hepatocytes as well as in PBMCs before IFN-α-based therapy is associated with resistance to this therapy

[32, 43]. We have also reported that a small number of CHC patients treated with IFN-α-based therapy revealed a tendency, in those who had a higher level of STAT1 (which is one of the ISGs) in the total NK cell population, to not respond well to the therapy in the early phase, such as in week 8 after its initiation [24]. In the present study, we did not observe a significant correlation between the STAT1 expression level in the NK cell subsets and the sensitivity to IFN-α based therapy, but we did find a tendency of those who had a higher level of STAT1 in the NK cell subsets to not respond well to the therapy in the early phase, such as in week 8 after its initiation (T. Miyagi et al. unpublished data). The number of evaluated patients, however, was small. More data on treated patients will be required to accurately evaluate the relationship between the STAT1 expression level in the NK cell subsets and the therapy outcome.

We have recently reported that NKG2D expression on NK cells could be down-regulated by the soluble major histocompatibility complex class I-related chain A (MICA), which was increased in patients with CHC compared with healthy controls [44]. In the present study, NKG2D expression levels on both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells from the CHC patients were significantly lower than those from the HS (Fig. 2). Thus, the lower NKG2D expression on either $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells in patients with CHC might be caused by the increased soluble MICA. In response to IFN-α treatment in vivo, the expression of NKG2A/CD94 was down-regulated in both subsets in the CHC patients. In vitro stimulation of NK cells with IFN-α did not down-regulate or up-regulate the messenger RNA expression of NKG2A/CD94 in NK cells (T. Miyagi et al. unpublished data). Thus, the lower expression of NKG2A/CD94 might be modulated not directly but indirectly by in vivo IFN-α treatment.

In the present study, we investigated how the NK cell subsets differed in frequency, phenotype, and cytokine response, and also how chronic HCV infection modified these differences. $CD56^{bright}$ NK cells had a relatively higher level of intracellular STAT1 expression than $CD56^{dim}$ NK cells in the HS. Both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells from the CHC patients displayed remarkably higher levels of STAT1 expression than those from the HS, without any significant differences between these subsets. Upon in vitro stimulation with cytokines such as IL-12, IFN-γ, and IFN-α, $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells phosphorylated STAT1/4 differently. These differences between the NK cell subsets in frequency, phenotype, and cytokine response were partly altered in the CHC patients, suggesting their possible association with the persistence of HCV infection and the resistance to IFN-α based therapy. These observations suggest the possibility of cellular or molecular targets for the treatment of chronic HCV infection.

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

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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-1β 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.[1] 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).[2–4] Aside from its angiogenic effect, FGF-2 has also been shown to act as a mitogen for HCC cell proliferation *via* an autocrine mechanism.[5] 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.[6] 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.[7,8] The liver contains a large compartment of innate immune cells (NK cells and NKT cells) and acquired immune cells (T cells),[9,10] 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.[11] 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

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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_2 and 37°C.

ELISA

The sera from CH patients ($n = 80$), LC patients ($n = 84$), HCC patients ($n = 112$, StageI/II $n = 51$ and StageIII/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 hepatocyts 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-1β (IL-1β) (50 ng/ml, Peprotech, Rocky Hill, NJ), human IL-6 (300 ng/ml, Peprotech), human transforming growth factor-β1 (TGF-β1) (50 ng/ml, R&D Systems) and human tumor necrosis factor-α (TNF-α) (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 μg/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′)2 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 β-actin (Hs:99999903_m1) mRNAs according to the manufacturer's instructions. β-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).[12] The cytolytic ability of NK cells against FGF-2-treated HepG2 and PLC/PRF/5 cells was assessed by 4-hr ^{51}Cr-release assay with or without antihuman MICA/B Ab (BD Biosciences) as previously described.[12] 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. 1*a*). 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 1*b*, 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 StageI/II patients and III/IV patients was not observed (data not shown). We compared the serum FGF-2 levels before and after the

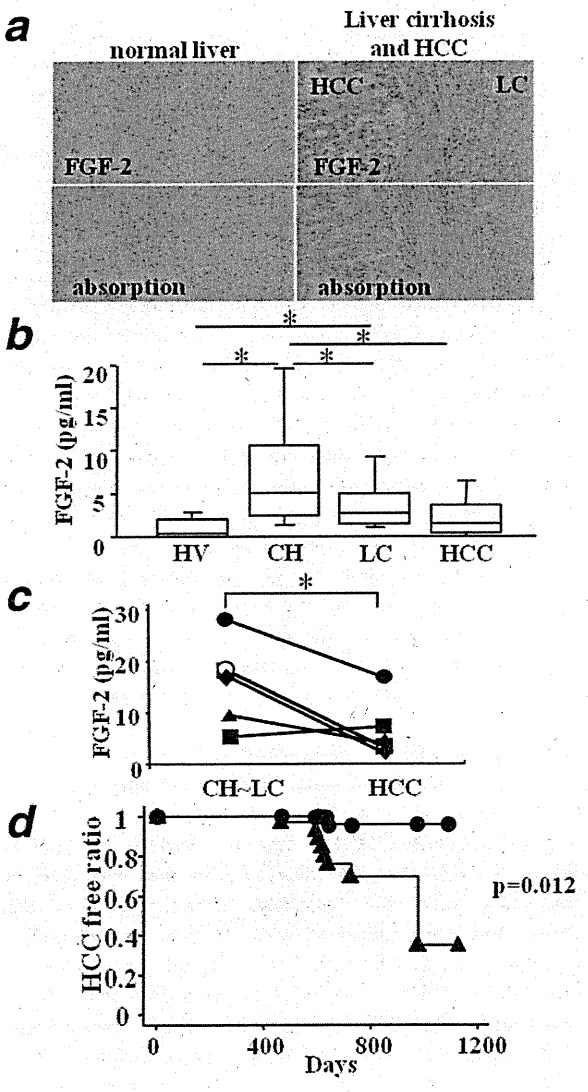

Figure 1. Expressions of FGF-2 in the liver of patients with chronic liver diseases and serum FGF-2 levels in chronic liver disease patients were associated with HCC incidence. (*a*) Immunohistochemical analysis of FGF-2 in normal liver tissues ($N = 2$), chronic hepatitis tissues ($N = 4$), liver cirrhosis (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 (≦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. 1*c*). 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 (≦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 1*d*, 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.[5] Some inflammatory cytokines, such as IL-1β, IL-6, TGF-β and TNF-α, are known to increase in CH patients.[13–15] 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 2*a*, IL-1β 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-1β and IL-6 were significantly higher than those in nontreated control HCC cells (Fig. 2*b*). We also examined FGF-2 levels in the supernatants of the HCC cells cocultured with inflammatory cytokines. FGF-2 levels of IL-1β- 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-1β, but not IL-6, were also significantly higher than those in nontreated control cells (Fig. 2*c*). These results suggested that both IL-1β and IL-6 were capable of inducing FGF-2 expression in HCC cells and normal hepatocytes. We also examined whether TGF-β1 and TNF-α 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. 3*a*), 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. 3*b*). 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. 3*b*). 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. 3*b*). 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. 3*c*). 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. 4*a*). 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

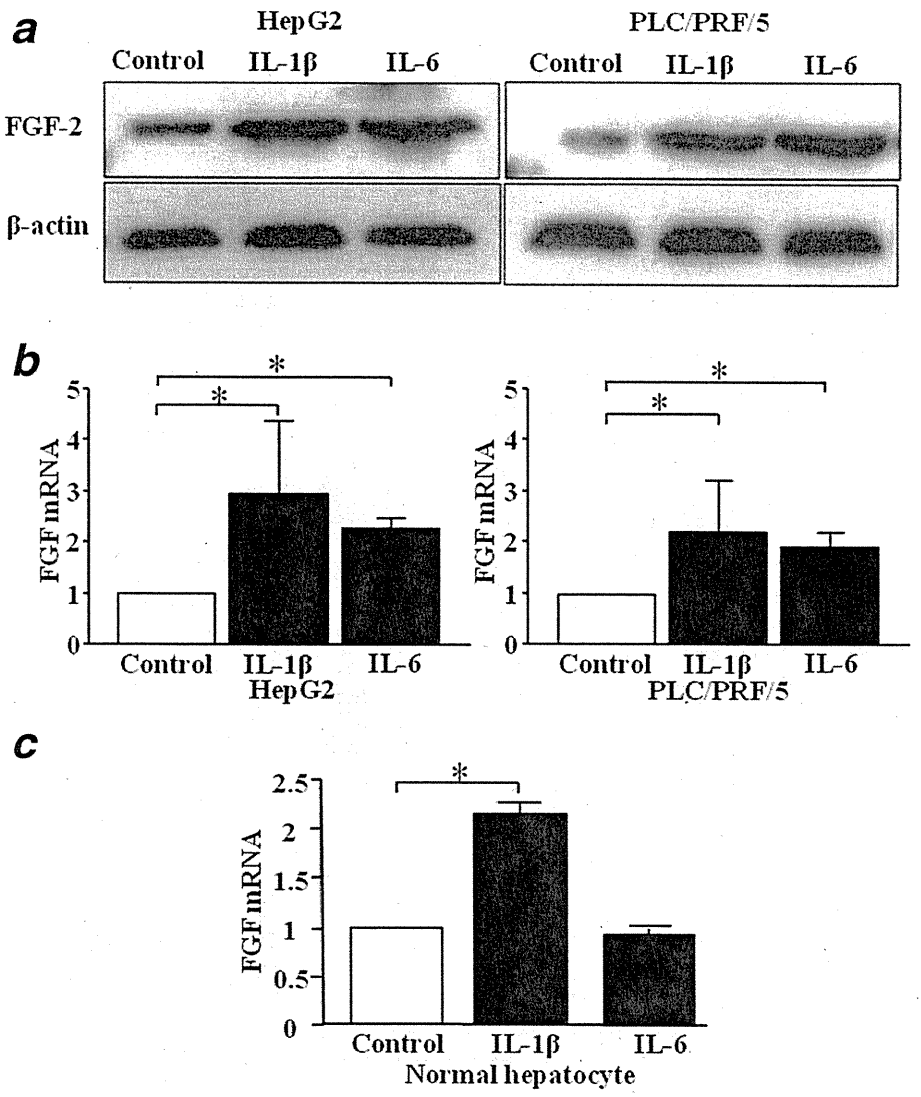

Figure 2. IL-1β and IL-6 increased FGF-2 expressions on human HCC cells and normal hepatocytes. To examine the effect of IL-1β and IL-6 on FGF-2 expression, HepG2 and PLC/PRF/5 cells (*a,b*) or normal hepatocytes (*c*) were cultured for 72 hr in the presence or absence of IL-1β (50 ng/ml) and IL-6 (300 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 β-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 β-actin expression. Results are expressed as mean ±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. 4*b*).

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. 5*a*). The cytolytic activity against FGF-2-treated HCC cells decreased to the control levels on addition of anti-MICA/B blocking antibody (Fig. 5*a*) but not on addition of isotype IgG antibody (Fig. 5*b*). These results demonstrated that adding FGF-2 enhanced the NK sensitivity of HCC cells *via*

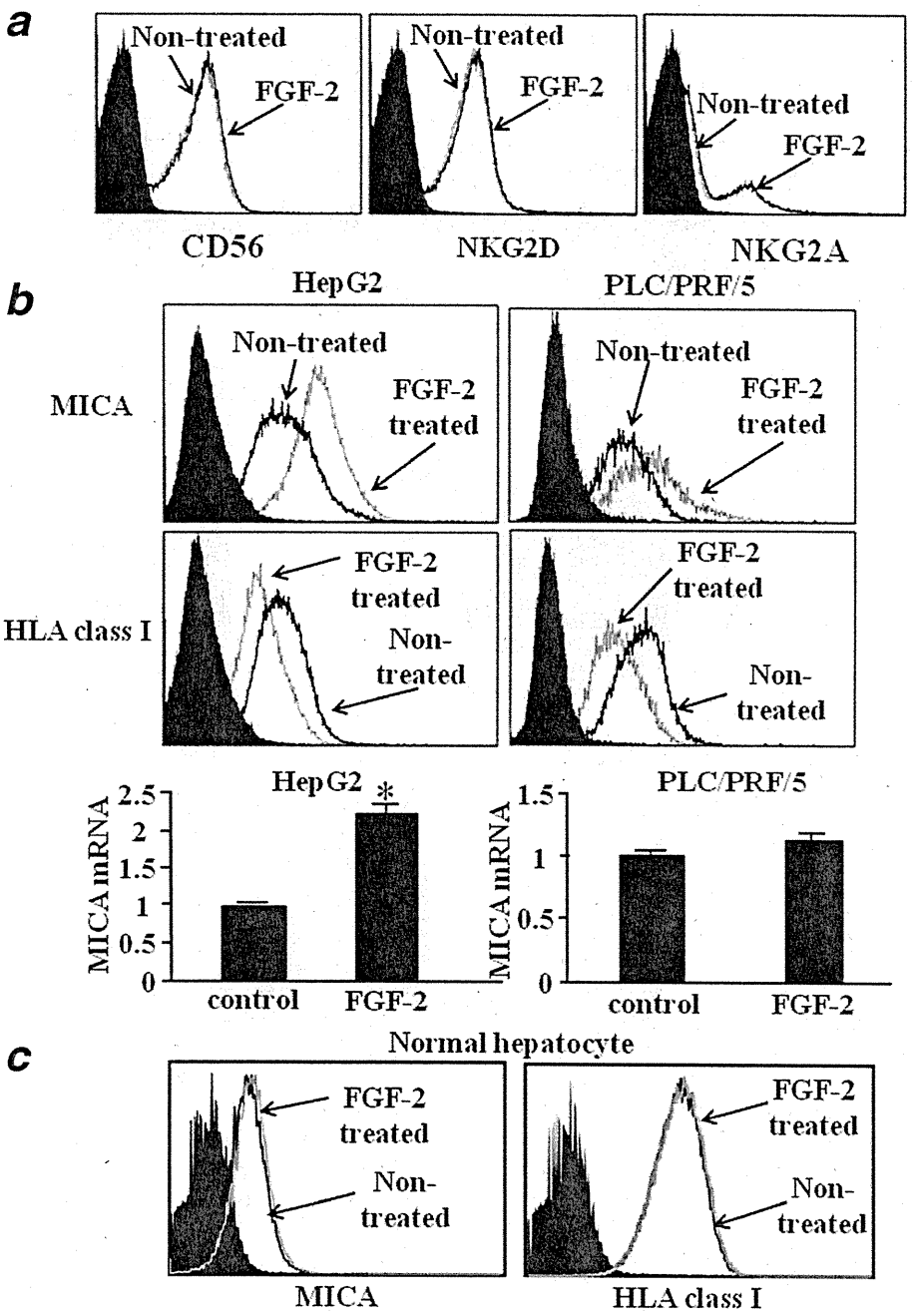

Figure 3. The expressions of NKG2D and NKG2A on FGF-2-treated NK cells and the expressions of MICA and HLA class I on FGF-2-treated hepatoma cells. (*a*) The expressions of NKG2D or NKG2A on FGF-2-treated or nontreated NK cells were evaluated. NK cells obtained from healthy volunteers (2×10^6 cells/well) 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. * $p < 0.05$.

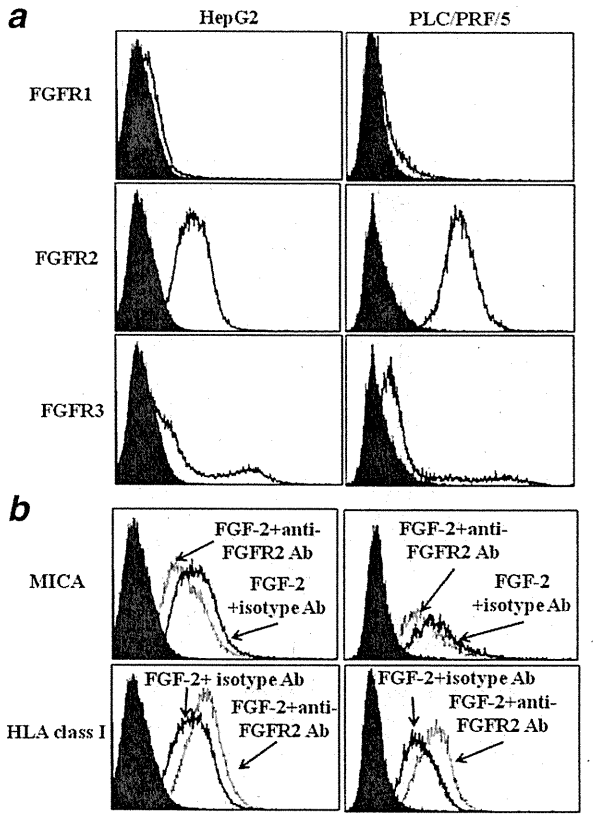

Figure 4. The expressions of FGF receptors on hepatoma cells. (*a*) The expressions of FGF receptors (FGFR1, FGFR2, and FGFR3) on both HepG2 and PLC/PRF/5 cells were evaluated by flow cytometry. Black line histograms: staining of each FGF receptors (FGFR1, FGFR2, FGFR3), shaded/black histograms: control 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 μg/ml) or isotype control Ab (murine isotype control IgG 10 μg/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.

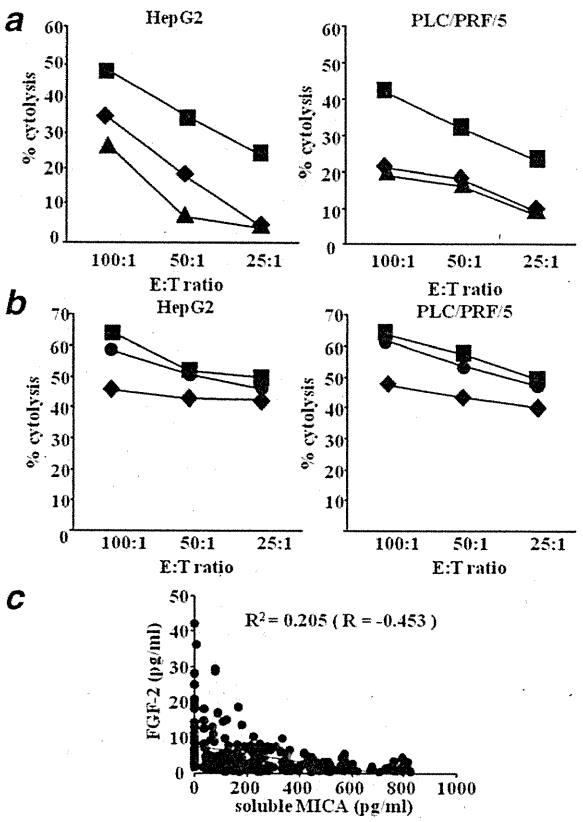

Figure 5. The cytolytic activity against FGF-2-treated HCC cells and the correlation between serum FGF-2 and soluble MICA in patients with chronic liver disease. (*a,b*) Both HepG2 and PLC/PRF/5 cells were cultured with or without FGF-2 protein (250 ng/ml) for 48 hr, and the cytolytic activities of NK 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 (◆) or FGF-2-treated HCC cells without (■) or with blocking Ab of MICA/B (6D4) (*a*, ▲) or isotype IgG Ab (*b*, ●). 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.

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. 5*c*). These results suggested that high FGF-2 levels in patients with chronic liver disease may prevent the shedding of MICA in liver tissues.

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.[6] 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.[16] 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.[1,2] As for HCC development, FGF-2 has been reported to augment vascular endothelial growth factor (VEGF)-mediated angiogenesis in HCC development.[17] 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-1β and IL-6, increased in CH or LC patients.[13–15] Aside from liver cells, IL-6 could induce FGF-2 expressions in basal cell carcinoma cell line[18] or Kaposi's sarcoma cell and human umbilical vein endothelial cells.[19] 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-1β and IL-6. Both IL-1β and IL-6 are produced mainly by local immune cells, including activated Kupffer cells.[20] 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,[11] 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.[21] 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.[12,22] 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.[2,5] 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 γδ T cells and all human CD8+ T cells and a subset of CD4+ T cells.[23] 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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Highly sensitive lens culinaris agglutinin-reactive α-fetoprotein is useful for early detection of hepatocellular carcinoma in patients with chronic liver disease

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Abstract. The fucosylated fraction of α-fetoprotein (AFP-L3) is a specific marker for hepatocellular carcinoma (HCC). However, conventional AFP-L3% (c-AFP-L3%) has not always been reliable in cases with low serum α-fetoprotein (AFP) levels. In this study, we evaluated the clinical utility of a newly developed assay, highly sensitive AFP-L3% (hs-AFP-L3%). Subjects included 74 patients with benign liver disease (BLD), including chronic hepatitis and cirrhosis, and 94 with HCC. Serum hs-AFP-L3% was significantly higher than c-AFP-L3% in patients with early-stage HCC (solitary or <20 mm in diameter). Additionally, hs-AFP-L3% was significantly increased in patients with well-differentiated HCC. In patients with serum AFP <20 ng/ml, the sensitivities of c-AFP-L3% and hs-AFP-L3% were 12.5 and 44.6%, respectively, at a cut-off value of 5%. In 59 BLD patients with serum AFP <20 ng/ml, the HCC-positive rate in patients with hs-AFP-L3% ≥5% was significantly higher compared to those with hs-AFP-L3% <5% during the follow-up period (median, 35 months; range, 5-48 months). Importantly, none of the BLD patients with both serum AFP <20 ng/ml and hs-AFP-L3% <5% developed HCC. These results indicated that hs-AFP-L3% is useful for early detection of HCC in BLD patients, even for those with serum AFP <20 ng/ml. Furthermore, since hs-AFP-L3% increases before HCC is detectable by various advanced imaging modalities, this assay may help identify BLD patients with a higher risk of HCC.

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Key words: α-fetoprotein, α-fetoprotein fucosylated fraction L3, hepatocellular carcinoma, hepatocarcinogenesis

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world, and the third most common cause of cancer-related death (1). Although it is more common in Asia and Africa, its incidence in the United States has increased over the past two decades, largely due to the spread of hepatitis C (HCV) infection, which is an underlying risk factor (2). Early detection of HCC increases the potential for curative treatment and improves prognosis. Several methods developed for the diagnosis of HCC, including evaluation of serum markers, ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI), have been tested clinically. α-fetoprotein (AFP) and des-γ carboxy prothrombin (DCP), serum proteins that are elevated in HCC, are the most widely used markers. Although routine screening offers the best chance for early tumor detection, the reported sensitivities and specificities of elevated serum AFP and DCP levels vary significantly (3-8). Furthermore, serum AFP levels increase in only 30-40% of patients with HCC, especially early in the disease process (5). Additionally, an increase in serum AFP is also seen in patients with non-cancerous conditions, including cirrhosis or exacerbation of chronic hepatitis (9). AFP-L3, the lectin lens culinaris agglutinin-bound fraction, is one of the three glycoforms of AFP, and is the major glycoform elevated in the serum of HCC patients. The reported sensitivities of AFP-L3 as a method of detecting HCC range from 75-97% with specificities of 90-92% (10,11). In cases of HCC, however, high percentage of AFP-L3 is closely associated with poor differentiation and biologically malignant characteristics, including portal vein invasion, of neoplastic cells (11,12). Therefore, it is not clear how useful this test is for the early detection of HCC. Additionally, measurement of AFP-L3 has not always been reliable for serum samples with low total AFP concentration, as determined by conventional lectin affinity system (LiBASys) (13).

Recently, a novel automated immunoassay for AFP-L3 has been developed. The new method uses on-chip electrokinetic reaction and separation by affinity electrophoresis (micro-total

analysis system; μ-TAS) (14). In patients with an AFP level of ≥20 μg/ml, μ-TAS AFP-L3% correlated well with LiBASys AFP-L3% (15). Furthermore, this system has enabled the accurate measurement of AFP-L3% at very low AFP concentrations. Therefore, in this retrospective study, we investigated the clinical utility of the new highly sensitive μ-TAS AFP-L3% assay for diagnosis of HCC in a population of patients with HCC or benign liver diseases (BLD), including chronic hepatitis or cirrhosis.

Patients and methods

Patients. Between December 2006 and September 2010, frozen serum samples were obtained from 94 patients with HCC, as well as from 74 patients with BLD, who had chronic hepatitis or liver cirrhosis, but not HCC (Table I). All patients met the eligibility criteria (availability of stored serum samples and written informed consent). Among the BLD patients, 20 were positive for hepatitis B surface antigen (HBsAg), 43 were positive for anti-hepatitis C virus (HCV) antibody, and 11 were negative for either HBsAg or anti-HCV antibody. The BLD patients were followed after serum sampling for 32.8±12.3 months (median, 35; range, 5-48); liver imaging was performed by US at 6- to 12-month intervals in most patients with chronic hepatitis, and CT, MRI, or US was performed at 3- to 6-month intervals in patients with liver cirrhosis.

HCC patients were diagnosed using imaging modalities such as US, MRI and CT during hepatic arteriography. Vascular invasion was evaluated by imaging modalities. In some cases that showed atypical features upon imaging, ultrasound-guided biopsies were performed. Based on imaging findings, tumor stage was ranked using the tumor-node-metastasis (TMN) staging system of the Liver Cancer Study Group of Japan (16,17): T1 (fulfilling the following three conditions: solitary, 2 cm, no vessel invasion), T2 (fulfilling two of the three conditions), T3 (fulfilling one of the three conditions), T4 (fulfilling none of the three conditions or showing presence of distant metastasis); N0 (no lymph node metastasis), N1 (metastasis to lymph nodes); M0 (no distant metastasis), M1 (distant metastasis); stage 1 (T1N0M0), stage II (T2N0M0), stage III (T3N0M0), and stage IV (T4N0M0 or any TN1M0, or any TN0-1M1).

Measurement of serum AFP and AFP-L3%. For the HCC group, AFP and AFP-L3% were measured in the same sample obtained at the time of HCC diagnosis, before any treatment. For the BLD without HCC group, measurements were made at the time of diagnosis of chronic liver disease. Highly sensitive AFP-L3% (hs-AFP-L3%) were measured by a microchip capillary electrophoresis and liquid-phase binding assay on a μ-TASWako i30 auto analyzer (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (15). Conventional AFP-L3% (c-AFP-L3%) was examined using a column chromatography and liquid-phase binding assay on a LiBASys auto analyzer (Wako Pure Chemical Industries, Ltd.) (13). The analytical sensitivity of the μ-TASWako i30 auto analyzer is 0.3 μg/ml AFP; the AFP-L3% can be measured when AFP-L3 is over 0.3 μg/ml. Although the analytical sensitivity of the LiBASys is 0.8 μg/ml AFP, AFP-L3% cannot be measured at AFP <10 ng/ml. Therefore, the correlation between μ-TAS-L3% and LiBA-L3% was poor at AFP <20 ng/ml.

Statistical analysis. We used the Mann-Whitney U test, Z test and Chi-square test for evaluation of the statistical significance of each finding. SPSS version 17.0J (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis; $p<0.05$ was considered to indicate statistical significance.

Results

Clinical feature of patients. The demographics, etiology of liver disease, hepatic functional reserve ranked by Child-Pugh classification, tumor stage, tumor size and tumor number of the study patients are summarized in Table I. The HCC group included 94 patients: 35 patients with stage I, 35 with stage II, 14 with stage III, and 10 with stage IV; thus, ~75% of HCC cases were stage I or II. The incidence of cirrhosis in HCC patients (55.3%) was significantly higher than in BLD (25.7%), whereas the hepatic reserve expressed by Child-Pugh classification of HCC patients was significantly preserved compared with BLD patients.

Serum AFP levels in patients with HCC were significantly higher than those with BLD (Table I and Fig. 1A). hs-AFP-L3% was measurable in 47.3 and 78.7% of patients with BLD and HCC, respectively, whereas c-AFP-L3% was detected in 31.1 and 63.8% of patients. Thus, hs-AFP-L3% was significantly higher than c-AFP-L3% in both BLD and HCC patients (Table I and Fig. 1B). Since a cut-off value of 5% has been reported to be useful for diagnosis of HCC using hs-AFP-L3% (18), the cut-off value for AFP-L3% was set at 5% in the present study. The sensitivity and specificity of hs-AFP-L3% were 57.0 and 63.5%, respectively, whereas those of c-AFP-L3% were 40.4 and 81.1%.

hs-AFP-L3% significantly increases in HCC patients at early stage. Next, we analyzed serum AFP levels, c-AFP-L3% and hs-AFP-L3%, and compared early and advanced stages of HCC (Fig. 2). When compared with HCC patients with stage I or II cancer, serum AFP levels were significantly increased in patients with stage III and IV disease (Fig. 2A). Both c-AFP-L3% and hs-AFP-L3% in HCC patients with advanced stages were also significantly higher than in patients with early stages (Fig. 2B). Although 86% of HCC patients with stage I (n=35) exhibited serum AFP <20 ng/ml, c-AFP-L3% and hs-AFP-L3% were measurable in 46 and 69% of these patients, respectively; hs-AFP-L3% was significantly higher than c-AFP-L3%. Consequently, in HCC patients at stage I, the sensitivity of c-AFP-L3% or hs-AFP-L3% at a cut-off level of 5% were 17.1 or 48.6%, respectively.

Next, we evaluated the relationship between AFP-L3% and tumor number or size (Fig. 3). hs-AFP-L3% was significantly higher than c-AFP-L3%, even in patients with single or small HCC (<20 mm in diameter) (Fig. 3). Conversely, when compared to HCC patients with solitary or small HCC, both c-AFP-L3% and hs-AFP-L3% were increased in cases with multiple or ≥20 mm HCC, and there was no statistical difference between c-AFP-L3% and hs-AFP-L3%. These results indicate that hs-AFP-L3% is a useful biomarker for detecting early-stage HCC.

An increase in hs-AFP-L3% is observed in both BLD and HCC patients with AFP <20 ng/ml. We analyzed c-AFP-L3%

Table I. Clinical features of patients with BLD and HCC.

	BLD (n=74)	HCC (n=94)	p-value
Age	56.23±13.88	65.76±12.98[a]	<0.001
Gender (male/female)	30/44	56/38[a]	0.015
CH/LC	55/19	42/52[a]	<0.001
HBV/HCV/NBNC	20/43/11	5/61/28[a]	<0.001
Child-Pugh class (A/B/C/unknown)	39/5/4/26	75/19/0/0[a]	<0.001
TNM stage (I/II/III/IV)		35/35/14/10	
Tumor size (mean ± SD)		22.35±16.42	
<20 mm/≥20 mm		58/36	
Tumor number (single/multiple)		50/44	
AFP (ng/ml)	46.17±163.6	2871.5±9882.7[a]	<0.001
c-AFP-L3%	2.96±6.45	18.19±26.95[a]	<0.001
hs-AFP-L3%	3.84±5.59	21.12±29.01[a]	<0.001
Platelet count (x$10^4/\mu$l)	14.98±6.82	11.39±4.73[a]	0.001
AST (IU/l)	70.55±95.87	55.78±22.92	0.099
ALT (IU/l)	85.38±144.71	48.28±24.13	0.783

BLD, benign liver disease; HCC, hepatocellular carcinoma; CH, chronic hepatitis; LC, liver cirrhosis; HBV, hepatitis B virus; HCV, hepatitis C virus; hs-AFP-L3%, hypersensitive-AFP-L3%; c-AFP-L3%, conventional-AFP-L3%.

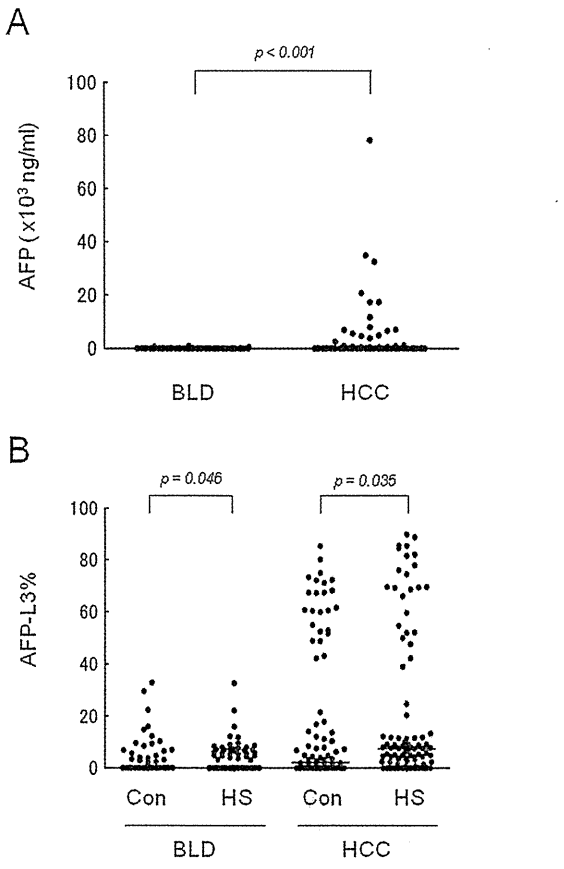

Figure 1. Serum levels of AFP, c-AFP-L3% and hs-AFP-L3% in patients with BLD or HCC. (A) Serum AFP concentrations in HCC patients (n=94) were significantly higher than those in BLD (n=74). (B) hs-AFP-L3% (HS) significantly increased in comparison with c-AFP-L3% (Con) in both BLD and HCC patients.

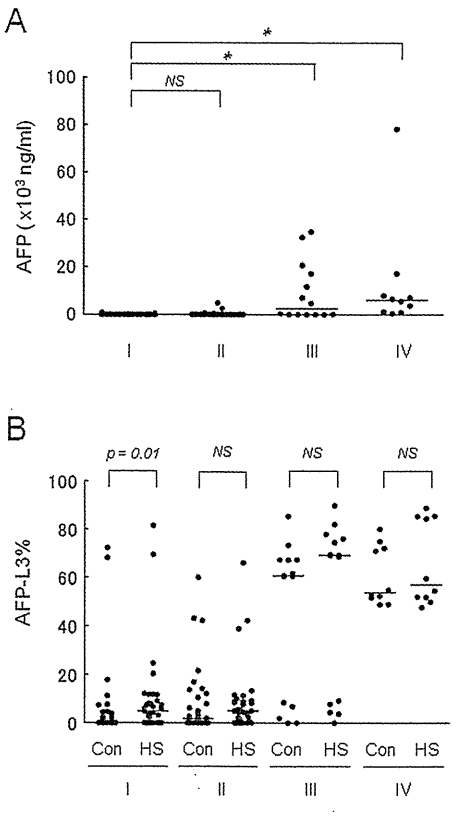

Figure 2. Serum levels of AFP, c-AFP-L3% and hs-AFP-L3% in patients with early or advanced HCC. (A) Serum AFP levels in HCC patients at stage III (n=14) or IV (n=10) were significantly higher than those at stage I (n=35) or II (n=35). *p<0.05. (B) hs-AFP-L3% (HS) was significantly higher than c-AFP-L3% (Con) in patients with HCC at stage I, whereas there was no significant difference between c- and hs-AFP-L3% in HCC patients at stages II, III and IV.

Table II. Clinical features of BLD and HCC patients with AFP <20 ng/ml.

	BLD (n=59)	HCC (n=56)	p-value
Age	56.78±13.51	68.88±12.05[a]	<0.001
Gender (male/female)	23/36	26/30	0.422
CH/LC	45/14	25/31[a]	0.001
HBV/HCV/NBNC	14/35/10	5/32/19[a]	0.008
Child-Pugh class (A/B/C/unknown)	31/4/1/23	50/6/0/0[a]	<0.001
TNM stage (I/II/III/IV)		30/21/5/0	
Tumor size (mean ± SD)		16.16±11.59	
<20 mm/≥ 20 mm		47/9	
Tumor number (single/multiple)		35/21	
AFP (ng/ml)	4.68±3.6	8.92±5.23[a]	<0.001
c-AFP-L3%	0.83±3.92	1.86±3.16[a]	0.002
hs-AFP-L3%	2.7±5.15	4.86±5.19[a]	0.003
Platelet count (x10^4/μl)	15.93±6.67	11.93±4.49[a]	0.001
AST (IU/l)	43.91±25.72	54.32±21.61[a]	0.003
ALT (IU/l)	49.21±51.7	48.66±24.41	0.184

BLD, benign liver disease; HCC, hepatocellular carcinoma; CH, chronic hepatitis; LC, liver cirrhosis; HBV, hepatitis B virus; HCV, hepatitis C virus; hs-AFP-L3%, hypersensitive-AFP-L3%; c-AFP-L3%, conventional-AFP-L3%.

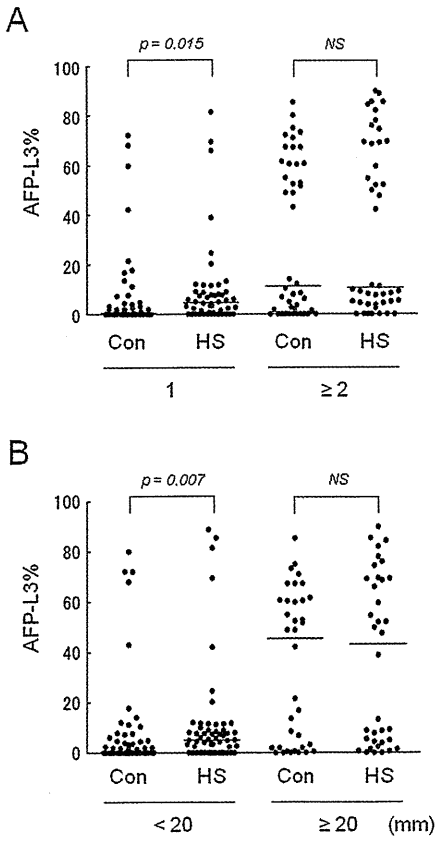

Figure 3. hs-AFP-L3% significantly increased in patients with solitary or small HCC, but not multiple or HCC ≥20 mm in diameter. (A) hs-AFP-L3% (HS) was significantly higher than c-AFP-L3% (Con) in patients with solitary HCC (n=50), but not in patients with multiple HCC (n=44). (B) hs-AFP-L3% significantly increased in comparison with c-AFP-L3% in patients with small HCC (<20 mm in diameter) (n=58), but not in patients with large HCC (≥20 mm) (n=36).

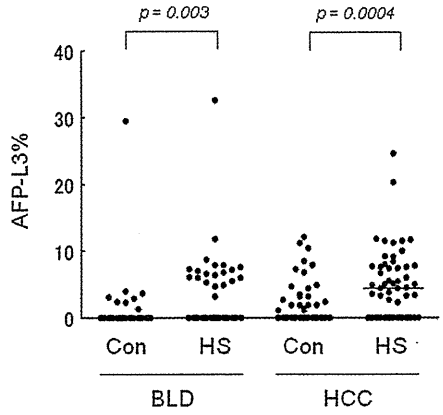

Figure 4. Higher levels of hs-AFP-L3% were observed in both BLD and HCC patients with serum AFP <20 ng/ml. c-AFP-L3% (Con) and hs-AFP-L3% (HS) in BLD and HCC patients with AFP <20 ng/ml (n=59 and 56, respectively) were analyzed. c-AFP-L3% was detectable in 13.6 and 39.3% of BLD and HCC patients, respectively, whereas hs-AFP-L3% was measurable in 33.9 and 64.3% of BLD and HCC patients, respectively; hs-AFP-L3% was significantly higher than c-AFP-L3%.

and hs-AFP-L3% in BLD and HCC patients with AFP <20 ng/ml (Table II). Forty-seven of 56 (83.4%) HCC patients exhibited small HCCs (<20 mm in diameter); 35 patients (62.5%) exhibited solitary tumors. c-AFP-L3% was detectable in 13.6 and 39.3% of BLD and HCC patients, respectively. Conversely, hs-AFP-L3% was measurable in 33.9 and 64.3% of BLD and HCC patients, respectively, and the levels of hs-AFP-L3% were significantly higher than those of c-AFP-L3% [BLD: mean ± SD (range) 0.83±3.92 (1.3-29.5) vs. 2.70±5.15%, p=0.003, and HCC: 1.86±3.16 (1.1-12.1) vs. 4.86±5.19% (2.3-24.6), p=0.004] (Fig. 4). The sensitivity and specificity of hs-AFP-L3%