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 $p < 0.05$.

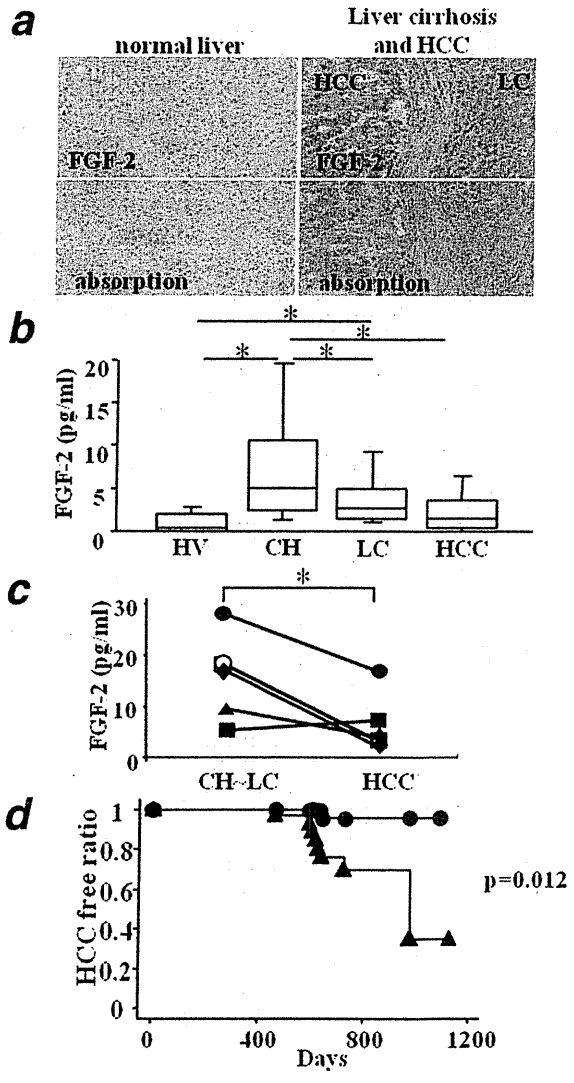

Figure 1. Expressions of FGF-2 in the liver of patients with chronic liver diseases and serum FGF-2 levels in chronic liver disease patients were associated with HCC incidence. (*a*) Immunohistochemical analysis of FGF-2 in normal liver tissues ($N = 2$), chronic hepatitis tissues ($N = 4$), liver cirrhosis (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.

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

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

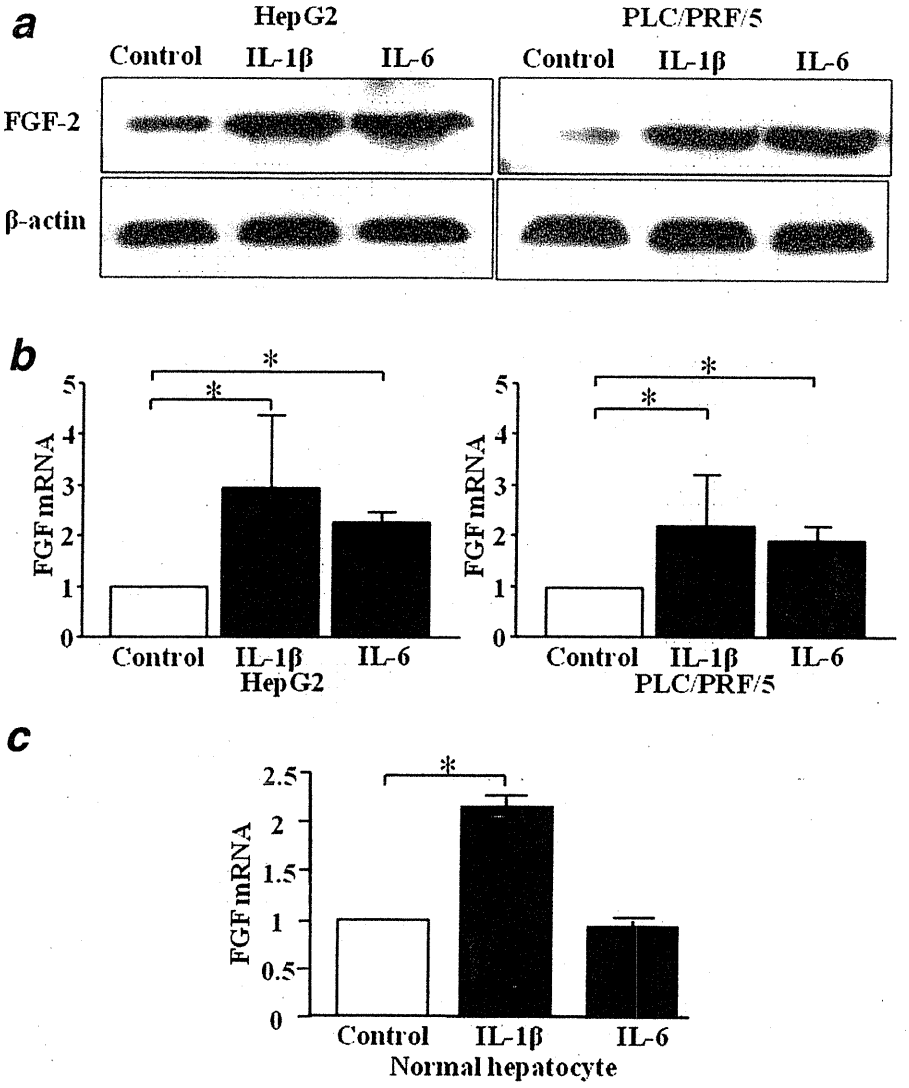

Figure 2. IL-1β and IL-6 increased FGF-2 expressions on human HCC cells and normal hepatocytes. To examine the effect of IL-1β and IL-6 on FGF-2 expression, HepG2 and PLC/PRF/5 cells (*a,b*) or normal hepatocytes (*c*) were cultured for 72 hr in the presence or absence of IL-1β (50 ng/ml) and IL-6 (300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

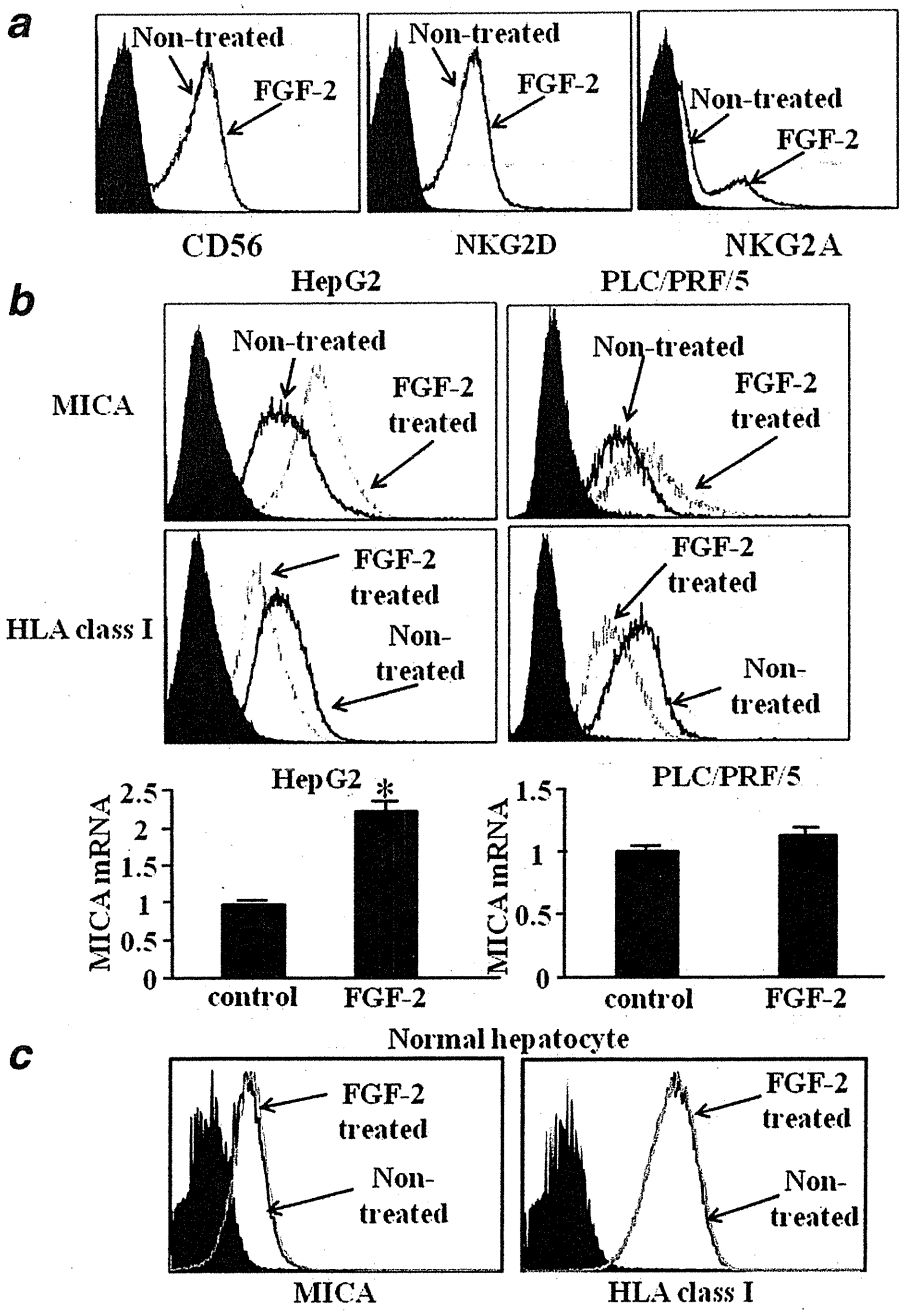

Figure 3. The expressions of NKG2D and NKG2A on FGF-2-treated NK cells and the expressions of MICA and HLA class I on FGF-2-treated hepatoma cells. (*a*) The expressions of NKG2D or NKG2A on FGF-2-treated or nontreated NK cells were evaluated. NK cells obtained from healthy volunteers (2×10^6 cells/well)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

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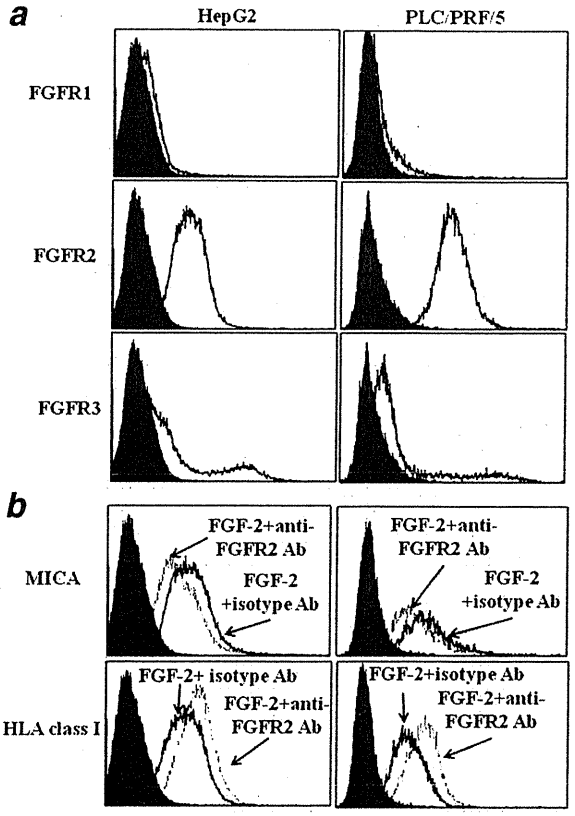

Figure 4. The expressions of FGF receptors on hepatoma cells. (*a*) The expressions of FGF receptors (FGFR1, FGFR2, and FGFR3) on both HepG2 and PLC/PRF/5 cells were evaluated by flow cytometry. Black line histograms: staining of each FGF receptors (FGFR1, FGFR2, FGFR3), shaded/black histograms: control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

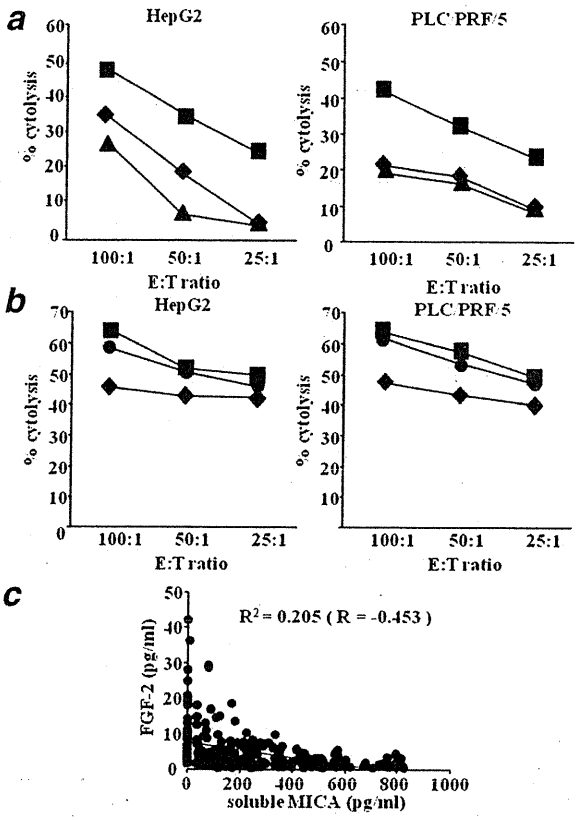

Figure 5. The cytolytic activity against FGF-2-treated HCC cells and the correlation between serum FGF-2 and soluble MICA in patients with chronic liver disease. (*a,b*) Both HepG2 and PLC/PRF/5 cells were cultured with or without FGF-2 protein (250 ng/ml) for 48 hr, and the cytolytic activities of NK 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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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Efficacy of pegylated interferon plus ribavirin combination therapy for hepatitis C patients with normal ALT levels: a matched case–control study

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Abstract
Background The antiviral effect of pegylated interferon (Peg-IFN) plus ribavirin combination therapy in chronic hepatitis C (CHC) patients with normal alanine aminotransferase (ALT) levels (N-ALT) has been reported to be equivalent to that for patients with elevated ALT levels (E-ALT). However, the actual antiviral effect in N-ALT patients remains obscure because efficacy can be overestimated in patients with an advantageous background.
Methods In this study, 386 patients were extracted, for a matched case–control study, from 1320 CHC patients treated with Peg-IFN alpha-2b plus ribavirin combination therapy; 193 N-ALT patients [116 with hepatitis C virus genotype 1 (HCV-1), 77 with HCV genotype 2 (HCV-2)] were matched with 193 E-ALT patients by a propensity score method using the variables of age, sex, IFN treatment history, body mass index, and platelet counts.
Results On multivariate analysis for sustained virological response (SVR) in N-ALT patients, younger age, low HCV RNA level at baseline, and HCV-2 were significant factors. The matched case–control study showed that the SVR rates of N-ALT patients were equivalent to those of E-ALT patients; at 49 and 40% in the HCV-1 group ($P = 0.146$), and 78 and 81% in the HCV-2 group ($P = 0.691$). However, in N-ALT patients with non-SVR, approximately 40% showed ALT elevation at 24 weeks post-treatment.
Conclusion Our findings indicate that the antiviral effect of Peg-IFN plus ribavirin therapy in N-ALT patients is comparable to that for E-ALT patients irrespective of their advantageous background; however, the application of this therapy for N-ALT patients, especially for those with HCV-1, should be considered carefully.

N. Hiramatsu and Y. Inoue contributed equally to this work.

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Introduction

In patients with hepatitis C virus (HCV) infection, alanine aminotransferase (ALT) levels fluctuate and sometimes biochemical remission is maintained. Approximately 20% of patients with normal ALT levels (N-ALT) show ALT elevation and fibrosis progression within 3–5 years [1–5], and consequently, 70–80% of N-ALT patients have mild to moderate fibrosis on liver biopsy. N-ALT patients have been excluded from conventional interferon (IFN) therapy, because their sustained virological response (SVR) rates on conventional IFN monotherapy have been reported to be only 6–15% [6–9], and ALT levels were noted to increase during or after treatment in 47–62% of the patients. The incidence of ALT flares has raised concerns regarding the risk of conventional IFN therapy compared with a small benefit. However, a large randomized controlled trial has demonstrated that combination therapy with pegylated interferon (Peg-IFN) and ribavirin produced SVR rates in N-ALT patients with chronic hepatitis C (CHC) that were comparable to those of patients with elevated ALT levels (E-ALT) [10]. Thus, such treatment is now being considered for N-ALT patients with CHC [11].

Comparison of the characteristics of N-ALT and E-ALT patients has shown that the mean age of N-ALT patients was lower than that of E-ALT patients, and females and HCV genotype 2 patients were predominant among N-ALT patients [4, 7, 12–17]. In the American Association for the Study of Liver Disease guideline, the pretreatment predictors of achieving SVR with Peg-IFN plus ribavirin combination therapy for CHC patients are HCV genotype 2 or 3 infection, low viral load (<600 KIU/ml), female gender, and age less than 40 years [11]. Considering these characteristics, N-ALT patients with CHC can be said to have an advantageous background, and their response to antiviral therapy, including Peg-IFN plus ribavirin combination therapy, can be overestimated. Therefore, patient background, especially factors affecting the treatment efficacy of the combination therapy, needs to be matched between study groups in order to compare the treatment efficacies in N-ALT patients with CHC and E-ALT patients with CHC accurately. In this study, we evaluated, by a matched case–control study approach, whether the antiviral efficacy in N-ALT patients with CHC, reported to be equal to that in E-ALT patients with CHC, could be obtained without their advantageous background, and whether the factors contributing to SVR in N-ALT patients were the same as those in E-ALT patients. In addition, ALT flares after treatment in N-ALT patients without SVR were examined.

Patients and methods

Patient selection and study design

The subjects were 1320 consecutive CHC patients, 1015 with HCV genotype 1 (HCV-1) and 305 with HCV genotype 2 (HCV-2) who had undergone combination therapy with Peg-IFN alpha-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (REBETOL; Schering-Plough) at standard doses for 48 weeks (patients with HCV-1) or for 24 weeks (patients with HCV-2) at 30 medical institutions participating in the Osaka Liver Forum between December 2004 and December 2007. Peg-IFN alpha-2b and ribavirin dosages were based on body weight according to the manufacturer's instructions and were modified based on the manufacturer's instructions according to the severity of adverse hematologic effects. In the 1 month preceding treatment, none of the patients had received any IFN formulations or other types of drugs for liver supporting therapy. Before starting treatment, all patients had positive anti-HCV and a detectable level of HCV RNA according to a polymerase chain reaction (PCR)-based assay (COBAS Amplicor HCV Monitor Test v2.0; Roche Diagnostics, Branchburg, NJ, USA). None of the patients showed evidence of dual infection with hepatitis B virus or human immunodeficiency virus, or other forms of liver diseases such as alcoholic liver disorder, autoimmune hepatitis, or drug-induced liver injury.

In this study, a normal serum ALT level was defined as ALT $\leq$30 IU/l at the start of the combination therapy, as, in the guidelines for treatment of hepatitis C in N-ALT patients in Japan, ALT levels of $\leq$30 IU/l are regarded as an indicator of no or little inflammation in the liver, and patients whose ALT levels are $\leq$30 IU/l are recommended to be followed without antiviral therapy, especially if the platelet count is $\geq 15 \times 10^4/mm^3$.

Among the 1320 consecutive CHC patients, the antiviral effect in 193 N-ALT patients (116 with HCV-1, 77 with HCV-2) was compared with that in 193 E-ALT patients (116 with HCV-1, 77 with HCV-2) who were matched by a propensity score method based on age, sex, IFN treatment history, body mass index (BMI), and platelet counts. BMI was calculated as weight (kg)/[height (m)]^2.

HCV RNA was determined at week 4, week 12, end of treatment (EOT), and 24 weeks after EOT. HCV RNA was also determined at week 24 for HCV-1 patients. HCV RNA was monitored by the PCR Amplicor method with a detection limit of 50 IU/ml. (COBAS Amplicor HCV v2.0;

Roche Diagnostics). Complete early virological response (cEVR) and end-of-treatment response (ETR) were defined as undetectable HCV RNA at week 12 and EOT, respectively.

Written informed consent was obtained from each patient, and the study protocol was reviewed and approved according to the ethical guidelines of the 2004 Declaration of Helsinki by institutional review boards at the respective sites.

Propensity score

Propensity score methods are used to create balanced covariates and reduce selection bias in a matched case–control study. Propensity scores were calculated using a multivariate logistic regression model that had ALT levels as a dependent variable and other covariates as independent variables, and the model was utilized for matching between the N-ALT patients with CHC (the case group) and the E-ALT patients with CHC (the control group). Data analyses were conducted using SAS, version 9.2 (SAS Institute, Cary, NC, USA).

Statistical analysis

Continuous variables are reported as the mean with standard deviation (SD) or median levels, while categorical variables are shown as the count and proportion. Statistical significance was assessed by Student's t test (mean), the Mann–Whitney U test (median), and the χ^2 test for independent samples, and the paired t test for paired samples. For all tests, two-sided P values were calculated, and the results were considered statistically significant if $P < 0.05$. Variables that achieved statistical significance on univariate analysis were subjected to multivariate logistic regression analysis. Stepwise and multivariate logistic regression models were used to explore the independent factors that could be used to predict SVR. Statistical analysis was performed using the SPSS program for Windows, version 15.0 J (SPSS, Chicago, IL, USA).

Results

Baseline characteristics of all CHC patients according to HCV genotype and ALT levels before matching

The baseline characteristics of 1320 patients at the commencement of combination therapy with Peg-IFN and ribavirin are shown in Table 1, according to HCV genotype and ALT levels before matching. Of the 116 N-ALT patients with HCV-1 there were 36 males and 80 females (69%), with a mean age of 54 ± 11 years. Eighty-five (73%) were IFN-naïve. In terms of liver histology, 66 (73%) patients had

Table 1 Demographic characteristics of patients with normal ALT and patients with elevated ALT

	HCV genotype 1			HCV genotype 2		
	Normal ALT ($n = 116$)	Elevated ALT ($n = 899$)	P value	Normal ALT ($n = 77$)	Elevated ALT ($n = 228$)	P value
Sex: male/female	36/80	512/387	<0.001	32/45	121/107	0.081
Age (years)	54 ± 11	56 ± 10	0.136	51 ± 13	52 ± 13	0.423
Body mass index (kg/m^2)	22.9 ± 3.1	23.3 ± 3.2	0.131	23.0 ± 2.9	23.3 ± 3.2	0.424
Past IFN therapy: naïve/experienced (relapser/non-responder)[a]	85/31 (18/5)	547/352 (131/154)	0.011	58/19 (9/4)	175/53 (21/10)	0.876
Histology (METAVIR)[b]						
Activity: 0–1/2–3	66/25	296/338	<0.001	43/6	69/94	<0.001
Fibrosis: 0–1/2–4	67/24	330/304	<0.001	42/7	101/62	0.002
HCV RNA (KIU/ml)[c]	1800	1700	0.793	2200	1100	<0.001
White blood cell (/mm^3)	5220 ± 1507	5137 ± 1582	0.595	5538 ± 1687	5338 ± 1725	0.377
Neutrophil (/mm^3)	2770 ± 1074	2595 ± 1078	0.108	3017 ± 1180	2688 ± 1230	0.047
Hemoglobin (g/dl)	13.6 ± 1.5	14.2 ± 1.4	<0.001	13.8 ± 1.6	14.2 ± 1.4	0.071
Platelet (×10^4/mm^3)	19.9 ± 5.7	16.2 ± 5.3	<0.001	20.5 ± 4.5	17.8 ± 5.8	<0.001
ALT (IU/l)	24 ± 5	88 ± 62	<0.001	22 ± 5	97 ± 67	<0.001

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon

[a] Status was unknown in 8 patients in the normal ALT group and 67 in the elevated ALT group with HCV genotype 1; and in 6 in the normal ALT group and 22 in the elevated ALT group with HCV genotype 2

[b] Data missing in 25 patients in the normal ALT group and in 265 in the elevated ALT group with HCV genotype 1; and in 28 in the normal ALT group and in 65 in the elevated ALT group with HCV genotype 2

[c] Values are expressed as medians

mild activity (activity, 0–1) and 67 (74%) had mild fibrosis (fibrosis, 0–1) by the METAVIR system. Mean white blood cell counts, hemoglobin levels, and platelet counts were 5220 ± 1570 /mm^3, 13.6 ± 1.5 g/dl, and 19.9 ± 5.7 × 10^4/mm^3. In 899 E-ALT patients compared to N-ALT patients, the proportions of female and IFN-naïve patients were significantly lower, at 43% ($P < 0.001$) and 61% ($P = 0.011$), respectively. Higher scores for activity ($P < 0.001$) and fibrosis ($P < 0.001$) were observed in E-ALT patients. E-ALT patients had higher hemoglobin levels and lower platelet counts than N-ALT patients, at 14.2 ± 1.5 g/dl ($P < 0.001$) and 16.2 ± 5.3 × 10^4/mm^3 ($P < 0.001$), respectively. Mean ALT levels were 24 ± 5 IU/l in N-ALT patients and 88 ± 62 IU/l in E-ALT patients ($P < 0.001$).

Of the 77 N-ALT patients with HCV-2, 32 were males and 45, females (58%). Their mean age was 51 ± 13 years and 58 (75%) were IFN-naïve. In terms of liver histology, 43 (88%) patients had mild activity (activity, 0–1) and 42 (86%) had mild fibrosis (fibrosis, 0–1). Compared to the 228 E-ALT patients, the N-ALT patients had higher HCV RNA levels (median 2200 vs. 1100 KIU/ml, $P < 0.001$). Higher scores for activity ($P < 0.001$) and fibrosis ($P = 0.002$) were observed in E-ALT patients. Neutrophils and platelet counts in N-ALT patients were higher than those in E-ALT patients, at 3017 ± 1180 versus 2688 ± 1230 /mm^3 ($P = 0.047$) and 20.5 ± 4.5 × 10^4 versus 17.8 ± 5.8 × 10^4/mm^3 ($P < 0.001$), respectively. Mean ALT levels were 22 ± 5 IU/l in N-ALT patients and 97 ± 67 IU/l in E-ALT patients ($P < 0.001$).

Prognostic factors for SVR in the N-ALT patients

For all N-ALT patients (HCV-1, 116; HCV-2, 77), univariate analysis for factors associated with achieving SVR was performed for the following variables: sex, age, BMI, history of past IFN therapy, histology, baseline HCV RNA level, HCV genotype, white blood cell count, neutrophil count, hemoglobin level, platelet count, and ALT level (Table 2). The results indicated that age, fibrosis, baseline HCV RNA level, and HCV genotype contributed to SVR. Next, multivariate logistic regression analysis was performed for all N-ALT patients ($n = 193$), using these factors except for fibrosis, as there were many missing samples. The multivariate analysis showed that younger age [by 10-year increase: odds ratio (OR) 0.552; 95% confidence interval (CI) 0.404–0.756; $P < 0.001$] and lower baseline HCV RNA level (by 100-KIU/ml increase: OR 0.976; 95% CI 0.954–0.998; $P = 0.037$), as well as HCV genotype (genotype 2 vs. genotype 1: OR 3.724; 95% CI 1.859–7.463; $P < 0.001$) were independently associated with SVR (Table 3).

Table 2 Factors associated with SVR in patients with normal ALT—univariate analysis

Factor	SVR ($n = 117$)	Non-SVR ($n = 76$)	P value
Sex: male/female	43/74	25/51	0.645
Age (years)	50 ± 13	57 ± 9	<0.001
Body mass index (kg/m^2)	22.8 ± 3.3	23.1 ± 2.6	0.511
Past IFN therapy: naïve/experienced	88/29	55/21	0.737
Histology (METAVIR)[a]			
Activity: 0–1/2–3	67/14	42/17	0.148
Fibrosis: 0–1/2–4	69/12	40/19	0.022
HCV genotype: 1/2	57/60	59/17	<0.001
HCV RNA (KIU/ml)[b]	1700	2100	0.040
White blood cell (/mm^3)	5461 ± 1426	5170 ± 1798	0.213
Neutrophil (/mm^3)	2968 ± 1167	2709 ± 1032	0.126
Hemoglobin (g/dl)	13.7 ± 1.4	13.7 ± 1.6	0.970
Platelet (×10^4/mm^3)	20.4 ± 4.8	19.8 ± 5.8	0.388
ALT (IU/l)	23 ± 5	24 ± 5	0.384

SVR sustained virological response, *ALT* alanine aminotransferase, *IFN* interferon, *HCV* hepatitis C virus

[a] Data missing in 36 patients in the SVR group and in 17 in the non-SVR group

[b] Values are expressed as medians

Table 3 Factors associated with SVR in patients with normal ALT—multivariate analysis

Factor	Category	Odds ratio	95% CI	P value
Age	By 10 years	0.552	0.404–0.756	<0.001
HCV genotype	1/2	3.724	1.859–7.463	<0.001
HCV RNA	By 100 KIU/ml	0.976	0.954–0.998	0.037

The number of patients used for this multivariate analysis was 193 (SVR, $n = 117$; non-SVR, $n = 76$)

SVR sustained virological response, *ALT* alanine aminotransferase, *CI* confidence interval, *HCV* hepatitis C virus

Comparison of patient characteristics between patients with normal ALT and those with elevated ALT matched by a propensity score method

The baseline characteristics of CHC patients matched by a propensity score method at the commencement of combination therapy with Peg-IFN and ribavirin were compared between N-ALT patients and E-ALT patients (see Table 4). There were 116 CHC patients with HCV-1 in each of the groups of N-ALT and E-ALT patients. The two groups were well matched by propensity score methods and there was no significant difference, except in ALT values (mean value, N-ALT, 24 ± 5 IU/l vs. E-ALT, 78 ± 53 IU/l, $P < 0.001$). Similarly, with CHC patients with HCV-2, there were no significant differences, except for ALT levels (mean value, N-ALT, 22 ± 5 IU/l vs. E-ALT, 80 ± 58 IU/l, $P < 0.001$), activity scores [0–1, N-ALT, 88% (43/49) vs. E-ALT, 49% (25/51), $P < 0.001$], and HCV RNA levels (median value, N-ALT, 2200 KIU/ml vs. E-ALT, 1000 KIU/ml, $P < 0.001$).

Treatment efficacy of combination therapy with Peg-IFN and ribavirin in CHC patients

Antiviral effects of the combination therapy with Peg-IFN and ribavirin were evaluated by rapid virological response (RVR), cEVR, ETR, SVR, and relapse rates, as shown in Table 5. Among patients with HCV-1 in the N-ALT and E-ALT patients, respectively, RVR rates were 6% (6/98) and 6% (6/102), cEVR rates were 53% (62/116) and 43% (50/116), and ETR rates were 72% (84/116) and 58% (67/116) ($P = 0.019$). SVR and relapse rates in N-ALT patients were 49% (57/116) and 32% (27/84). These rates in E-ALT patients were 40% (46/116) and 31% (21/67). In the patients with HCV-2, RVR, cEVR, ETR, SVR, and relapse rates were 68% (41/60), 90% (69/77), 96% (74/77), 78% (60/77), and 19% (14/74) for N-ALT patients, and 62% (36/58), 91% (70/77), 91% (70/77), 81% (62/77), and 11% (8/70) for E-ALT patients, respectively. Comparisons between N-ALT and E-ALT patients with HCV-1 or HCV-2 showed no significant differences in RVR, cEVR, ETR, SVR, and relapse rates, except in ETR rates in patients with HCV-1.

Changes in ALT levels during combination therapy and follow-up periods in N-ALT patients with SVR and those with non-SVR

Changes in ALT levels in N-ALT patients during the combination therapy and follow-up periods were evaluated according to the treatment response (Fig. 1). In patients with HCV-1, the mean baseline ALT level in the SVR

Table 4 Comparison of characteristics between patients with normal ALT and patients with elevated ALT matched by a propensity score method

	HCV genotype 1			HCV genotype 2		
	Normal ALT ($n = 116$)	Elevated ALT ($n = 116$)	*P* value	Normal ALT ($n = 77$)	Elevated ALT ($n = 77$)	*P* value
Sex: male/female	36/80	32/84	0.564	32/45	30/47	0.742
Age (years)	54 ± 11	55 ± 11	0.746	51 ± 13	50 ± 13	0.742
Body mass index (kg/m^2)	22.9 ± 3.1	22.6 ± 2.9	0.536	23.0 ± 2.9	22.8 ± 2.9	0.780
Past IFN therapy: naïve/experienced (relapser/non-responder)[a]	85/31 (18/5)	80/36 (13/18)	0.469	58/19 (9/4)	57/20 (7/4)	0.853
Histology (METAVIR)[b]						
Activity: 0–1/2–3	66/25	49/35	0.056	43/6	25/26	<0.001
Fibrosis: 0–1/2–4	67/24	59/25	0.736	42/7	36/15	0.068
HCV RNA (KIU/ml)[c]	1800	1700	0.896	2200	1000	<0.001
White blood cell (/mm^3)	5220 ± 1507	5329 ± 1626	0.569	5538 ± 1687	5530 ± 1780	0.977
Neutrophil (/mm^3)	2770 ± 1074	2702 ± 1094	0.641	3017 ± 1180	2755 ± 1189	0.189
Hemoglobin (g/dl)	13.6 ± 1.5	13.7 ± 1.4	0.542	13.8 ± 1.6	14.0 ± 1.4	0.592
Platelet (×10^4/mm^3)	19.9 ± 5.7	19.4 ± 7.1	0.562	20.5 ± 4.5	20.6 ± 5.5	0.911
ALT (IU/l)	24 ± 5	78 ± 53	<0.001	22 ± 5	80 ± 58	<0.001

ALT alanine aminotransferase, *IFN* interferon, *HCV* hepatitis C virus

[a] Data unknown in 8 patients in the normal ALT group and in 5 in the elevated ALT group with HCV genotype 1; and in 6 in the normal ALT group and 9 in the elevated ALT group with HCV genotype 2

[b] Data missing in 25 patients in the normal ALT group and in 32 in the elevated ALT group with HCV genotype 1; and in 28 in the normal ALT group and 26 in the elevated ALT group with HCV genotype 2

[c] Values are expressed as medians

Table 5 Antiviral effect for patients with normal ALT and those with elevated ALT according to HCV genotype

		Normal ALT	Elevated ALT	*P* value
HCV genotype 1		$n = 116$	$n = 116$	
	Undetectable HCV RNA rate			
	At week 4 (RVR)[a]	6% (6/98)	6% (6/102)	1.000
	At week 12 (cEVR)	53% (62/116)	43% (50/116)	0.287
	At week 48 (ETR)	72% (84/116)	58% (67/116)	0.019
	Post-24 weeks (SVR)	49% (57/116)	40% (46/116)	0.146
	Relapse rate	32% (27/84)	31% (21/67)	0.916
HCV genotype 2		$n = 77$	$n = 77$	
	Undetectable HCV RNA rate			
	At week 4 (RVR)[b]	68% (41/60)	62% (36/58)	0.563
	At week 12 (cEVR)	90% (69/77)	91% (70/77)	0.723
	At week 24 (ETR)	96% (74/77)	91% (70/77)	0.191
	Post-24 weeks (SVR)	78% (60/77)	81% (62/77)	0.691
	Relapse rate	19% (14/74)	11% (8/70)	0.212

ALT alanine aminotransferase, *HCV* hepatitis C virus, *RVR* rapid virological response, *cEVR* complete early virological response, *ETR* end-of-treatment response, *SVR* sustained virological response

[a] Data missing in 18 patients in the normal ALT group and in 14 in the elevated ALT group with HCV genotype 1

[b] Data missing in 17 patients in the normal ALT group and in 19 in the elevated ALT group with HCV genotype 2

group ($n = 57$) was similar to that in the non-SVR group ($n = 59$) (mean ± standard error of the mean (SEM): SVR group, 24.5 ± 0.6 IU/l; non-SVR group, 24.2 ± 0.7 IU/l; $P = 0.694$). Transitions of ALT levels were not significantly different between SVR and non-SVR groups during the therapy. However, in the SVR group, the ALT level fell to 15.1 ± 0.7 IU/l at 24 weeks after treatment completion ($P < 0.001$, compared to the baseline level), while in the non-SVR group, higher ALT levels were observed after treatment compared to the baseline level; the ALT level rose to the peak value of 36.2 ± 3.6 IU/l at post-12 weeks ($P = 0.001$), and slightly fell to 31.3 ± 2.6 IU/l at post-24 weeks ($P = 0.007$) (Fig. 1a). In comparison with the SVR group, the non-SVR group showed significant differences in mean ALT levels at post-4, -12, and -24 weeks ($P = 0.002$, <0.001, and <0.001, respectively). At post-48 weeks in the non-SVR group, the ALT level was 30.4 ± 2.9 IU/l, which was still higher than the baseline level ($P = 0.025$).

Similarly, in patients with HCV-2, baseline ALT levels in the SVR group ($n = 60$) and the non-SVR group ($n = 17$) were equivalent (mean ± SEM; SVR, 21.8 ± 0.7 IU/l; non-SVR, 22.5 ± 1.1 IU/l; $P = 0.622$), and there was no significant difference in transitions of the ALT levels during therapy. However, after treatment, in the non-SVR group, ALT levels tended to rise in comparison with those at baseline; they rose to 74.9 ± 26.9 IU/l at post-12 weeks ($P = 0.068$) and fell to 35.7 ± 10.2 IU/l at post-24 weeks ($P = 0.196$). On the other hand, in the SVR group, ALT levels fell significantly, to 16.4 ± 1.3 IU/l at post-12 weeks ($P < 0.001$) and 15.2 ± 1.2 IU/l at post-24 weeks ($P < 0.001$) (Fig. 1b). Comparison of ALT levels between the SVR and non-SVR groups after treatment showed that mean ALT levels in the non-SVR group tended to be high at post-4, -12 and, -24 weeks ($P = 0.045$, 0.051, and 0.066, respectively). At post-48 weeks in the non-SVR group, the ALT level was 32.4 ± 8.9 IU/l, which tended to be high compared with the baseline ALT level, although no significant difference was found ($P = 0.248$).

Next, the ALT levels in N-ALT patients were examined according to the treatment response at 24 weeks after completion of the combination therapy. In HCV-1 patients with SVR, ALT levels remained below the upper limit of normal (ULN) for this study (<30 IU/l) in 55 (98%) patients, and ALT elevation <2 × ULN occurred in only one (2%) patient (ALT 32 IU/l). On the other hand, in patients with non-SVR, ALT levels remained stable in 34 (60%) patients but increased to <2 × ULN in 20 (35%) patients, and to ≥2 × ULN in 3 (5%) patients (ALT 62, 79, and 135 IU/l). Similarly, in HCV-2 patients with SVR, ALT levels remained stable in 56 (95%) patients, and ALT elevation rarely occurred [<2 × ULN, 2 (3%) patients; ≥2 × ULN, one (2%) patient (ALT 68 IU/l)]. In contrast, in patients with non-SVR, ALT levels remained normal in 10 (67%) patients but increased to <2 × ULN in 4 (27%) patients and to ≥2 × ULN in one (6%) patient (ALT 174 IU/l).

Discussion

N-ALT patients with CHC are known to show demographic and virological features associated with higher

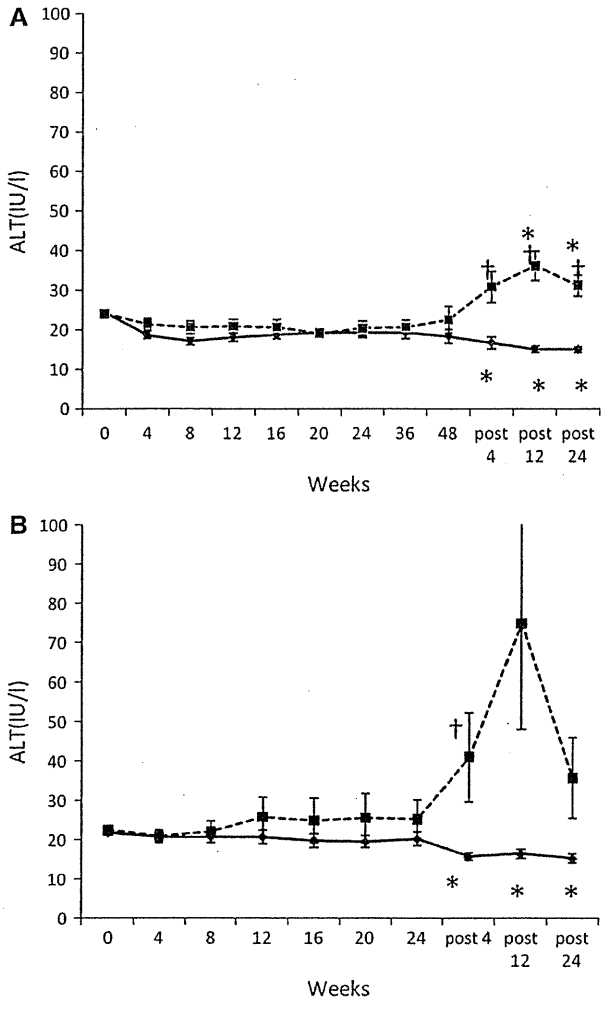

Fig. 1 Changes in serum alanine aminotransferase (*ALT*) levels (±standard error of the mean) according to response in patients with normal ALT levels with chronic hepatitis C treated with pegylated interferon and ribavirin. *Solid lines* show ALT levels in patients with a sustained virological response (SVR), and *dashed lines* show these levels in patients with a non-SVR. *Single-asterisks* denote a statistically significant difference ($P < 0.05$) in mean ALT levels between baseline and each time point of the follow-up period. *Daggers* denote a statistically significant difference between SVR and non-SVR groups. **a** Patients infected with hepatitis C virus genotype 1 (HCV-1). The number of patients was 57 in the SVR group and 59 in the non-SVR group. **b** HCV-2 patients. The number of patients was 60 in the SVR group and 17 in the non-SVR group

response rates to Peg-IFN and ribavirin combination therapy [4, 7, 12–17]. In the present study, N-ALT patients were younger and had higher platelet counts than E-ALT patients, thus giving N-ALT patients an advantage in antiviral efficacy in comparison with E-ALT patients in our cohort. However, the preponderance of females was greater in N-ALT patients with HCV-1 in this study, giving N-ALT patients a disadvantage. Accordingly, a direct comparison was made between these two patient groups after matching E-ALT patients with N-ALT patients using propensity score methods to reduce the bias due to differences in patient backgrounds. As a result, the efficacy of the combination therapy in N-ALT patients was revealed to be still equivalent to that in E-ALT patients, irrespective of their advantageous background. Moreover, in N-ALT patients with HCV-1, not only the ETR rate, but also the SVR rate tended to be higher than these rates in E-ALT patients (49% in N-ALT patients vs. 40% in E-ALT patients). Accordingly, N-ALT patients with HCV-1 can achieve a better treatment response in comparison with E-ALT patients, but further study is needed to clarify this.

In the present study, multivariate logistic regression analysis showed that achieving SVR was strongly influenced by HCV genotype and baseline HCV RNA level in N-ALT patients, which was consistent with findings of multicenter studies with E-ALT patients [18–21]. Therefore, decisions for treatment and the treatment regimen for N-ALT patients can mirror those recommended for E-ALT patients. The results of our multivariate analysis also revealed that patient age influenced the achievement of SVR in N-ALT patients. This offers support for the decision to offer antiviral treatment to younger N-ALT patients.

Among patients in our study who achieved SVR with the combination therapy, ALT levels after treatment decreased significantly, as shown in Fig. 1. However, approximately 40% of the non-SVR patients had increased ALT levels of up to $<2 \times$ ULN, and about 5% of patients had increased ALT levels of $\geq 2 \times$ ULN at 24 weeks after completion of the combination therapy, regardless of HCV genotype. When N-ALT patients are commencing the combination therapy, these patients should be told about the possibility of ALT exacerbation [6–9], although it is difficult to know whether this is drug-induced or due to the natural course. It is also difficult to state which patient characteristics make ALT elevation more likely to occur after the treatment.

Taking the findings obtained in the present study together, in N-ALT patients with HCV genotype 2, earlier treatment with Peg-IFN plus ribavirin combination therapy is desirable, as better efficacy was found for younger patients, with an SVR rate of approximately 80% being attained with this combination therapy, and few direct-acting antiviral agents (DAAs) have been developed for genotype 2. On the other hand, N-ALT patients with HCV genotype 1 should consider awaiting the DAAs, because SVR cannot be attained in about half of these patients, and the ALT level rises after treatment in about 40% of patients with non-SVR.

From the aspect of long-term prognosis, we need to verify, by prospective study, that viral eradication is really required for N-ALT patients because the incidence of hepatocellular carcinoma and liver-related mortality in

N-ALT patients has not been clarified. Deuffic-Burban et al. [22] calculated the impact of Peg-IFN plus ribavirin on morbidity and mortality in N-ALT patients using the Markov model and concluded that antiviral therapy in N-ALT patients would decrease morbidity and mortality rates. However, the treatment of N-ALT patients with CHC still remains an area of investigation, particularly with respect to the benefit-to-risk ratio of treatment. To help determine the indications for antiviral therapy in N-ALT patients, the liver histology should be evaluated before treatment. The presence of significant hepatic fibrosis (≥F2 by the METAVIR classification [23]) reflects continuous hepatic inflammation over a period of time and suggests a future risk of liver-related disease progression. Antiviral therapy may be appropriate for these patients. On the other hand, periodic follow up without antiviral therapy is recommended for patients in stages F0-1, because most of such patients show a low risk for progression to cirrhosis and the development of hepatocellular carcinoma [24].

This study had some limitations. First, the factors of viral mutation and host genetic mutation, which have been reported recently to affect the efficacy of Peg-IFN plus ribavirin combination therapy, could not be measured, and evaluation of the serum HCV RNA levels by a real-time PCR method, which is more sensitive to the measurement of serum HCV RNA levels, could not be done in the patients enrolled in this study, because we had few stored patient serum samples. Detailed examinations using the real-time PCR method in patients who are matched based on the factors of viral mutation and host genetic mutation as well as background factors will be needed for further study. Second, we excluded the factor of fibrosis from the multivariate analysis for factors associated with SVR in N-ALT patients, because data for fibrosis were lacking in 53 of the 193 patients in this study. Accordingly, the present study could not demonstrate whether fibrosis was associated with SVR in N-ALT patients. Finally, in this study, we investigated the antiviral efficacy of Peg-IFN plus ribavirin combination therapy for patients with N-ALT at the start of the therapy, not for patients with 'persistently' normal ALT. Accordingly, this study does not show the efficacy of this treatment in patients with persistently normal ALT. However, we believe that the results obtained in this study can be useful for pre-treatment prediction in outpatients who may not be followed by the reason of having normal ALT levels.

We have shown, in this matched case–control study using a propensity score method, that the therapeutic effect of combination therapy with Peg-IFN alpha-2b and ribavirin in N-ALT patients with CHC is comparable to that for E-ALT patients, irrespective of their advantageous background. Further work is needed to verify that HCV eradication can improve the prognosis of N-ALT patients.

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

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Differential alteration of CD56^{bright} and CD56^{dim} natural killer cells in frequency, phenotype, and cytokine response in chronic hepatitis C virus infection

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Abstract
Background Natural killer (NK) cells play an important role in immune responses to virus infection. The cell population consists of CD56^{bright} (bright-subset) and CD56^{dim} (dim-subset) subsets that possess armed functions of cytokine production and cytolysis, respectively. How these subsets are involved in chronic hepatitis C virus infection (CHC) remains obscure.
Methods We investigated the frequency, phenotype, and cytokine response of these subsets in blood from CHC patients and healthy subjects (HS).
Results Dim-subset, but not bright-subset, showed lower frequency in the patients than in HS. Bright-subset from the patients more frequently expressed the NKG2A/CD94 inhibitory receptor than that from HS, while both subsets from the patients expressed lower levels of the NKG2D activating receptor. Both subsets from the patients displayed a significantly higher level of the signal transducer and activator of transcription (STAT) 1, compared with the HS. Upon stimulation with interferon-α, bright-subset activated less STAT4, required for interferon-γ production, and dim-subset activated more STAT1, required for cytolysis, in the patients than in HS.
Conclusions These results indicate alterations of NK cell subsets in frequency, phenotype, and cytokine response in CHC, which might be associated with the immune pathogenesis of CHC.

Keywords NK cells · CD56^{bright} · CD56^{dim} · HCV · Chronic hepatitis

Abbreviations

NK	Natural killer
IFN	Interferon
IL	Interleukin
HCV	Hepatitis C virus
CHC	Chronic hepatitis C virus infection
CHB	Chronic hepatitis B virus infection
PBMC	Peripheral blood mononuclear cell
STAT	Signal transducer and activator of transcription
pSTAT	Phosphorylated-signal transducer and activator of transcription
HS	Healthy subjects
ISG	Interferon-stimulated gene
MICA	Major histocompatibility complex class I-related chain A

Introduction

Natural killer (NK) cells play an important role in innate immune responses to a variety of viral infections by directly killing infected cells with cytotoxic molecules such

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as perforin and granzyme [1]. The cells also have great ability to secrete a key cytokine, interferon (IFN)-γ, which activates subsequent adaptive immune responses as well as inhibiting viral replication [1, 2]. The activation of NK cells is regulated by several cytokines, such as interleukin (IL)-12, IL-15, and IFN-α [1, 3]. It is also regulated by a balance of activating and inhibitory signals, respectively transmitted by activating and inhibitory receptors that recognize ligands on the cell surface of potential target cells [1, 3]. The NK cell population consists of two subsets in the context of the CD56 surface expression level: $CD56^{bright}$ and $CD56^{dim}$ subsets [4, 5]. It has been reported that $CD56^{dim}$ NK cells are strongly cytolytic armed effector cells, and that $CD56^{bright}$ NK cells are focused on the production of cytokines such as IFN-γ [4, 5]. However, how these subsets are distinguished in the context of immune responses to virus infections remains to be fully elucidated.

Hepatitis C virus (HCV) causes persistent infection in more than 70% of infected patients. Whereas some patients show a carrier-like state, most develop chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, which is why HCV infection is a worldwide health problem [6]. The administration of IFN-α is a well-established anti-viral therapy for HCV infection. More than 90% of patients with acute HCV infection respond to IFN-α-based therapy, while the response rate falls to around 50% for patients with chronic HCV infection (CHC) [7–10], suggesting a mechanism by which persistent HCV infection leads to resistance to IFN-α-based therapy. The NK cell number has been demonstrated to decrease in patients with CHC [11–16], while it is controversial whether NK cell functions are impaired in patients with CHC [17–19]. It thus remains unclear whether the perturbation of NK cells, such as that of $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells, is involved in the persistence of CHC as well as the resistance to therapy.

In the present study, we investigated how the NK cell subsets differ in frequency, phenotype, and response to cytokine stimulation. We also examined how chronic HCV infection modifies those differences. We found clear differences between the NK cell subsets in the response to cytokine stimulation as well as in the frequency and phenotype, which were altered in the CHC patients. This alteration might be associated with the immune pathogenesis of CHC.

Subjects, materials, and methods

Subjects

Eleven patients with CHC (HCV RNA genotype 1) and eleven healthy volunteers were enrolled in this study from October 2007 to March 2008. Flow cytometric analysis was carried out consecutively for these subjects. Since some of the flow cytometric data were lacking for two subjects in the patient group, they were excluded from the final analysis. The demographic data of these analyzed subjects are shown in Table 1. There was no significant difference in demographic variables (age/sex) between these two groups. The patients were histologically diagnosed as having mild or moderate chronic hepatitis and were evaluated for the degree of liver inflammation and fibrosis according to the METAVIR scoring system [20]. In addition, six patients with chronic hepatitis B virus infection (CHB) (male/female 4/2, age range (median) 31–63 years (38), alanine aminotransferase (ALT) range (median) 24–496 IU/l (70), hepatitis B virus (HBV)-DNA viral load range (median) 5.6 to >9.0 log copies/ml (7.3), hepatitis B envelope antigen (HBeAg)+/− 3/3) were enrolled in this study and consecutive flow cytometric analyses were performed. None of the subjects in the patient group displayed any evidence of other types of liver diseases. The study was approved by the Human Research Ethics Committee of Osaka University Hospital.

Isolation of peripheral blood mononuclear cell populations

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized peripheral blood by Ficoll–Hypaque density gradient centrifugation as described elsewhere [21].

Flow cytometric analysis

The staining of prepared cells was performed as described [21–26]. Briefly, for the NK receptor staining, cells were

Table 1 Characteristics of patients and subjects

	CHC	HS
Number	9	11
Sex: M/F	4/5	8/3
Age, years, range (median)	44–74 (60)	45–72 (50)
ALT, IU/l, range (median)	14–136 (43)	ND
Activity (A 0–1/2–3)	(7/2)	ND
Fibrosis (F 0–1/2–3)	(5/4)	ND
Viral load, kIU/ml, range (median)	130 to >5000 (3400)	ND
Viral genotype	1	ND

Serum hepatitis C virus (HCV) RNA levels were quantitatively determined using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 kIU/mL; Roche Diagnostics, Branchburg, NJ, USA)

CHC patients with chronic hepatitis C virus infection, *HS* healthy subjects, *ALT* alanine aminotransferase, *ND* not determined

stained with biotin-conjugated anti-CD56 antibody (B159), allophycocyanin-conjugated anti-CD3 antibody (UCHT1) and phycoerythrin-conjugated anti-NKG2D (1D11), phycoerythrin-conjugated anti-NKG2A (Z199) or fluorescein isothiocyanate-conjugated anti-CD94 antibody (HP-3D9), or the corresponding isotype control, followed by staining with peridinin chlorophyll protein-conjugated streptavidin (BD Biosciences, San Jose, CA, USA). For intracellular staining of the signal transducer and activator of transcription (STAT) or phosphorylated-STAT (pSTAT), cells were stained with biotin-conjugated anti-CD56 antibody (B159) and (1), (2) Alexa Fluor® 647-conjugated or (3), (4) fluorescein isothiocyanate-conjugated anti-CD3 antibody (UCHT1), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and cold pure methanol, and then stained with (1) phycoerythrin-conjugated anti-pSTAT1 (pY701) antibody (4a) and Alexa Fluor® 488-conjugated anti-pSTAT4 (pY693) antibody (38/p-Stat4) or (2) Alexa Fluor® 488-conjugated anti-pSTAT4 (pY693) antibody (38/p-Stat4), or (3) Alexa Fluor® 647-conjugated anti-pSTAT1 (pY701) antibody (4a) or (4) phycoerythrin-conjugated anti-STAT1 antibody (1/Stat1), or the corresponding isotype control, followed by staining with peridinin chlorophyll protein-conjugated streptavidin (BD Biosciences). All antibodies except for anti-NKG2A antibody (Beckman Coulter, Brea, CA, USA) were purchased from BD Biosciences. The stained cells were analyzed with a FACSCalibur (BD Biosciences), and the data were processed using the FlowJo program (Tree Star, Ashland, OR, USA).

In vitro stimulation of PBMCs

Prepared PBMCs were resuspended at 2×10^7 cells/ml in RPMI 1640 (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (Japan Bioserum, Hiroshima, Japan) for in vitro stimulation with cytokines. Natural human IFN-α, recombinant human IFN-γ, and IL-12 were used at concentrations of 1,000 IU/ml, 50 ng/ml, and 10 ng/ml, respectively. For cells left unstimulated, media were added. The cells were incubated at 37°C in 5% CO_2 for 90 min, and then collected for further analyses. Natural human IFN-α was a generous gift from Otsuka Pharma (Tokyo, Japan). Recombinant human IFN-γ and IL-12 were obtained from PBL Biomedical Laboratories (Piscataway, NJ, USA) and R&D Systems (Minneapolis, MN, USA), respectively.

Statistical analysis

The statistical significance of differences between two groups or that of changes in the indicated variables in response to IFN-α treatment was determined by applying an unpaired or paired Student's t-test, respectively. The statistical significance was defined as $p < 0.05$.

Results

Frequency of $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells

The NK cell number has been demonstrated to decrease in patients with CHC [11–16]. We examined the proportion of $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells in PBMCs from the CHC patients and those from the healthy subjects (HS). We defined CD 56^{bright} NK cells or $CD56^{dim}$ NK cells as CD56 bright CD3-negative cells or CD56 dim CD3-negative cells, respectively, by flow cytometry (Fig. 1a). The proportion of $CD56^{bright}$ NK cells was much lower than that of $CD56^{dim}$ NK cells in both the HS and patient groups (Fig. 1b). The proportion of $CD56^{dim}$ NK cells from the CHC patients was significantly lower than that from the HS, while that of $CD56^{bright}$ NK cells did not show a significant difference between these groups.

Expression level of activating or inhibitory NK receptors on $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells

The activation of NK cells is partly regulated by the balance of signals transmitted by activating and inhibitory NK receptors [1, 3]. We therefore examined the expression level of activating or inhibitory NK receptors such as NKG2D or NKG2A/CD94 on $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells, by flow cytometry. We found lower expression of NKG2D, an activating receptor, on $CD56^{bright}$ NK cells than on $CD56^{dim}$ NK cells (Fig. 2). In contrast, the expression of NKG2A/CD94, an inhibitory receptor, on $CD56^{bright}$ NK cells was higher than that on $CD56^{dim}$ NK cells. The expression levels of NKG2D on both $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells from the CHC patients were significantly lower than those from the HS. However, the expression level of NKG2A/CD94 on $CD56^{bright}$ NK cells from the CHC patients was significantly higher than that from the HS, while that on $CD56^{dim}$ NK cells was not significantly different between these groups. In addition, no significant correlation was observed between the expression levels of the activating or inhibitory NK receptors and the level of viral load or the histological level of liver inflammation or fibrosis in the CHC patients (data not shown).

STAT1 expression levels in $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells

We have recently reported that NK cells displayed lower intracellular STAT1 expression than other immune cells

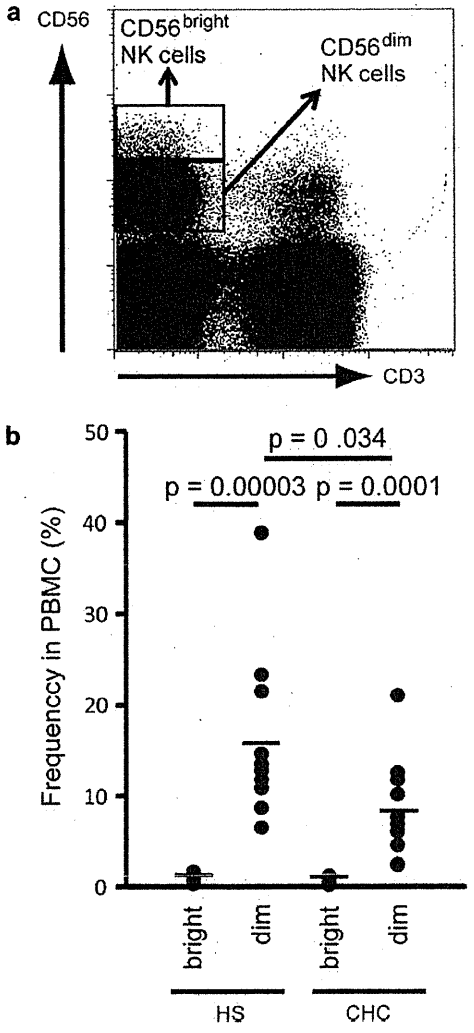

Fig. 1 Frequency of natural killer (*NK*) cell subsets in peripheral blood mononuclear cells (*PBMCs*). Frequencies of $CD56^{bright}$ NK and $CD56^{dim}$ NK cell subsets in PBMCs were evaluated by flow cytometry. PBMCs were obtained from patients with chronic hepatitis C virus (HCV) infection (*CHC*) ($n = 9$) and healthy subjects (*HS*) ($n = 11$). **a** $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells were defined as CD56 bright $CD3^-$ cells or CD56 dim $CD3^-$ cells, respectively, by flow cytometry. CD56 dim and CD56 bright were divided at a threshold where most CD3+ cells lost CD56 expression. **b** The frequency of $CD56^{bright}$ subset (bright) or $CD56^{dim}$ subset (dim) was evaluated by flow cytometry, electronically gating on CD56 bright $CD3^-$ cells or CD56 dim $CD3^-$ cells. Comparisons of the frequencies of those NK cell subsets in PBMCs between bright and dim subsets or between the CHC patients and the HS are shown 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

such as T cells or NKT cells, and that the level was higher in patients with CHC [24]. We therefore examined the expression level of STAT1 in the NK cell subsets, $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells. The expression level of STAT1 in $CD56^{bright}$ NK cells was clearly higher than that in $CD56^{dim}$ NK cells in the HS (Fig. 3a, b). The expression level of STAT1 in either $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells from the CHC patients was significantly higher than that from the HS, and no significant difference was observed in the STAT1 expression levels between $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells in the CHC patients. In addition, no significant correlation was observed between the STAT1 expression level in these NK cell subsets and the level of viral load or the level of liver inflammation or fibrosis in the CHC patients (data not shown). We also examined the expression level of STAT1 in the NK cell subsets from patients with CHB, which is also capable of causing chronic hepatitis. The expression level of STAT1 in either $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells in the CHB patients was modestly, but not significantly, higher than that of the HS (positive cell rate, mean ± SD; 35.0 ± 26.8% in $CD56^{bright}$ subset and 28.7 ± 18.1% in $CD56^{dim}$ subset in the CHB patients).

Activation of STAT1/4 occurring in response to cytokines in $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells

We have previously reported altered IFN-α-signaling in the total NK cell population from CHC patients [24]. We next examined the cytokine signaling in $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells. As IFN-α can phosphorylate both STAT1 and STAT4, IFN-γ can phosphorylate STAT1, and IL-12 can phosphorylate STAT4 in NK cells [1, 3], we evaluated the phosphorylation level of STAT1/4 that occurred in response to IFN-α, IFN-γ, or IL-12 in $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells.

In response to IL-12, the majority of $CD56^{bright}$ NK cells phosphorylated STAT4, while only some of the $CD56^{dim}$ NK cells did so (Fig. 4a). In response to IFN-γ, almost no $CD56^{bright}$ NK cells phosphorylated STAT1, while some of the $CD56^{dim}$ NK cells did so. These patterns were observed in both the HS and the patient group. Statistical analysis demonstrated that the phosphorylation level of STAT4 occurring in response to IL-12 in $CD56^{bright}$ NK cells was significantly higher than that in $CD56^{dim}$ NK cells in both the HS and the CHC patient group, while that of STAT1 occurring in response to IFN-γ in $CD56^{bright}$ NK cells was significantly lower than that in $CD56^{dim}$ NK cells in both groups (Fig. 4b). However, the phosphorylation level of STAT4 or STAT1, respectively, occurring in response to IL-12 or IFN-γ did not show any significant difference between these groups in either $CD56^{bright}$ NK cells or $CD56^{dim}$ NK cells.

In response to IFN-α, the whole population of $CD56^{bright}$ NK cells or CD 56^{dim} NK cells phosphorylated both STAT4 and STAT1 in both subject groups (Fig. 4a). Statistical analysis, however, demonstrated that