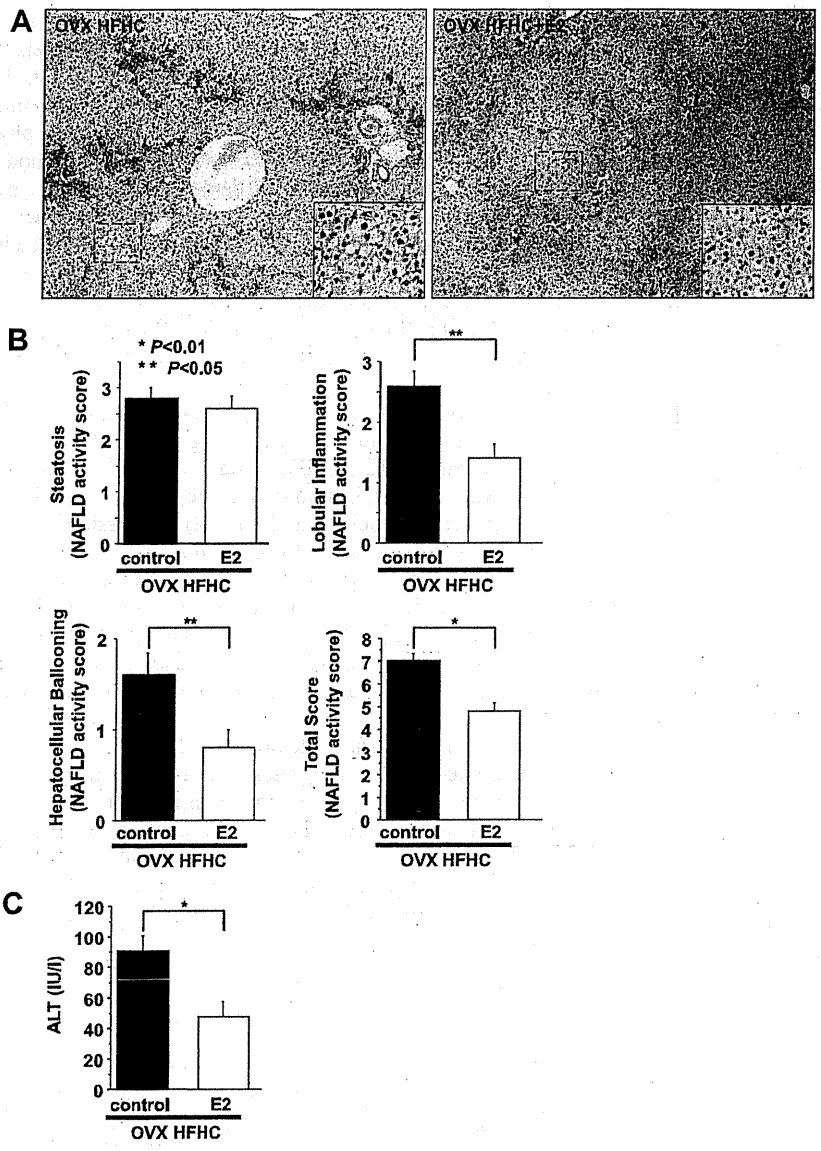

Fig. 5. Treatment with estrogen protects liver injury in OVX mice fed the HFHC diet. *A*: H&E staining of mice liver sections. OVX-HFHC+E2; estrogen pellet-implanted OVX-HFHC mice. *Insets*: magnified views of typical hepatocytes in mice (dashed squares indicate the magnified areas). Original magnification was ×100. *B*: NAFLD activity scores in mice. The graphs represent the scores for hepatic steatosis, lobular inflammation, hepatocellular ballooning, and total NAFLD activity. *C*: serum ALT levels in mice. Solid bars, OVX-HFHC mice (control), open bars: OVX-HFHC+E2. Data are means ± SD. $*P < 0.01$, $**P < 0.05$.

TNF or fatty acid synthase. In addition, dietary cholesterol induced hepatic MCP1 gene expression (48), and monocyte CCR2 expression was increased in hypercholesterolemic patients compared with normocholesterolemic controls (14). Cholesterol directly stimulates the CCR2 promoter activity through its cholesterol response element (7, 14), and, in this study, elevated serum cholesterol in OVX-HFHC mice increased, not only hepatocyte MCP1 expression, but also monocyte CCR2 expression in mice. Enhanced MCP1 expression in mice liver recruited CCR2-positive monocytes into the liver, and these recruited monocytes transformed into inflammatory macrophages.

The accumulation of macrophages at sites of inflammation and injury in the liver is thought to be mediated by chemokines such as MCP1. Accumulated inflammatory macrophages in mice liver produce various inflammatory cytokines, such as TNF-α and IFN-γ, leading to persistent liver injury. Estrogen at physiological concentrations inhibits the spontaneous secretion of these inflammatory cytokines in whole blood cultures (43). Moreover, hydrogen peroxide-induced TNF-α and MCP1 expressions were attenuated by estradiol in peritoneal macrophages from female mice (18), whereas estrogen inhibited MCP1 expression in human endometrial stromal cells, and tamoxifen, an estrogen antagonist, reversed the inhibition of MCP1 by estrogen (2). Finally, estrogen significantly decreased mice monocyte CCR2 expression and decreased chemotaxis of monocytes toward MCP1 (21). These findings suggest that estrogen itself may have hepatoprotection against inflammation by preventing macrophage accumulation and inhibiting proinflammatory cytokine production.

Estrogen has both anti-inflammatory and antifibrotic effects in the liver. During liver fibrogenesis, hepatic stellate cells (HSC) play a central role (11). HSC possess functional ER-β, but not ER-α, to respond directly to estrogen exposure. In addition, estrogen prevented reactive oxygen species and TGF-β production in cultured rat HSC by suppressing NADH/

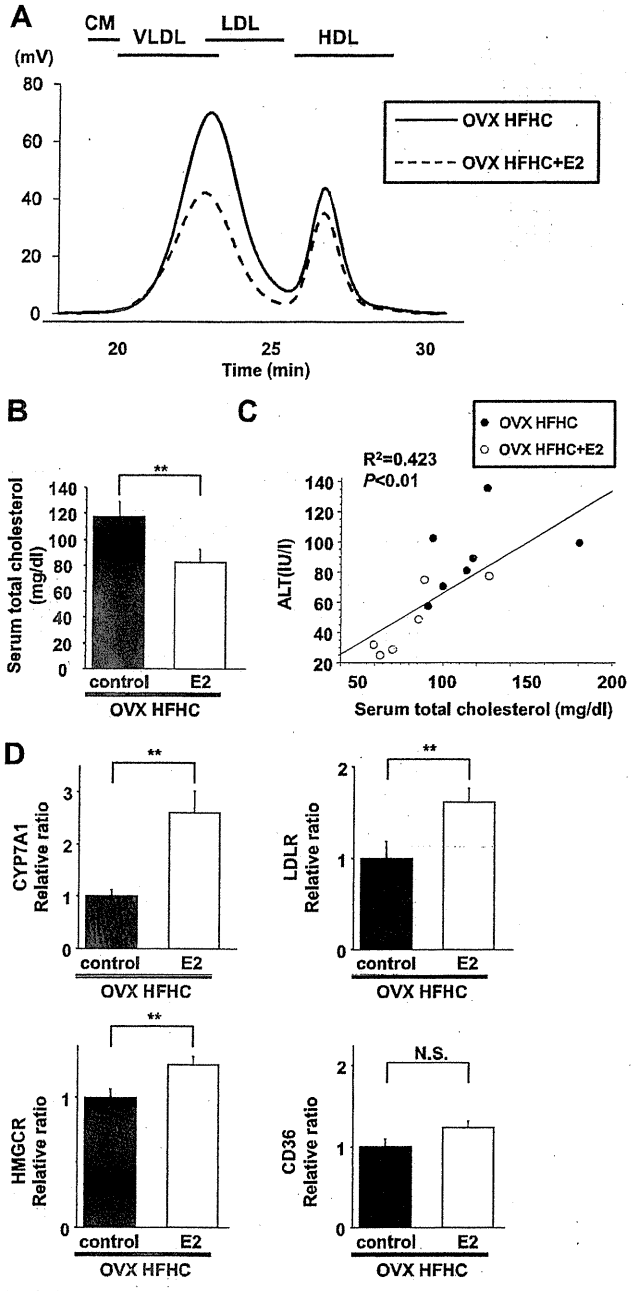

Fig. 6. Cholesterol lipoprotein distribution and serum cholesterol levels in mice treated with or without estrogen. *A*: serum cholesterol lipoprotein distribution analyzed by HPLC in mice. *B*: serum cholesterol levels in mice. Data are means ± SD. $^{**}P < 0.05$. *C*: correlations between serum ALT and cholesterol levels in mice. *D*: hepatic cholesterol metabolism-related gene expression changes in mice. Liver GAPDH-normalized CYP7A1, LDLR, HMGCR, and CD36 gene expressions. Data are means ± SD. $^{*}P < 0.01$, $^{**}P < 0.05$, N.S., not significant.

NADPH oxidase activity (20). In our study, fibrogenic genes, TGF-β1 and collagen Iα1, were upregulated in OVX-HFHC mice, and estrogen treatment improved the hepatic collagen Iα1 expression.

NASH is reported to be correlated with atherosclerotic cardiovascular diseases independently of Mets (47). In this study, we used HFHC diet (containing 15% cocoa butter, 1.25% cholesterol, and 0.5% cholate) to induce steatohepatitis in mice. This diet is known to be an atherogenic diet. This HFHC diet is also known to induce liver steatosis, inflammation, and fibrosis (34). This model seems to be a more physiological dietary model of NASH than existing animal models, which require genetic defects or the depletion of nutrients, such as methionine and choline deficiency diet-induced model. Moreover, the liver pathology induced by HFHC diet feeding involves steatohepatitis with hepatocellular ballooning, a necessary histological feature defining human NASH. These results indicate that the HFHC diet-induced NASH model in mice is one of the better experimental models of human NASH.

It was reported that mice fed HFHC diet were smaller than control mice, and insulin sensitivity and glucose tolerance were similar between HFHC-fed mice and NC diet-fed mice (29, 34). However, hepatic insulin resistance, as assessed by the liver insulin receptor substrate-2 (IRS-2) expression, was increased in HFHC-fed mice (34). In this study, both body weight and insulin resistance, as assessed by HOMA-IR, in HFHC diet-fed mice did not increase compared with in NC diet-fed mice, and IRS-2 gene expression was decreased in HFHC diet-fed mice liver (Table 2). These results indicated that hepatic insulin resistance would exist in HFHC-fed mice in our study.

In conclusion, our study demonstrated that estrogen deficiency promoted NASH progression and that estrogen treatment improved NASH progression in mice fed HFHC diet. Extrapolation of our results into the clinical setting suggests that estrogen deficiency and estrogen deficiency-induced hypercholesterolemia in postmenopausal women could potentially exacerbate NASH progression through enhanced liver inflammatory macrophage infiltration.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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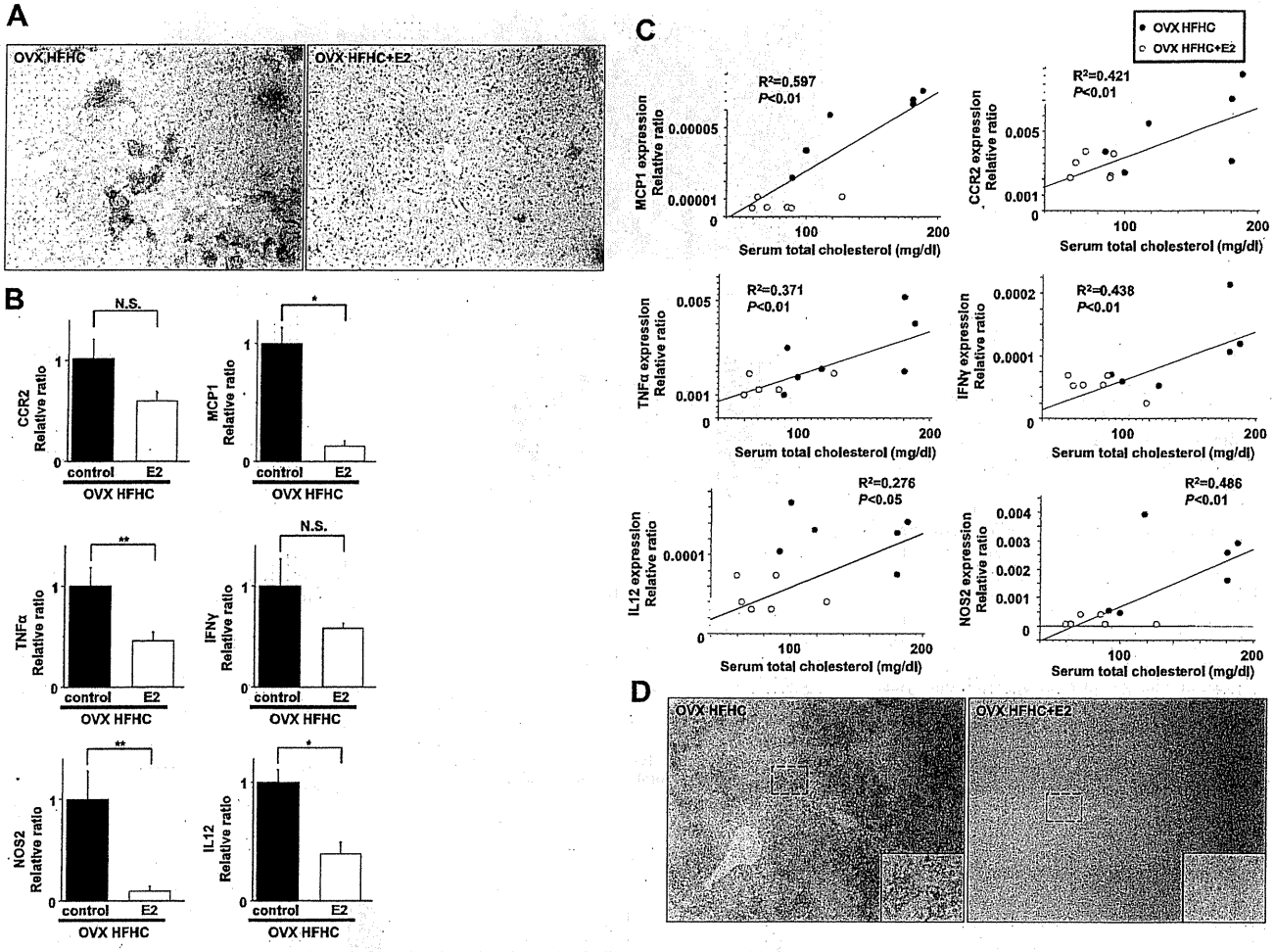

Fig. 7. OVX-exacerbated liver macrophage infiltration in mice fed HFHC diet was attenuated by estrogen treatment. *A*: F4/80 immunohistochemistry of mice livers. Original magnification, ×200. *B*: hepatic inflammation-related gene expression changes in mice livers. Liver GAPDH-normalized CCR2, MCP1, TNF-α, IFN-γ, NOS2, and IL12 gene expression determined by real-time RT-PCR. Data are means ± SD. $*P < 0.01$, $**P < 0.05$. N.S., not significant. *C*: correlations between serum cholesterol levels and liver inflammation-related gene expressions. *D*: MCP1 immunohistochemistry of mice livers. *Insets*: magnified views of mice livers (dashed squares indicate the magnified areas). Original magnification, ×100.

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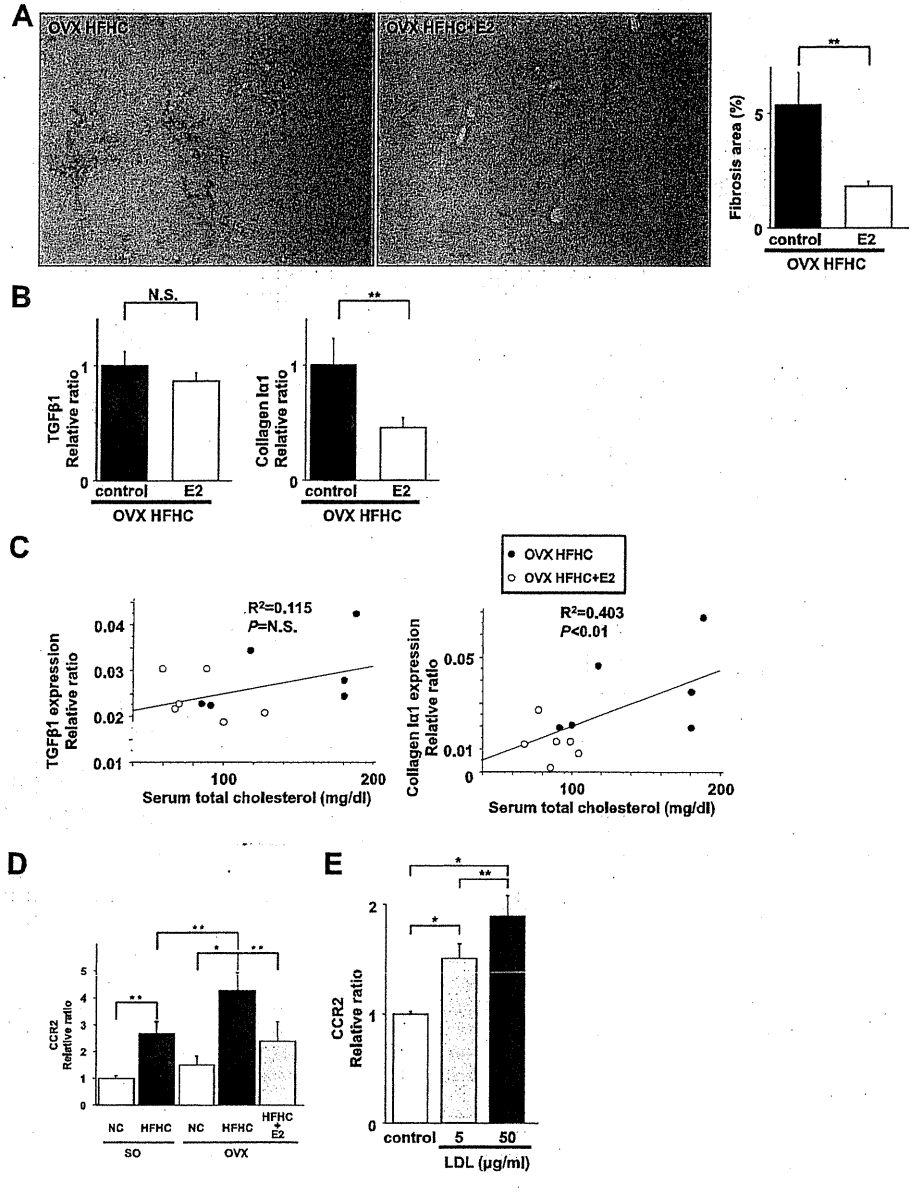

Fig. 8. Amelioration of hepatic fibrosis in OVX-HFHC mice treated with estrogen. *A*: picrosirius red staining of mice livers. Original magnification, ×100. Histographic representation of quantified data. Areas of fibrosis were measured as described in MATERIALS AND METHODS. The degree of fibrosis was expressed as the percentage of the total area measured. *B*: gene expression changes related to fibrosis. Liver GAPDH-normalized TGF-β1 and collagen Iα1 gene expression was determined by real-time RT-PCR. Data are means ± SD. $^{**}P < 0.05$; N.S., not significant. *C*: correlation between serum cholesterol level and fibrosis-related genes (TGF-β1, collagen Iα1). *D*: CCR2 gene expression changes in mice spleen monocytes from 5 groups in this study. *E*: CCR2 gene expression changes in THP1 monocytes treated with LDL. CCR2 gene expression changes were determined by real-time RT-PCR. Data are means ± SD. $^{*}P < 0.01$, $^{**}P < 0.05$.

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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

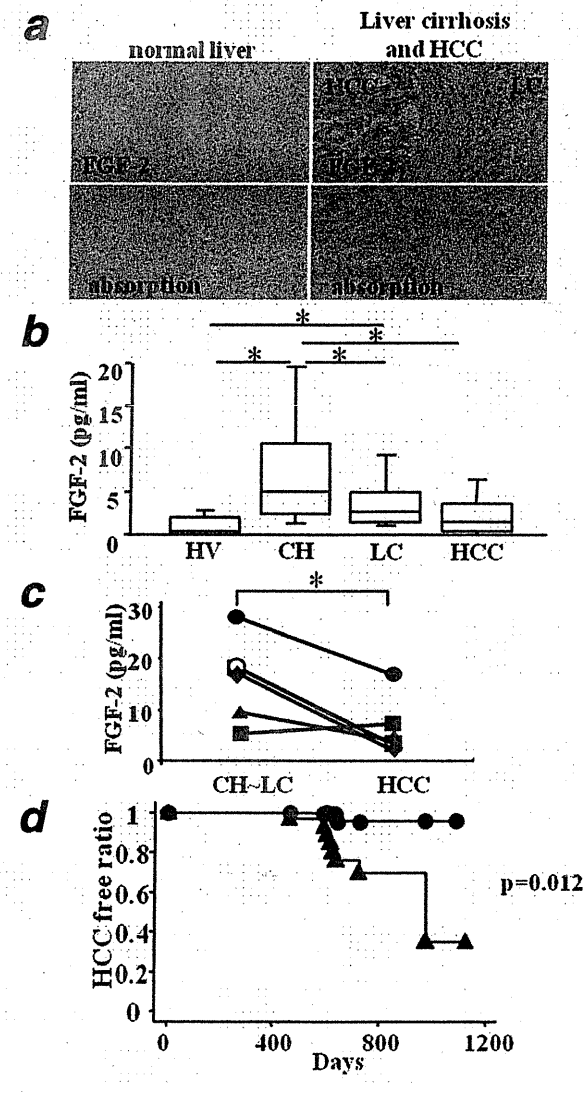

Figure 1. Expressions of FGF-2 in the liver of patients with chronic liver diseases and serum FGF-2 levels in chronic liver disease patients were associated with HCC incidence. (*a*) Immunohistochemical analysis of FGF-2 in normal liver tissues ($N = 2$), chronic hepatitis tissues ($N = 4$), liver cirrhosis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

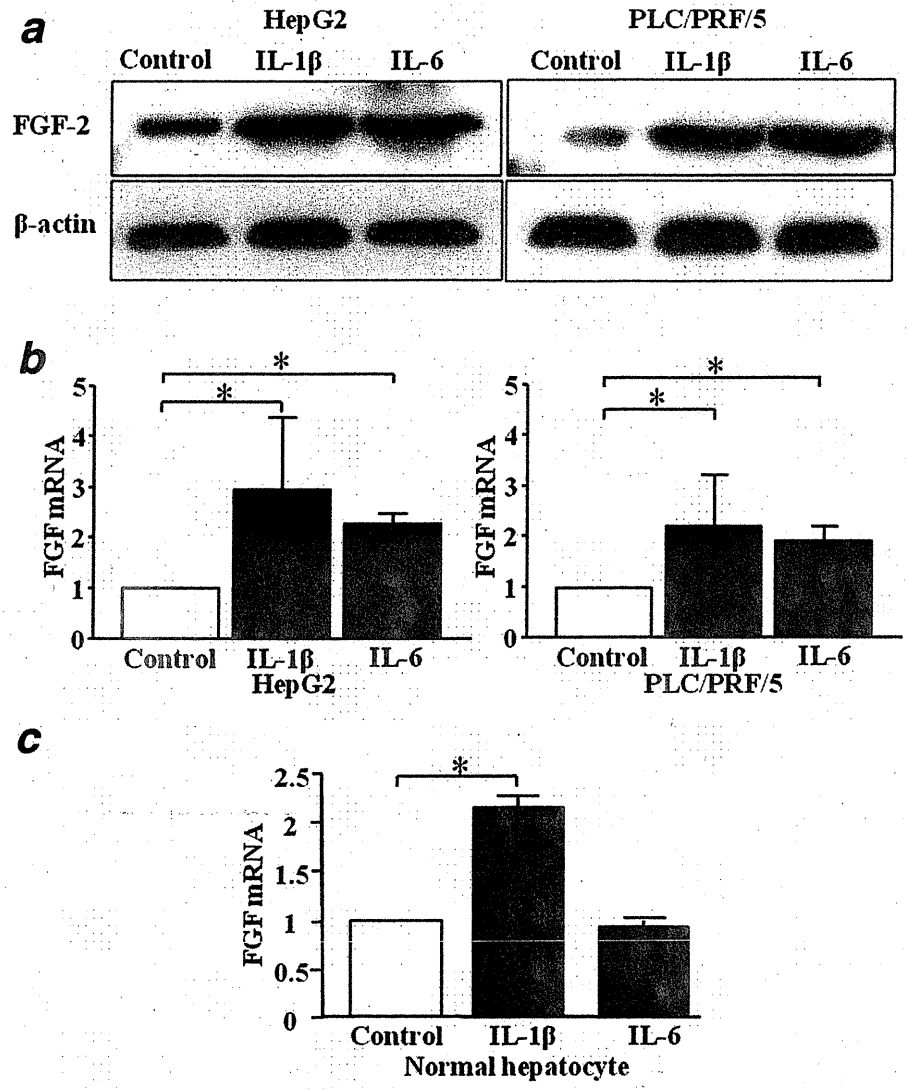

Figure 2. IL-1β and IL-6 increased FGF-2 expressions on human HCC cells and normal hepatocytes. To examine the effect of IL-1β and IL-6 on FGF-2 expression, HepG2 and PLC/PRF/5 cells (*a,b*) or normal hepatocytes (*c*) were cultured for 72 hr in the presence or absence of IL-1β (50 ng/ml) and IL-6 (300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

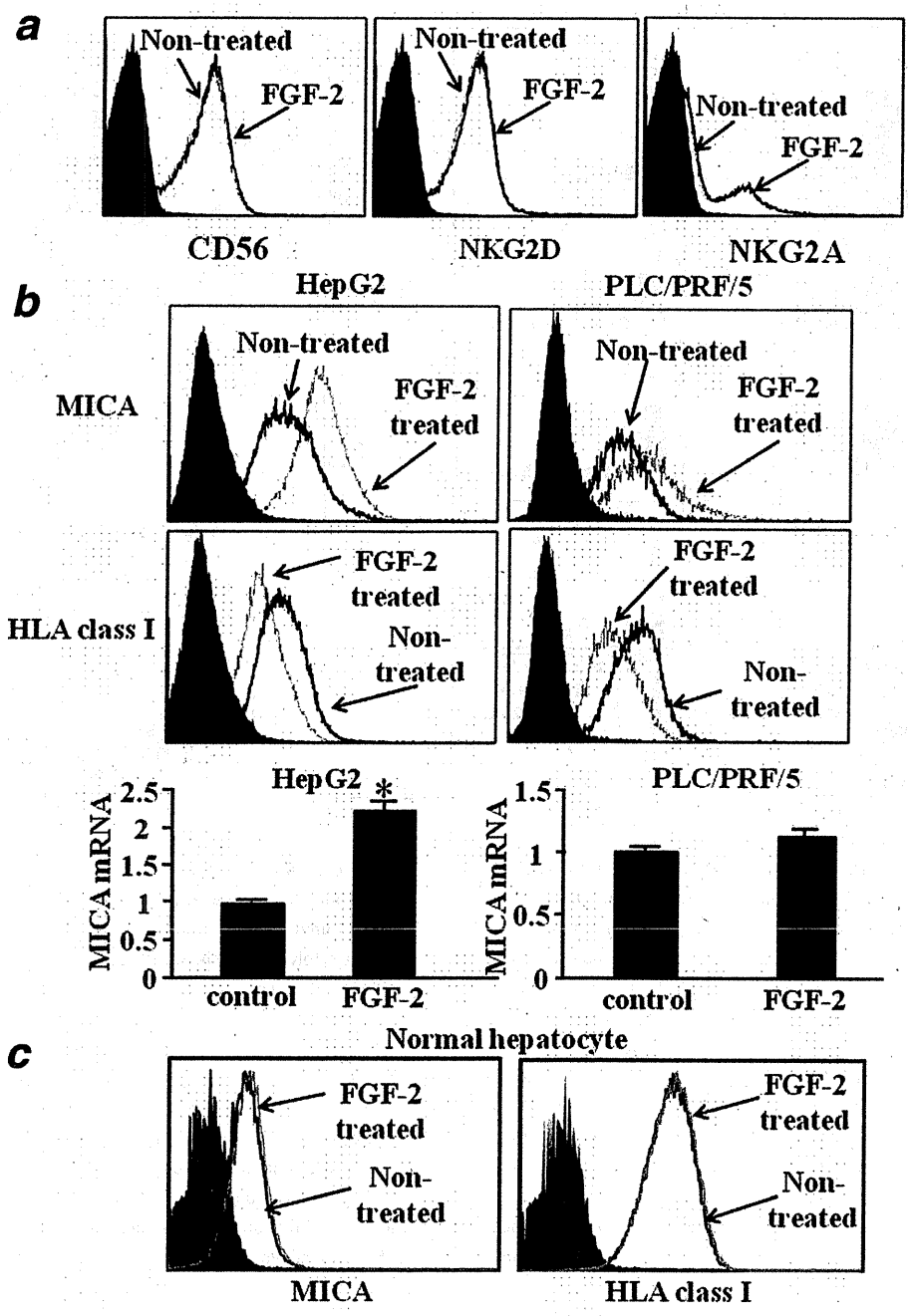

Figure 3. The expressions of NKG2D and NKG2A on FGF-2-treated NK cells and the expressions of MICA and HLA class I on FGF-2-treated hepatoma cells. (*a*) The expressions of NKG2D or NKG2A on FGF-2-treated or nontreated NK cells were evaluated. NK cells obtained from healthy volunteers (2×10^6 cells/well)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

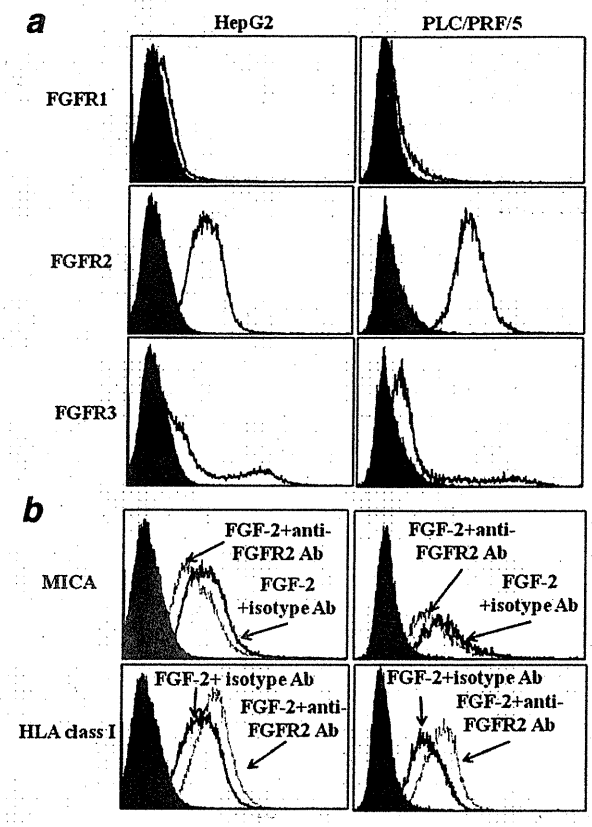

Figure 4. The expressions of FGF receptors on hepatoma cells. (*a*) The expressions of FGF receptors (FGFR1, FGFR2, and FGFR3) on both HepG2 and PLC/PRF/5 cells were evaluated by flow cytometry. Black line histograms: staining of each FGF receptors (FGFR1, FGFR2, FGFR3), shaded/black histograms: control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

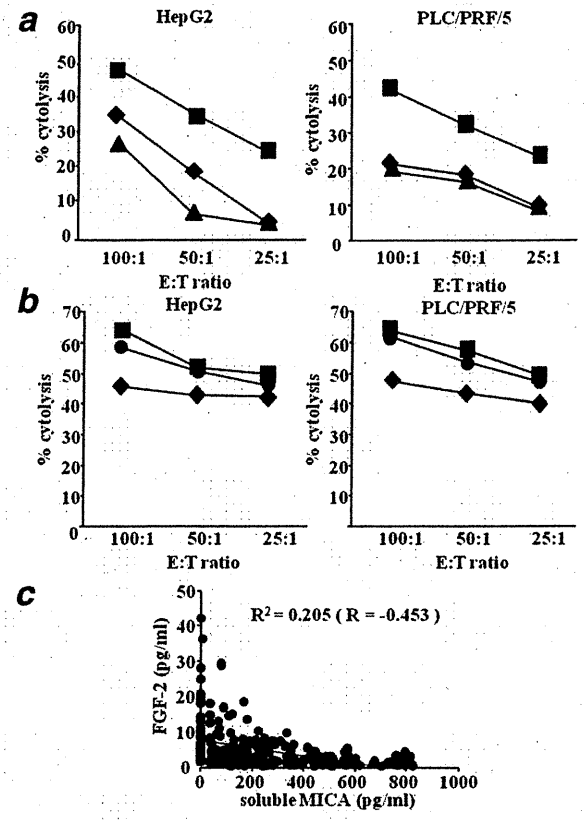

Figure 5. The cytolytic activity against FGF-2-treated HCC cells and the correlation between serum FGF-2 and soluble MICA in patients with chronic liver disease. (*a,b*) Both HepG2 and PLC/PRF/5 cells were cultured with or without FGF-2 protein (250 ng/ml) for 48 hr, and the cytolytic activities of NK 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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The rs8099917 Polymorphism, When Determined by a Suitable Genotyping Method, Is a Better Predictor for Response to Pegylated Alpha Interferon/Ribavirin Therapy in Japanese Patients than Other Single Nucleotide Polymorphisms Associated with Interleukin-28B▽†

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We focused on determining the most accurate and convenient genotyping methods and most appropriate single nucleotide polymorphism (SNP) among four such polymorphisms associated with interleukin-28B (IL-28B) in order to design tailor-made therapy for patients with chronic hepatitis C virus (HCV) patients. First, five different methods (direct sequencing, high-resolution melting analysis [HRM], hybridization probe [HP], the InvaderPlus assay [Invader], and the TaqMan SNP genotyping assay [TaqMan]) were developed for genotyping four SNPs (rs11881222, rs8103142, rs8099917, and rs12979860) associated with IL-28B, and their accuracies were compared for 292 Japanese patients. Next, the four SNPs associated with IL-28B were genotyped by Invader for 416 additional Japanese patients, and the response to pegylated interferon/ribavirin (PEG-IFN/RBV) treatment was evaluated when the four SNPs were not in linkage disequilibrium (LD). HRM failed to genotype one of the four SNPs in five patients. In 2 of 287 patients, the results of genotyping rs8099917 by direct sequencing differed from the results of the other three methods. The HP, TaqMan, and Invader methods were accurate for determination of the SNPs associated with IL-28B. In 10 of the 708 (1.4%) patients, the four SNPs were not in LD. Eight of nine (88.9%) patients whose rs8099917 was homozygous for the major allele were virological responders, even though one or more of the other SNPs were heterozygous. The HP, TaqMan, and Invader methods were suitable to determine the SNPs associated with IL-28B. The rs8099917 polymorphism should be the best predictor for the response to the PEG-IFN/RBV treatment among Japanese chronic hepatitis C patients.

Hepatitis C virus (HCV) infection is a global health problem, with worldwide estimates of 120 to 130 million carriers (7). Chronic HCV infection can lead to progressive liver disease, resulting in cirrhosis and complications, including decompensated liver disease and hepatocellular carcinoma (25). The current standard of care treatment for suitable patients with chronic HCV infection consists of pegylated alpha 2a or 2b interferon (PEG-IFN) given by injection in combination with oral ribavirin (RBV), for 24 or 48 weeks, dependent on HCV genotype. Large-scale treatment programs in the United States and Europe showed that 42 to 52% of patients with HCV genotype 1 achieved a sustained virological response (SVR) (3, 8, 13), and similar results were found in Japan. This treatment is associated with well-described side effects (such as a flu-like syndrome, hematologic abnormalities, and neuropsychiatric events) resulting in reduced compliance and fewer patients completing treatment (2). It is valuable to predict an individual's response before treatment with PEG-IFN/RBV to avoid these side effects, as well as to reduce the treatment cost. The HCV genotype, in particular, is used to predict the response: patients with HCV genotype 2 or 3 have a relatively high rate of SVR (70 to 80%) with 24 weeks of treatment, whereas those infected with genotype 1 have a much lower rate of SVR despite 48 weeks of treatment (8).

Recently, we reported from genome-wide association stud-

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TABLE 1. Characteristics of the patients examined

Parameter	Result for:	
	1st stage (n = 292)	2nd stage (n = 416)
Age (yr)	57.2 ± 10.2	56.6 ± 10.9
No. of patients male/female	145/147	194/222
No. (%) of patients in institution[a]:		
1	18 (6.2)	0 (0)
2	178 (61.0)	0 (0)
3	57 (19.5)	0 (0)
4	39 (13.3)	0 (0)
5	0 (0)	249 (59.9)
6	0 (0)	94 (22.6)
7	0 (0)	52 (12.5)
8	0 (0)	21 (5.0)

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ies (GWAS) that several highly correlated common single nucleotide polymorphisms (SNPs), located in the vicinity of the lambda 3 interferon (IFN-λ3), coded for by the interleukin-28B (IL-28B) gene on chromosome 19, are implicated in nonvirological response (NVR) to PEG-IFN/RBV among patients with HCV genotype 1 (21). At almost exactly the same time as our report, the association between response to PEG-IFN/RBV and SNPs associated with IL-28B was reported from the results of GWAS by two other groups (6, 19). Determination of these SNPs associated with IL-28B before PEG-IFN/RBV treatment will provide extremely valuable information, because the patients predicted as showing NVR to PEG-IFN/RBV treatment could avoid the treatment. There are two questions to be asked before using these SNPs in clinical practice: (i) which methods for genotyping these SNPs are efficient, and (ii) which SNP is most informative in cases where the SNPs are not in linkage disequilibrium (LD)? We have developed five different methods for detecting the SNPs associated with IL-28B and compared their accuracies to establish the most efficient genotyping method. The response to PEG-IFN/RBV treatment was evaluated, when the SNPs associated with IL-28B were not in LD, to determine the best SNP to predict the response to PEG-IFN/RBV treatment.

MATERIALS AND METHODS

Study population. Samples were obtained from 708 Japanese chronic hepatitis C patients and divided into groups of 292 patients (145 males and 147 females; mean age, 57.2 years) and 416 patients (194 males and 222 females; mean age, 56.6 years) for the first and second stages (Table 1). In the first stage, we focused on analyzing the effective methods for determining the genotypes of four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) associated with IL-28B (Fig. 1A). Figure 2 shows the locations of these four SNPs in chromosome 19; rs11881222 and rs8103142 are located in the IL-28B gene, and rs12979860 and rs8099917 are located downstream from the IL-28B gene. The results of genotyping the four SNPs by five different methods, described below, were compared and evaluated for consistency. For this first stage, the 292 chronic hepatitis C patients were recruited from the National Center for Global Health and Medicine; Hokkaido University Hospital, Tonami General Hospital, and Shin-Kokura Hospital in Japan (Table 1). From the results of the first stage, the InvaderPlus assay was chosen as one of the best methods to determine the genotypes of the four SNPs associated with IL-28B and was used for genotyping 416 patients (Fig.

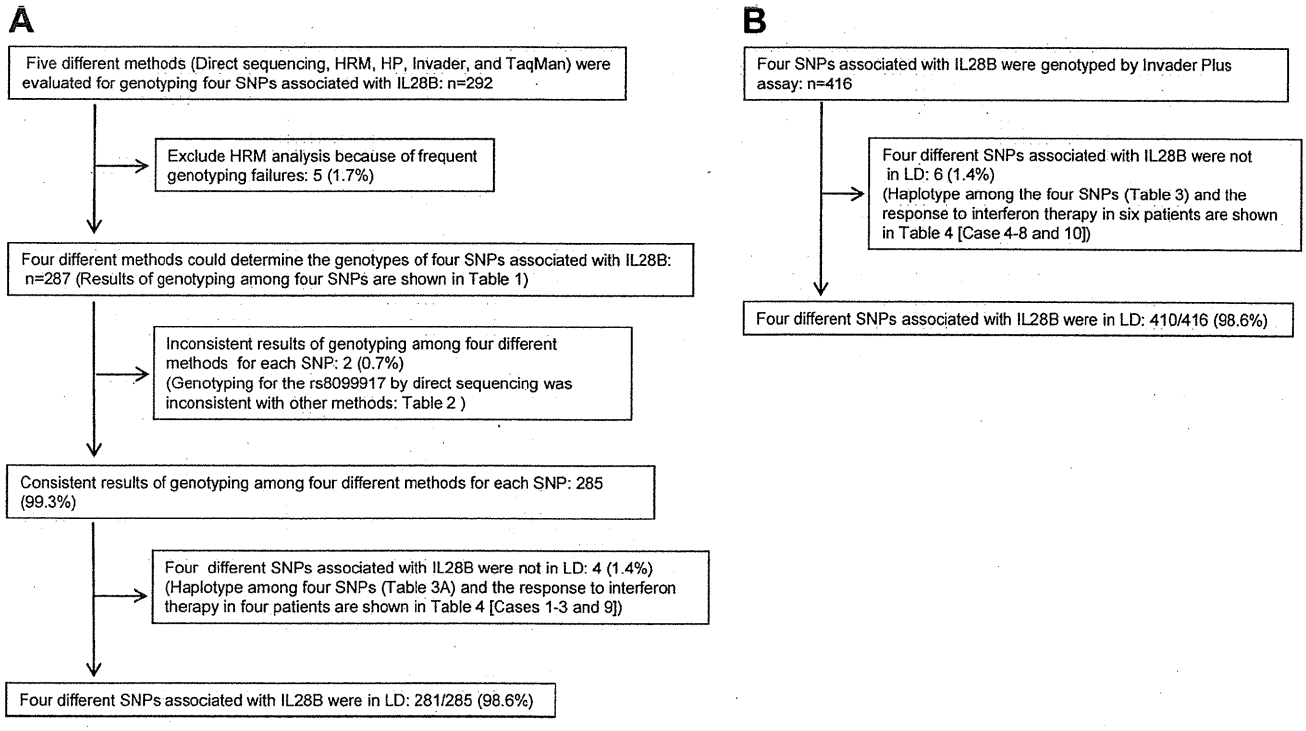

FIG. 1. Schema for the flowchart of the examinations.

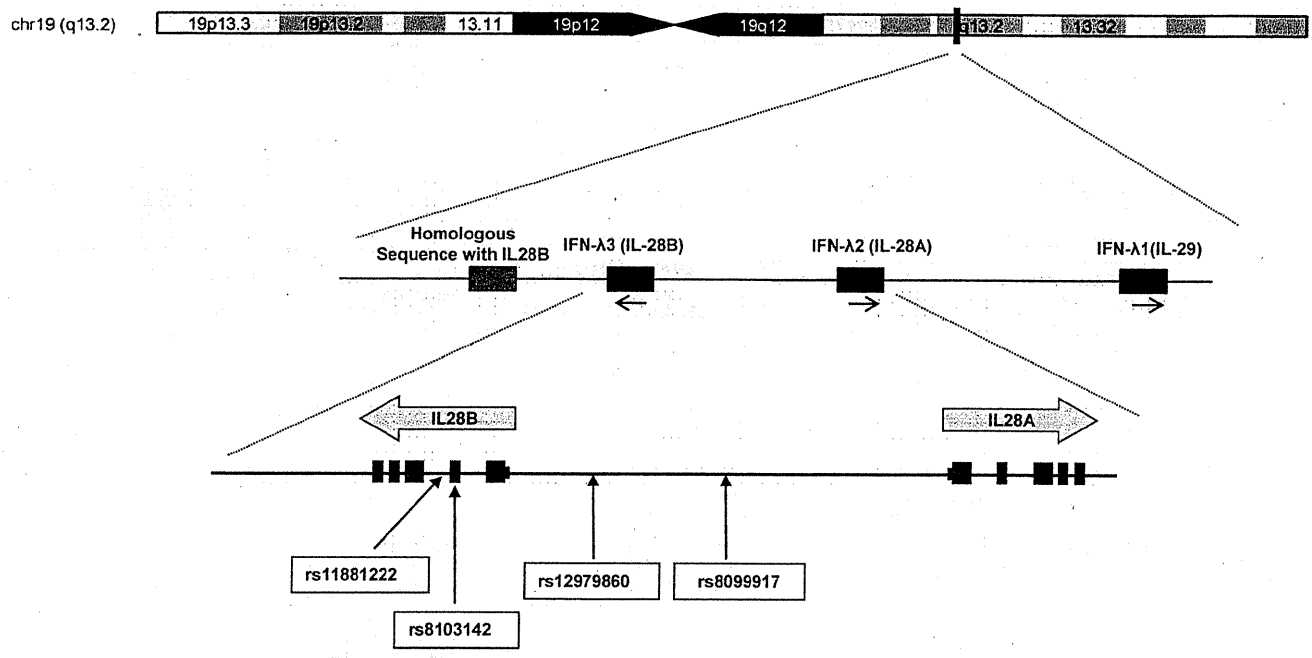

FIG. 2. Location of interferon lambda genes and the four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) associated with IL-28B. chr19, chromosome 19.

1B), recruited from NHO Nagasaki Medical Center, Nagoya City University Hospital, Nagoya Daini Red Cross Hospital, and Kawasaki Medical University Hospital in Japan, in the second stage (Table 1). We then focused on 10 patients whose four SNPs were found in the first and second stages not to be in LD and investigated the response to PEG-IFN/RBV treatment in detail for these patients. Informed consent was obtained from each patient who participated in the study. This study was conducted in accordance with provisions of the Declaration of Helsinki.

Definition of treatment responses. Nonvirological response (NVR) was defined as less than a 2-log-unit decline in the serum level of HCV RNA from the pretreatment baseline value within the first 12 weeks or detectable viremia 24 weeks after treatment. Virological response (VR) was defined in this study as the achievement of sustained VR (SVR) or transient VR (TVR); SVR was defined as undetectable HCV RNA in serum 6 months after the end of treatment, whereas TVR was defined as a reappearance of HCV RNA in serum after treatment was discontinued in a patient who had undetectable HCV RNA during the therapy or had achieved a more than 2-log-unit decline within the first 12 weeks after treatment.

DNA extraction. Whole blood was collected from all participants and centrifuged to separate the buffy coat. Genomic DNA was extracted from the buffy coat with Genomix (Talent SRL, Italy).

Five different genotyping methods. Four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) (Fig. 2) were determined in 292 patients by five different genotyping methods. We developed the five methods (direct sequencing, high-resolution melting analysis [HRM], hybridization probe (HP), Invader-Plus assay (Invader), and the TaqMan SNP genotyping assay (TaqMan) to determine the genotypes of the rs11881222 and rs8103142 polymorphisms. We also developed four different methods (direct sequencing, HRM, HP, and Invader) to determine the genotypes of the rs12979860 and rs8099917 polymorphisms. The genotype of rs12979860 was also determined by the TaqMan genotyping method developed by Duke University, and the genotype of rs8099917 was also determined with the TaqMan predesigned SNP genotyping assay. Figures 3,

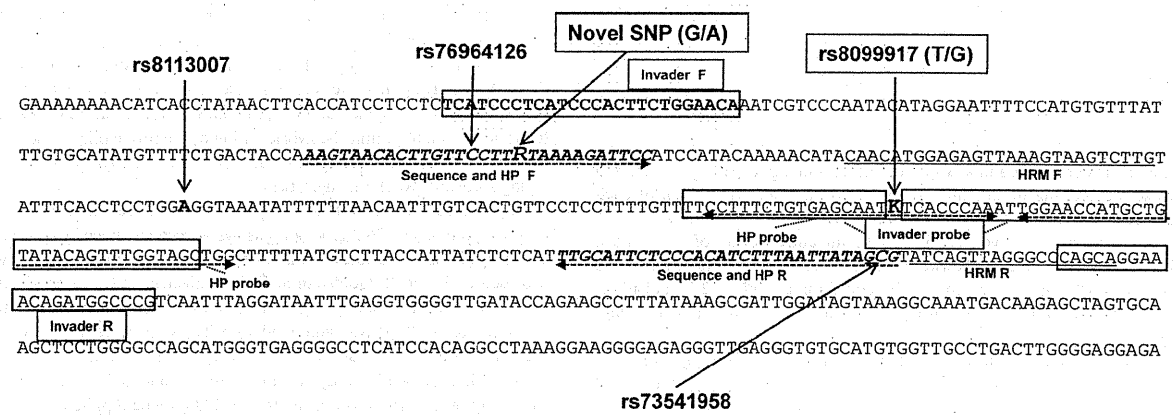

FIG. 3. The nucleotide sequence around rs8099917 is shown. Primers and probes for four different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay) to determine rs8099917 polymorphism are shown. F, forward primer; R, reverse primer.

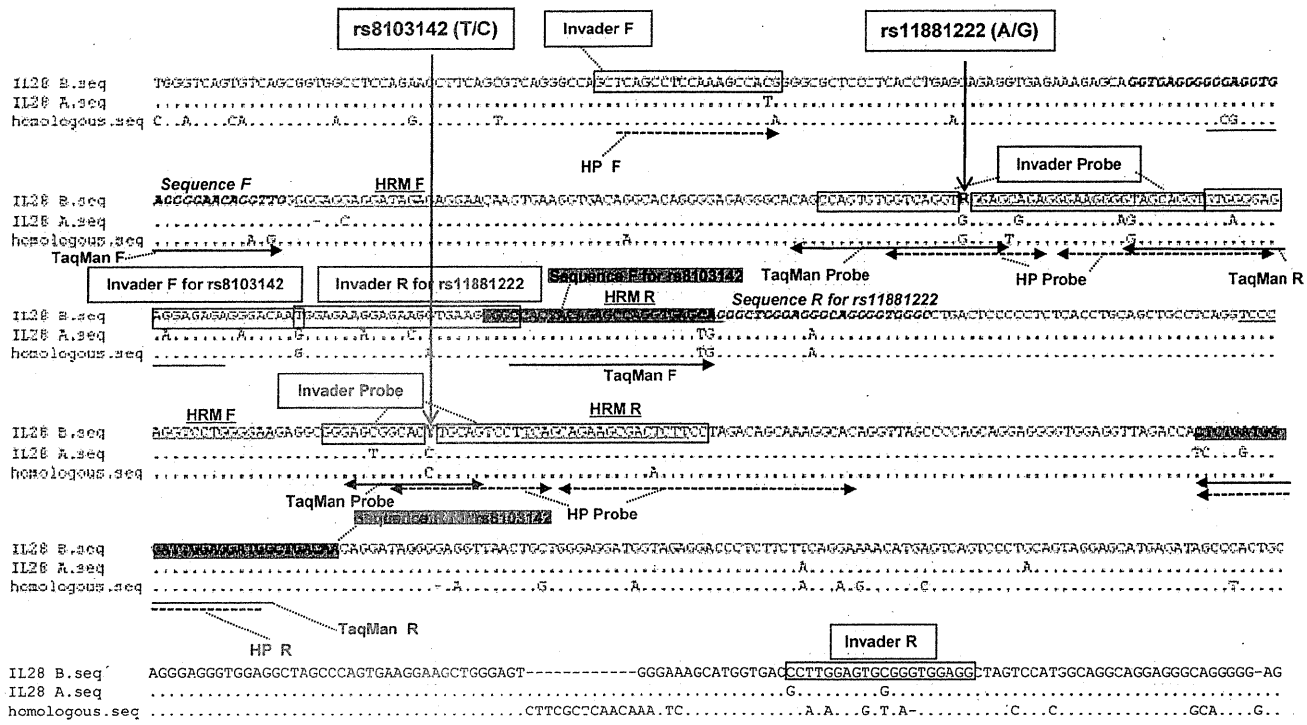

FIG. 4. The nucleotide sequence around rs11881222 and rs8103142 is shown. Primers and probes for five different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay; TaqMan, TaqMan assay) to determine rs11881222 and rs8103142 polymorphisms are shown. F, forward primer; R, reverse primer.

4, and 5 show the primers and probes for each genotyping method. Because the sequence of IL-28B is very similar to those of IL-28A, IL-29, and a homologous sequence upstream of IL-28B, we had to design the primers and probe for each method to distinguish IL-28B from the other sequences. First, primers were designed with Visual OMP Nucleic Acid software, and then we confirmed that the candidate primers should not amplify sequences other than the target region by using UCSC Genome Browser. Next, we confirmed that the amplicon was resolved as a single band, when the PCR products amplified by the primers under evaluation were electrophoresed. Finally, we had to optimize each set of primers and probe for each method (Fig. 3 to 5; see the table in the supplemental material).

Direct sequencing. PCR was carried out with 12.5 μl AmpliTaq Gold 360 master mix (Applied Biosystems), 10 pmol of each primer, and 10 ng of genomic DNA under the following thermal cycler conditions: stage 1, 94°C for 5 min; stage 2, 94°C for 30 s, 65°C for 30 s, 72°C for 45 s, for a total of 35 cycles; and stage 3, 72°C for 7 min. For sequencing, 1.0 μl of the PCR products was incubated with the use of a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). After ethanol purification, the reaction products were applied to the Applied Biosystems 3130xl DNA analyzer.

HRM analysis. HRM analysis was performed on a LightCycler 480 (LC480; Roche Diagnostics) as described previously (5, 15, 24). We designed pairs of primers flanking each SNP (Fig. 3 to 5) to amplify DNA fragments shorter than 200 bp. PCR was performed in a 20-μl volume containing 10 μl LightCycler 480 high-resolution melting master mix (Roche Applied Science), 4 pmol of each primer, and 10 ng genomic DNA. The cycling conditions were as follows: SYBR green I detection format, 1 cycle of 95°C for 10 min and 50 cycles of 95°C for 5 s, 60°C for 10s, and 72°C for 20 s, followed by an HRM step of 95°C for 1 min, 40°C for 1 min, and 74°C for 5 s and continuous acquisition to 90°C at 25 acquisitions per 1°C. HRM data were analyzed with Gene Scanning software (Roche Diagnostics).

Hybridization probe. We designed oligonucleotide primers and hybridization probes for the four SNPs (Fig. 3 to 5). All assays were performed with the LC480 as described previously (4, 18). The amplification mixture consisted of 4 μl of 5× reaction mixture (LightCycler 480 genotyping master; Roche Diagnostics), 5 pmol of each oligonucleotide primer, 3.2 pmol of each oligonucleotide probe, and 10 ng of template DNA in a final volume of 20 μl. Samples were amplified as follows: 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 20 s. The generation of target amplicons for each sample was monitored between the annealing and elongation steps at 610 and 640 nm. Samples positive for target genes were identified by the instrument at the cycle number where the fluorescence attributable to the target sequences exceeded that measured as background. Those scored as positive by the instrument were confirmed by visual inspection of the graphical plot (cycle number versus fluorescence value) generated by the instrument.

InvaderPlus assay. The InvaderPlus assay, which combines PCR and the Invader reaction (11, 12), was performed with the LC480. The enzymes used in InvaderPlus are native *Taq* polymerase (Promega Corporation, Madison, WI) and Cleavase enzyme (Third Wave Technologies, Madison, WI). The reaction is configured to use PCR primers with a melting temperature (T_m) of 72°C and Invader detection probe with a target-specific T_m of 63°C. The Invader oligonucleotide overlaps the probe by one nucleotide, forming at 63°C an overlap flap substrate for the Cleavase enzyme. The first step of InvaderPlus is PCR target amplification, in which the reaction is subjected to 18 cycles of a denaturation step (95°C for 15 s) and hybridization and extension steps (70°C for 1 min). At the end of PCR cycling, the reaction mixture is incubated at 99°C for 10 min to inactivate the *Taq* polymerase. Next, the reaction temperature is lowered to 63°C for 15 to 30 min to permit the hybridization of the probe oligonucleotide and the formation of the overlap flap structure. Data were analyzed by endpoint genotyping software (Roche Diagnostics).

TaqMan assay. The rs8099917 polymorphism was determined by using TaqMan predesigned SNP genotyping assays, as recommended by the manufacturer. The TaqMan assay for determination of the genotype of rs12979860 was kindly provided by David B. Goldstein at Duke University. We designed primers and probes for TaqMan genotyping assays for the other two SNPs. Each genomic DNA sample (20 ng) was amplified with TaqMan universal PCR master mix reagent (Applied Biosystems, Foster City, CA) combined with the specific TaqMan SNP genotyping assay mixture, corresponding to the SNP to be genotyped. The assays were carried out using the LC480 (Roche Applied Science) and the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analyzed by endpoint genotyping software (Roche Diagnostics).

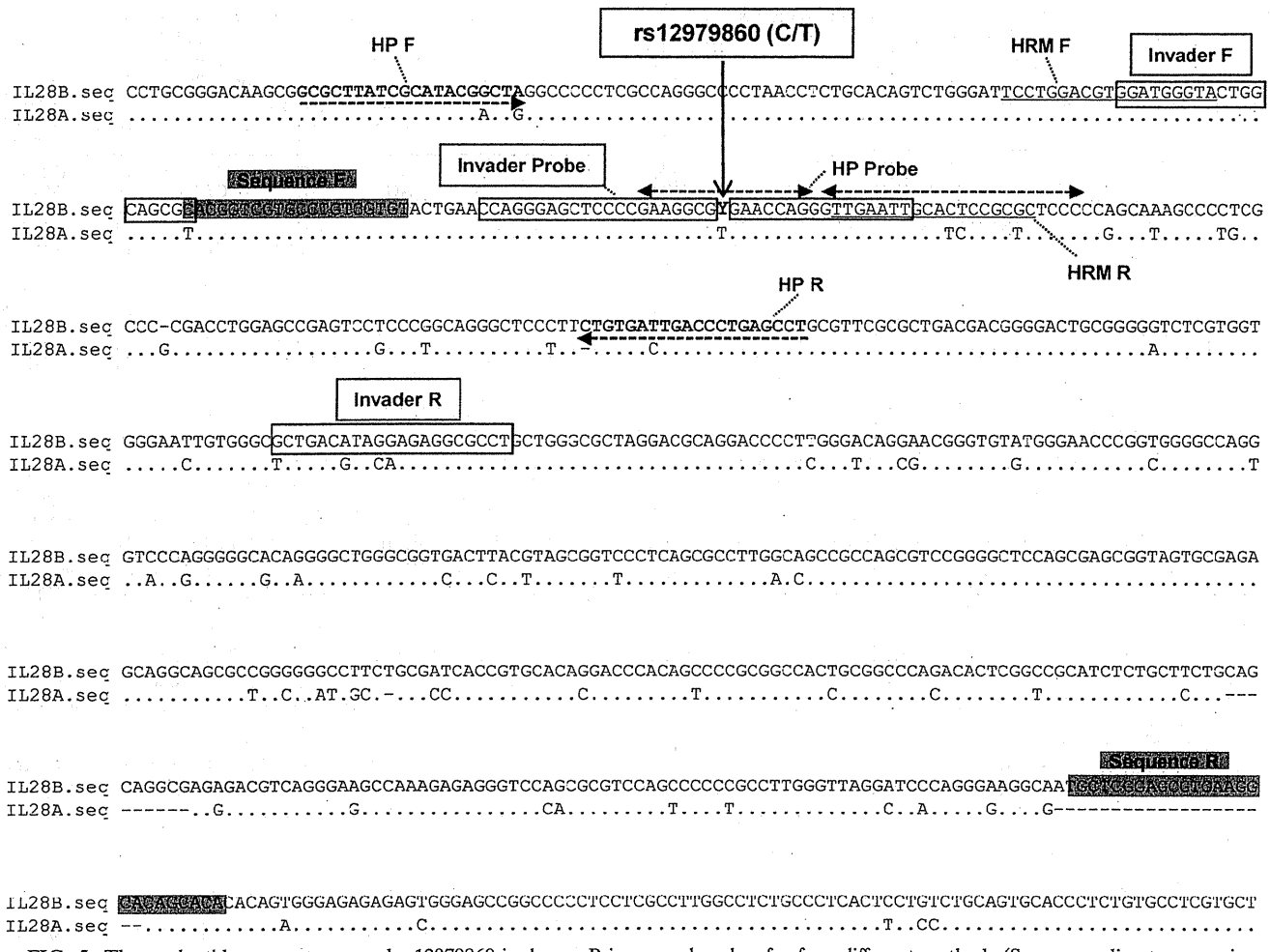

FIG. 5. The nucleotide sequence around rs12979860 is shown. Primers and probes for four different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay) to determine rs12979860 are shown. F, forward primer; R, reverse primer.

RESULTS

Genotyping for four SNPs associated with IL-28B was unsuccessful by HRM in five cases. Figure 1A shows the patients' flowchart of the first stage. Genotyping of four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) was attempted by five different methods (direct sequencing, HRM, HP, Invader, and TaqMan) for 292 patients. In five cases, one of the four SNPs could not be genotyped by HRM. Therefore, we excluded the HRM method from further study. The genotyping failures by HRM involved two cases for rs11881222, two cases for rs8103142, and one case for rs8099917.

Consistencies of four different methods to determine genotypes for four SNPs associated with IL-28B. Consistencies among the results of genotyping by the remaining four methods were 100%, except for the results for rs8099917 (Table 2). For rs8099917, the results determined by direct sequencing were inconsistent with the other three methods in two cases (Tables 2 and 3). The HP, TaqMan, and Invader methods were accurate and reliable for genotyping the four SNPs associated with IL-28B. Invader was chosen for genotyping in the second stage, because the analysis time was the shortest and the sen-

TABLE 2. Determination of four SNPs associated with IL-28B by four different methods[a]

SNP	Genotype	No. (%) of cases with genotype by:			
		Direct sequencing	HP	Invader	TaqMan
rs11881222	AA	199 (69.3)	199 (69.3)	199 (69.3)	199 (69.3)
	AG	84 (29.3)	84 (29.3)	84 (29.3)	84 (29.3)
	GG	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
rs8103142	TT	199 (69.3)	199 (69.3)	199 (69.3)	199 (69.3)
	TC	84 (29.3)	84 (29.3)	84 (29.3)	84 (29.3)
	CC	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
rs12979860	CC	198 (69.0)	198 (69.0)	198 (69.0)	198 (69.0)
	CT	85 (29.6)	85 (29.6)	85 (29.6)	85 (29.6)
	TT	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
rs8099917	TT	204 (71.1)	202 (70.4)	202 (70.4)	202 (70.4)
	TG	79 (27.5)	81 (28.2)	81 (28.2)	81 (28.2)
	GG	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)

[a] There was 100% consistency for rs11881222, rs8103142, and rs12979860, and there was 99.3% consistency for rs8099917.

TABLE 3. Inconsistency in two cases between rs8099917 genotyping by direct sequencing and three other methods

Case no.	rs8099917 genotype by[a]:			
	Direct sequencing	HP	Invader	TaqMan
1	**T/T**	T/G	T/G	T/G
2	**T/T**	T/G	T/G	T/G

[a] Homozygous genotypes are highlighted in boldface.

sitivity was the greatest of the three methods (HP, TaqMan, and Invader), as reported previously (20).

Genotyping error for rs8099917 by direct sequencing due to novel SNP. In two cases, the results of genotyping for rs8099917 by direct sequencing were inconsistent with the results by the other methods (Table 3). Direct sequencing determined the genotype for rs8099917 as T/T in cases 1 and 2; however, the other three genotyping methods (HP, Invader, and TaqMan) determined the genotypes for rs8099917 as T/G in both cases. Further study using alternative primers for direct sequencing revealed that the correct genotypes were T/G and revealed a novel minor SNP present in the forward primer binding site in these two cases (data on file) and which interfered with the PCR amplification step (Fig. 3).

Distribution of haplotypes among four SNPs associated with IL-28B. In the first stage, the four SNPs were in LD in 281 (98.6%) of 285 cases and not in LD in the remaining 4 (1.4%). The first stage revealed five different haplotypes (no. 1 to 5 in Table 4). In haplotypes 1 to 3, the four SNPs were in LD (haplotype 1, homozygous of the major allele among 4 SNPs; n = 198 [69.5%]; haplotype 2, heterozygous among 4 SNPs; n = 79 [27.7%]; and haplotype 3, homozygous of the minor allele among 4 SNPs; n = 4 [1.4%]). In haplotype 4 (3 cases) rs11881222, rs8103142, rs12979860, and rs8099917 were AG, TC, CT, and TT, respectively. In haplotype 5 (one case), rs11881222, rs8103142, rs12979860, and rs8099917 were AA, TT, CT, and TT, respectively. Genotyping by the Invader method of the four SNPs associated with IL-28B in 416 patients in the second stage revealed that the four SNPs were not in LD in 6 cases (1.4%) (Table 4). A total of 410 (98.6%) of 416 cases were in LD for the four different SNPs. The second stage showed six different haplotypes (haplotypes 1 to 4, 6, and 7). Haplotypes 1 to 4 were detected in the first stage, but haplotypes 6 and 7 were not. The distribution of haplotypes was such that haplotypes 1, 2, 3, and 4 were found in 294 (70.7%), 110 (26.5%), 6 (1.4%), and 4 (1.0%) cases, respectively. In haplotype 6 (one case), rs11881222, rs8103142, rs12979860, and rs8099917 were AG, TT, CC, and TT, respectively. In haplotype 7 (one case), rs11881222, rs8103142, rs12979860, and rs8099917 were AA, TT, CT, and TG, respectively.

Response to PEG-IFN/RBV treatment in 10 cases in which the four SNPs associated with IL-28B were not in LD. In 7 (cases 1 to 7 [70%]) of the 10 cases where the four SNPs were not in LD, the haplotype was such that rs11881222, rs8103142, rs12979860, and rs8099917 were AG, TC, CT, and TT, respectively (Table 5). In nine cases (cases 1 to 9), rs8099917 was homozygous for the major allele, while one or more of the other SNPs were heterozygous. Eight (cases 1 to 8) of these

TABLE 4. Distribution of haplotypes among four SNPs associated with IL-28B in stages 1 and 2

Stage	Haplotype no.	Genotype for SNP:				No. (%) of cases with haplotype shown
		rs11881222	rs8103142	rs12979860	rs8099917	
1	1	AA	TT	CC	TT	198 (69.5)
	2	AG	TC	CT	TG	79 (27.7)
	3	GG	CC	TT	GG	4 (1.4)
	4	AG	TC	CT	TT	3 (1.0)
	5	AA	TT	CT	TT	1 (0.4)
2	1	AA	TT	CC	TT	294 (70.7)
	2	AG	TC	CT	TG	110 (26.5)
	3	GG	CC	TT	GG	6 (1.4)
	4	AG	TC	CT	TT	4 (1.0)
	6	AG	TT	CC	TT	1 (0.2)
	7	AA	TT	CT	TG	1 (0.2)

nine cases were viral responders who met the following criteria: HCV had disappeared during therapy, or HCV RNA had decreased more than 2 log copies/ml before 12 weeks after beginning of therapy, although some cases were under treatment or before determination of the final response to PEG-IFN/RBV. Case 9 was NVR due to poor adherence of PEG-IFN (<50% dose), even though rs8099917 was homozygous of the major allele. The haplotype of case 9 showed that rs11881222, rs8103142, rs12979860, and rs8099917 were AA, TT, CT, and TG, respectively. NVR in case 10 was reasonable from the genotypes of rs8099917 and rs12979860, because they were heterozygous, although rs11881222 and rs8103142 were homozygous for the major allele.

DISCUSSION

The relationship between SNPs associated with IL-28B and the response to PEG-IFN/RBV therapy for chronic hepatitis C was found by SNP array, using GWAS technology, by three different groups throughout the world, including our own, in 2009 (6, 19, 21). Following these reports, many studies have confirmed the association between the response to PEG-IFN/RBV and SNPs associated with IL-28B (14, 16). Therefore, it is obvious that these SNPs may be valuable for predicting the response to PEG-IFN/RBV therapy. Recently, it was reported that various SNPs were associated with development of disease and response to therapy and correlated with adverse effects. Several SNPs, such as the UGT1A1 polymorphism for the treatment with irinotecan (1, 17), have already been exploited in clinical practice to avoid severe adverse effects. These tailor-made therapies are expected to become more common in clinical practice in the near future (9). The next step toward tailor-made therapy for PEG-IFN/RBV therapy against chronic hepatitis C involved the development of simple, accurate, and inexpensive methods to determine the genotype of SNPs and determination of the best SNP where the four SNPs associated with IL-28B were not in LD, so that they may be applied in clinical practice.

Genotyping of IL-28B SNPs is quite different from other SNPs, because the sequence of IL-28B is very similar to those of IL-28A, IL-29, and an additional homologous sequence upstream of IL-28B (Fig. 2). We had to design primers and probes for each method to distinguish IL-28B specifically. We