Published OnlineFirst May 18, 2010; DOI:10.1158/0008-5472.CAN-09-4210

a FACSCalibur (BD Biosciences). Intracellular AFP, CK19, and albumin levels were examined using a BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences), anti-AFP mouse monoclonal antibody (Nichirei Biosciences Inc.), anti-CK19 mouse monoclonal antibody (DAKO), and rabbit polyclonal anti-albumin antibodies (Cell Signaling Technology), respectively.

Cell proliferation and colony formation assay

For cell proliferation assays, 2×10^3 cells were seeded in 96-well plates and cultured with 1% FBS DMEM (control), 1% DMEM with OSM (100 ng/mL), 5-FU (2 μg/mL), or OSM (100 ng/mL) and 5-FU (2 μg/mL) for 3 to 7 days without media changes. Cell viability was evaluated in quadruplicate using a CellTiter 96 AQueous kit (Promega). For colony formation assays, 1×10^3 cells were harvested in a one-well Culture Slide (BD Biosciences) and cultured with 1% FBS DMEM (control) with or without OSM (100 ng/mL). Culture medium was replaced every 3 days and the colonies were fixed with ice-cold 100% methanol and used for immunofluorescence 10 days after the initiation of treatment.

RNA interference

SiRNAs specific to OSMR (Silencer Select siRNA S17542) and a control siRNA (Silencer Select Negative Control no. 1) were obtained from Ambion (Applied Biosystems). To each well of a six-well plate, 2×10^5 cells were seeded 12 hours before transfection. Transfection was performed using LipofectAMINE 2000 (Invitrogen), according to the instructions of the manufacturer. A total of 100 pmol/L of siRNA duplex was used for each transfection.

Apoptosis assay

Cells were cultured in 1% FBS DMEM (control), 1% FBS DMEM with OSM (100 ng/mL), 5-FU (2 μg/mL), or OSM (100 ng/mL) and 5-FU (2 μg/mL) for 3 days in six-well plates or in culture slides (BD Biosciences). Annexin V binding to cell membranes was visualized using Annexin V-FITC antibodies and a FACSCalibur flow cytometer (BD Biosciences). Activation of caspase 3 was visualized by immunohistochemistry or immunofluorescence using anti-active caspase-3 polyclonal antibodies (Promega), as described by the manufacturer.

Animal studies

Six-week-old NOD/SCID mice (NOD/NCrCRl-*Prkdc*^{scid}) were purchased from Charles River Laboratories, Inc. The protocol was approved by the Kanazawa University Animal Care and Use Committee. One million tumor cells were suspended in 200 μL of DMEM and Matrigel (1:1), and a s.c. injection was performed. The incidence and size of subcutaneous tumors were recorded. Intratumoral injections of 50 μL of PBS (control), OSM (2 μg/tumor), 5-FU (250 μg/tumor), or OSM (2 μg/tumor) and 5-FU (250 μg/tumor) were initiated twice weekly 48 days after the injection of tumor cells when the average volume of four tumors in each group had reached 400 mm^3. For histologic evaluation, tumors were formalin-fixed and paraffin-embedded.

Statistical analyses

The association of OSMR expression and clinicopathologic characteristics in HCC was examined using either Mann-Whitney U or χ^2 tests. Student's t test was used to compare various test groups assayed by quantitative reverse transcription-PCR analysis. All analyses were performed using GraphPad Prism software.

Results

Distinct expression of OSMRs in HCC

Before exploring the effect of OSM on HCC, we examined the expression of its receptor, OSMR, in surgically resected HCC and adjacent noncancerous liver tissues by immunohistochemistry. Representative staining of OSMRs in tumor/nontumor tissues is shown in Fig. 1A. In general, cell surface and cytoplasmic immunoreactivity to OSMR were rarely detected in hepatocytes in chronic hepatitis liver (a), but were frequently detected in small hepatocyte-like cells in the stroma or transitional cells in the lobule of cirrhotic liver (b), as indicated by the arrows. Note that immunoreactivity to OSMR was not detected in bile duct epithelia or ductular reactions in which EpCAM$^+$ hepatic progenitor cells are thought to accumulate (Supplementary Fig. S1), suggesting that OSMRs might be expressed in hepatic progenitor cells committed to hepatocytes. Immunoreactivity to OSMRs was more strongly detected in HCC than in noncancerous liver (c), and the expression was heterogeneous in the tumor. Of note, OSMRs were detected in HCC cells at the invasive front area of the tumor (d) where CSCs are known to invade frequently (arrows).

Immunoreactivity to OSMR antibodies and EpCAM antibodies was detected in 66 (61.7%) and 38 (35.5%) of 107 HCC specimens, respectively. The clinicopathologic characteristics of OSMR$^+$ and OSMR$^-$ HCC cases are shown in Table 1. OSMR$^+$ HCC was characterized by high serum AFP values ($P = 0.009$), poorly differentiated morphology ($P < 0.0001$), and a high frequency of EpCAM$^+$ HCCs ($P = 0.024$), suggesting that the OSMR is expressed in HCC with stem/progenitor cell features. OSMR$^+$ HCC was also characterized by young onset of disease and male dominance, although these features did not reach statistical significance ($P = 0.052$ and 0.058, respectively). OSMR was more frequently detected in EpCAM$^+$ HCCs (76.3%) than in EpCAM$^-$ HCCs (53.7%). Expression of OSMR and EpCAM was further investigated by double immunofluorescence analysis, and immunoreactivity to OSMR was detected in both EpCAM$^+$ normal hepatic progenitors (Fig. 1B) and EpCAM$^+$ HCC cells (Fig. 1C). These data suggest that although OSMR is more widely expressed than EpCAM in HCC, OSMR is frequently expressed in EpCAM$^+$ normal hepatic progenitors and liver CSCs.

OSM induces hepatocytic differentiation of EpCAM+ HCC

Because OSMR was expressed in the majority of EpCAM$^+$ HCCs, we investigated the effect of OSM on EpCAM$^+$ HCC cell lines. First, we examined the expression of OSMR and its signal transducer glycoprotein 130 (gp130) in EpCAM$^+$ AFP$^+$ HCC cell lines HuH1 and HuH7 by immunofluorescence

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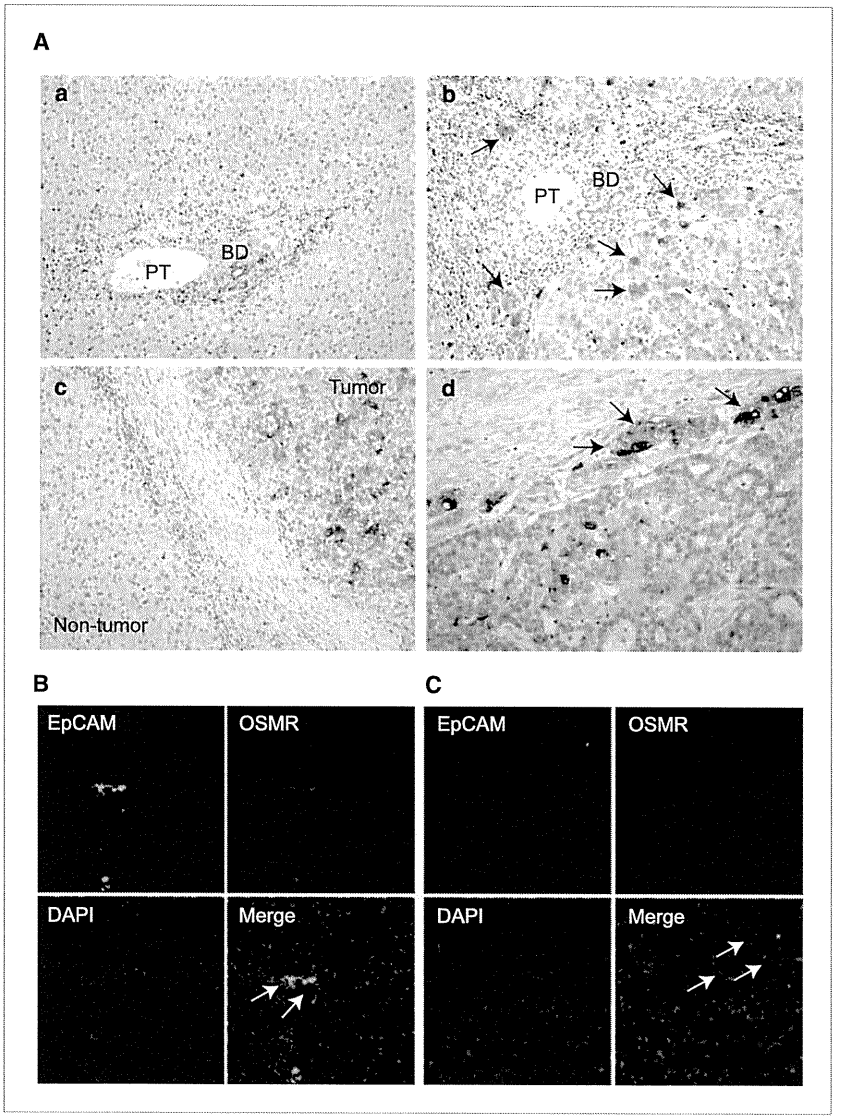

Figure 1. A, representative images of OSMR staining in noncancerous liver tissues and HCC tissues. Immunoreactivity to OSMR was not detected in hepatocytes in chronic hepatitis liver tissue (a) but was detected in a subset of small hepatocyte-like cells in the stroma or transitional cells in the lobule (b, arrows) of cirrhotic liver tissue. OSMR was more abundantly expressed in HCC than in noncancerous liver (c). $OSMR^+$ cancer cells were disseminated in the invasive front area of the tumor (d, arrows). PT, portal tract; BD, bile duct. B and C, double immunofluorescence analysis of EpCAM (green) and OSMR (red) expression in noncancerous (B) and HCC (C) tissues.

(Fig. 2A). Both gp130 and OSMR protein expressions were detected in these cells, consistent with the immunohistochemical data. Because OSM is known to induce the hepatocytic differentiation of hepatoblasts in a STAT3-dependent manner, we investigated the effect of OSM on phosphorylation of STAT3 in HuH1 and HuH7 cells by immunofluorescence and Western blotting. Incubation of HCC cells for 1 hour with OSM at a concentration of 100 ng/mL resulted in the induction and nuclear accumulation of phosphorylated STAT3 compared with controls (Fig. 2B and C). We examined the effect of OSM on the $EpCAM^+$ cell population in HuH1 and HuH7 cells. We first labeled HuH1 and HuH7 cells with CD326 (EpCAM) MicroBeads and FITC-conjugated anti-EpCAM antibodies (Clone Ber-EP4) and performed positive/negative selection using magnetic activated cell sorting to determine the appropriate gating criteria for EpCAM-high (designated as $EpCAM^+$) and EpCAM-low/negative (designated as $EpCAM^-$) cell population (Fig. 2D, top). It is interesting that OSM treatment (100 ng/mL for 72 hours) diminished the $EpCAM^+$ cell population from 50.7% to 10.1% in HuH1 and from 55.2% to 28.8% in HuH7 cells when the same constant gating criteria was applied (Fig. 2D, bottom).

We used RNA interference to investigate whether the decrease in $EpCAM^+$ cells by OSM treatment depends on the expression of OSMR. Transfection of siRNAs specific to *OSMR* (si-OSMR) resulted in the knockdown of target genes

Published OnlineFirst May 18, 2010; DOI:10.1158/0008-5472.CAN-09-4210

compared with the control (si-Control) in HuH1 and HuH7 cells 48 hours after transfection (Supplementary Fig. S2A). We further confirmed the decrease of OSMR protein expression by immunofluorescence and Western blotting 72 hours after transfection (Supplementary Fig. S2B and C). When we treated these HuH1 and HuH7 cells with OSM (100 ng/mL) for 1 hour, we observed the decrease of phosphorylated STAT3 by *OSMR* gene silencing compared with the control (Supplementary Fig. S2C). Furthermore, OSM-mediated decrease in the number of EpCAM$^+$ cells was inhibited by *OSMR* gene silencing (Supplementary Fig. S2D), suggesting that OSM exploits the diminution of EpCAM$^+$ cells through the activation of the OSMR signaling pathway in EpCAM$^+$ HCC.

We further examined the effect of OSM on hepatocytic differentiation by quantitative reverse transcription-PCR and fluorescence-activated cell sorting (FACS) analyses. OSM treatment in HuH1 cells reduced the expression of hepatic progenitor–related genes including *AFP, KRT19* (encoding CK19), and *TERT* (encoding telomerase reverse transcriptase; TERT; Fig. 3A). OSM treatment further reduced the expression of *BMI1* and *POU5F1* (encoding Oct4), which is known to be expressed and required for self-renewal in embryonic stem cells. OSM treatment also increased the expression of the hepatocyte marker, *CYP3A4*. Furthermore, OSM treatment reduced AFP$^+$ and CK19$^+$ cells and increased albumin$^+$ cells compared with the untreated controls, as evaluated by the geometric mean of the fluorescence intensities of whole cells analyzed by intracellular FACS (Fig. 3B). Similar results were obtained in HuH7 cells (data not shown) and, taken together, these data suggest that OSM induced the hepatocytic differentiation of EpCAM$^+$ HCCs.

Hepatocytic differentiation of EpCAM+ HCC by OSM augments cell proliferation

In general, normal stem cells are more quiescent than differentiated cells in terms of cell division. We therefore evaluated the effect of OSM on cell proliferation in HuH1 and HuH7 cells. It is interesting that OSM treatment for 10 days resulted in a larger colony formation following treatment with OSM (100 ng/mL) compared with untreated controls. Of note, the majority of cells comprising these larger colonies were EpCAM$^-$, or had low expression levels, whereas a subset of untreated control cells maintained high EpCAM expression (Fig. 3C). Similar results were obtained when cell proliferation was examined using a [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] tetrazolium assay and Ki-67 labeling index (Fig. 3D). OSM modestly enhanced cell proliferation (top) and increased Ki-67–positive cells (middle and bottom) compared with untreated controls in both HuH1 and HuH7 cells with statistical significance (Fig. 3D).

OSM treatment increases chemosensitivity of EpCAM+ HCC

The abovementioned data imply that although OSM may induce the hepatocytic differentiation of dormant EpCAM$^+$ liver CSCs, OSM treatment alone might instead enhance cell proliferation through expansion of amplifying differentiated cancer cells *in vitro*, raising the question of efficacy of differentiation therapy in EpCAM$^+$ HCC. Because rapidly amplifying cells are considered to be more sensitive to chemotherapeutic agents, we investigated the effect of combining OSM treatment with conventional chemotherapy to target both dormant CSCs and amplifying non-CSCs. We have shown that 5-FU treatment

Table 1. Clinicopathologic characteristics of OSMR$^+$ and OSMR$^-$ HCC cases used for immunohistochemical analyses

Variables	OSMR$^+$ (n = 66)	OSMR$^-$ (n = 41)	P*
Age (years, mean ± SE)	62.7 ± 1.3	66.4 ± 1.3	0.052
Sex (male/female)	55/11	27/14	0.058
Etiology (HBV/HCV/other)	25/35/6	8/30/3	0.10
Liver cirrhosis (yes/no)	43/23	26/15	1.0
AFP (ng/mL, mean ± SE)	6,453 ± 5901	1,039 ± 935	0.009
Histologic grade†			
I–II	3	16	
II–III	54	20	
III–IV	9	5	<0.0001
Tumor size (<3 cm/>3 cm)	30/36	15/26	0.42
Tumor-node-metastasis classification			
I/II	48	31	
III/IV	18	10	0.82
EpCAM (positive/negative)	29/37	9/32	0.024

*Mann-Whitney U test or χ^2 test.
†Edmondson-Steiner.

Published OnlineFirst May 18, 2010; DOI:10.1158/0008-5472.CAN-09-4210

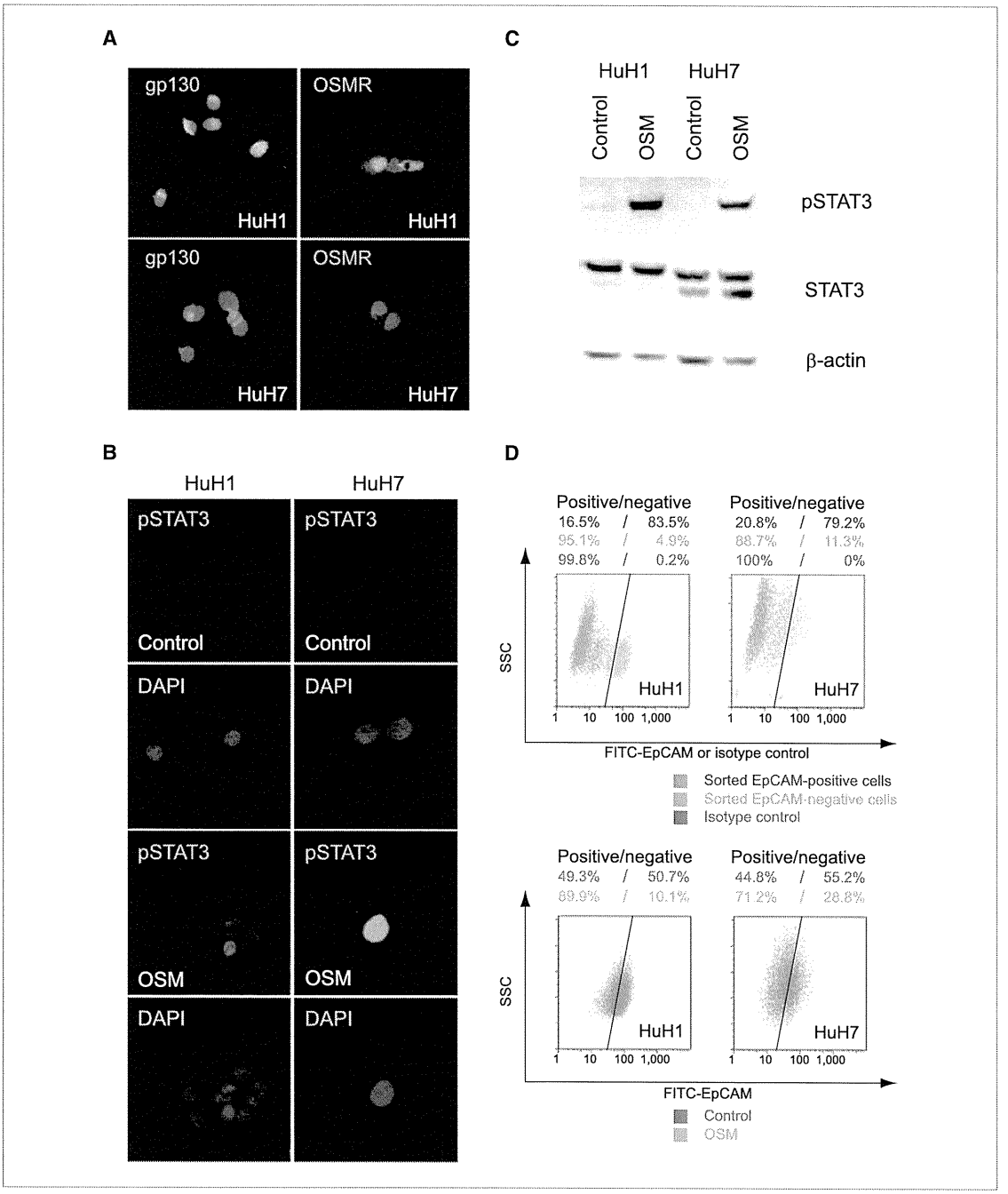

Figure 2. A, immunofluorescence analysis of gp130 and OSMR expression in HuH1 and HuH7 cell lines. B, immunofluorescence analysis of phosphorylated STAT3 expression in HuH1 and HuH7 cell lines stimulated by OSM (100 ng/mL for 1 hour) and controls. C, Western blotting analysis of whole or phosphorylated STAT3 protein expression in HuH1 and HuH7 cells stimulated by OSM (100 ng/mL for 1 hour) and controls. D, FACS analysis of HuH1 and HuH7 cells stained with FITC-conjugated anti-EpCAM antibodies. Top, EpCAM-high (designated as $EpCAM^+$; yellow) and EpCAM-low/negative cells (designated as $EpCAM^-$; blue) were enriched by magnetic activated cell sorting and labeled with FITC-conjugated anti-EpCAM antibodies or isotype control antibodies. Bottom, cells were cultured in 1% FBS DMEM with (green) or without OSM (100 ng/mL; orange) for 3 days and stained with FITC-conjugated anti-EpCAM antibodies.

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alone could diminish EpCAM⁻ non-CSCs which results in the enrichment of EpCAM⁺ CSCs in HCC (18). We therefore explored the effect of 5-FU in combination with OSM on EpCAM⁺ HCC cell proliferation and apoptosis *in vitro*.

When HuH1 and HuH7 cells were treated with OSM alone and cultured for 7 days, cell proliferation was modestly increased compared with untreated controls (Fig. 4A). In contrast, 5-FU treatment clearly inhibited cell proliferation.

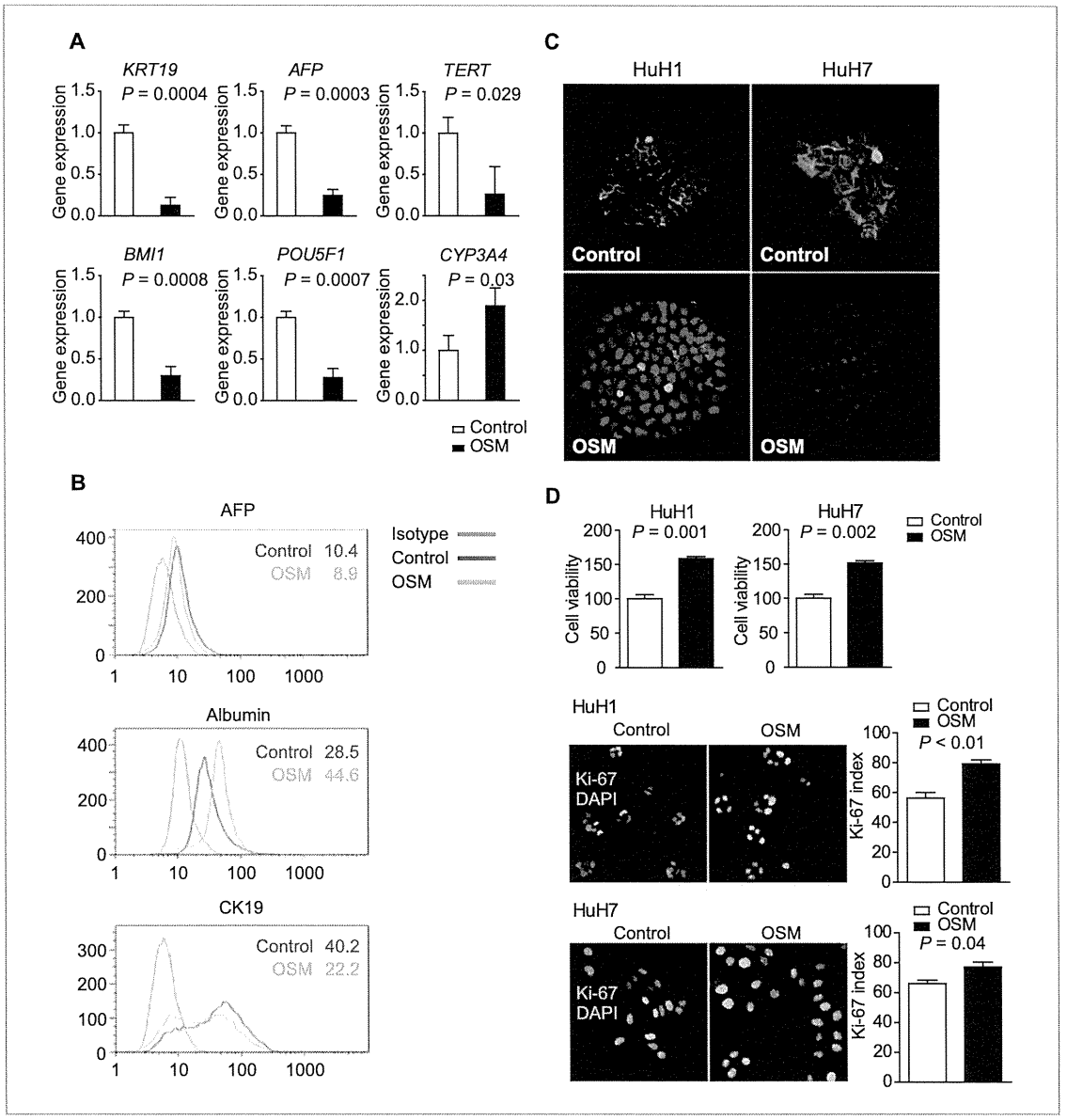

Figure 3. A, quantitative reverse transcription-PCR analysis of HuH1 cells cultured in 1% FBS DMEM with (black columns) or without (white columns) OSM (100 ng/mL) for 3 days. B, intracellular FACS analysis of HuH1 cells cultured in 1% FBS DMEM with (green line) or without (red line) OSM (100 ng/mL) for 3 days. The number in the figure indicates the geometric mean of the fluorescence intensity on a logarithmic scale. C, immunofluorescence analysis of HuH1 and HuH7 cell colonies cultured in 1% FBS DMEM with or without OSM (100 ng/mL) for 10 days. Colonies were fixed with 100% ice-cold methanol and stained with FITC-conjugated anti-EpCAM antibodies. D, top, cell proliferation assay of HuH1 and HuH7 cells cultured in 1% FBS DMEM with (black column) or without (white column) OSM (100 ng/mL) for 3 days. Middle and bottom, immunofluorescence analysis of HuH1 and HuH7 cells cultured in 1% FBS DMEM with or without OSM (100 ng/mL) for 3 days. Cells were fixed with 100% ice-cold methanol and stained with anti–Ki-67 antibodies.

Published OnlineFirst May 18, 2010; DOI:10.1158/0008-5472.CAN-09-4210

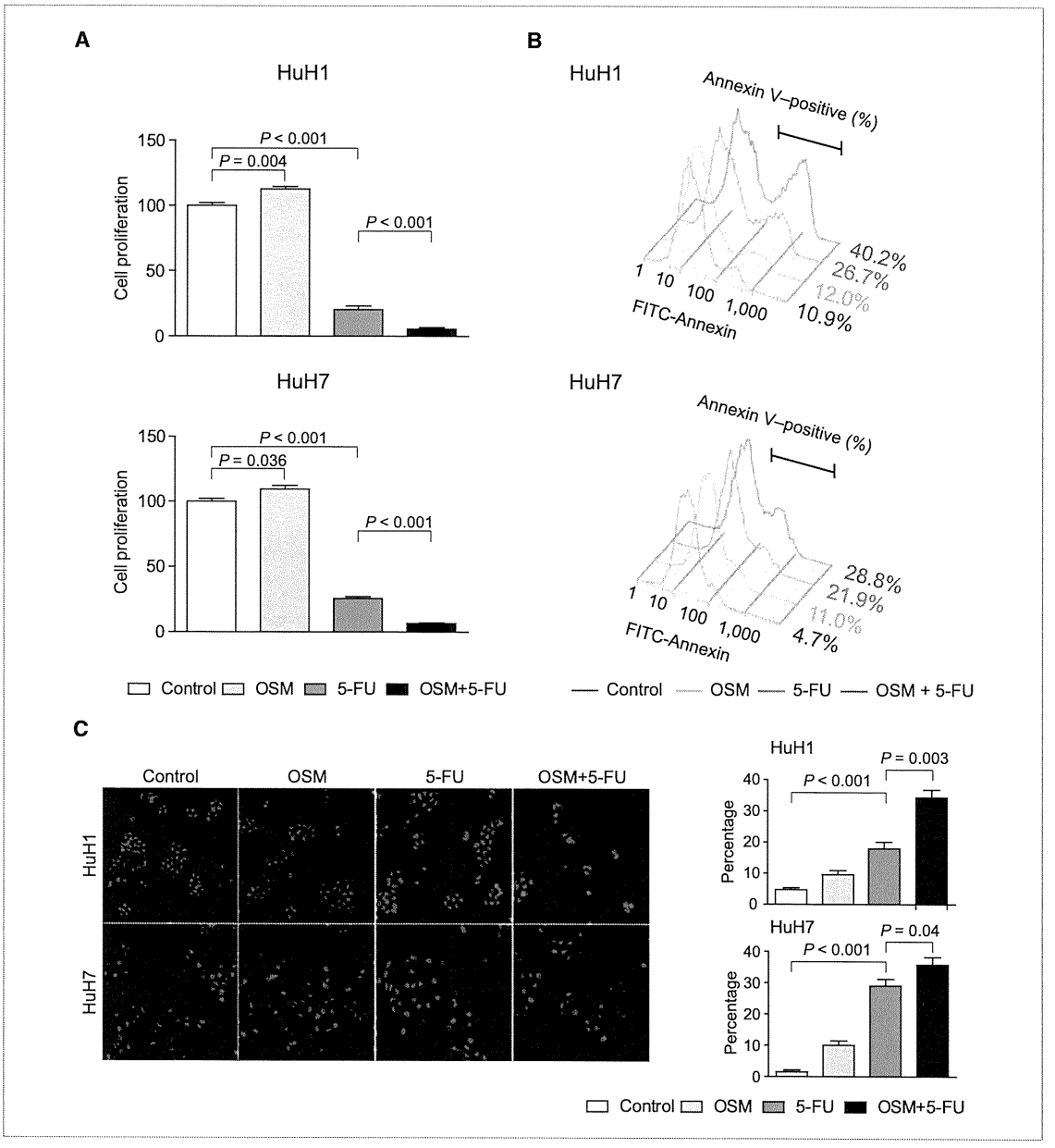

Figure 4. A, cell proliferation assay of HuH1 and HuH7 cells cultured in 1% FBS DMEM with OSM (100 ng/mL; light gray columns), 5-FU (2 μg/mL; gray columns), OSM (100 ng/mL) and 5-FU (2 μg/mL; black columns), or PBS as control (white columns) for 7 days. B, FACS analysis of HuH1 and HuH7 cells stained with FITC-conjugated anti–Annexin V antibodies. Cells were cultured in 1% FBS DMEM with OSM (100 ng/mL; green line), 5-FU (2 μg/mL; blue line), OSM (100 ng/mL) and 5-FU (2 μg/mL; red line), or PBS as control (gray line) for 3 days. C, left, immunofluorescence analysis of HuH1 and HuH7 cells stained with anti–active caspase 3 antibodies. Cells were cultured in 1% FBS DMEM with OSM (100 ng/mL), 5-FU (2 μg/mL), OSM (100 ng/mL) and 5-FU (2 μg/mL), or PBS control for 3 days. Right, bar graphs indicating the percentages of active caspase 3–positive cells.

Noticeably, the combination of OSM and 5-FU effectively suppressed cell proliferation in HuH1 and HuH7 cells (Fig. 4A). We further investigated the effects of OSM and 5-FU on apoptosis, evaluated by Annexin V binding to cell membranes and the activation of caspase 3 (Fig. 4B and C). Although OSM treatment alone had a small effect on the induction of apoptosis, 5-FU treatment induced Annexin V^+ and activated caspase 3^+ cells more than in the control. The

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combination of OSM and 5-FU most strongly induced apoptosis in both HuH1 and HuH7 cells with statistical significance.

Finally, we investigated the effect of OSM on EpCAM$^+$ HCC *in vivo* using a primary HCC specimen and cell lines. Single-cell suspensions from primary EpCAM$^+$ HCC cells (1×10^6 cells) were injected into 6-week-old male NOD/SCID mice, and these cells formed subcutaneous tumors 48 days after transplantation. Subsequently, 50 μL of PBS, OSM (2 μg/tumor), 5-FU (250 μg/tumor), or OSM (2 μg/tumor) and 5-FU (250 μg/tumor) solution were injected directly into each tumor twice a week. Although OSM treatment alone showed weak tumor-suppressive effects, the changes in tumor size showed no significant difference compared with controls (Fig. 5A). Similarly, 5-FU treatment alone showed limited tumor-suppressive effects. However, the combination of OSM with 5-FU showed a marked inhibition of tumor growth compared with PBS control or 5-FU alone (P = 0.02 and 0.05, respectively). Immunohistochemical analysis of xenografted tumors showed that OSM treatment decreased the number of EpCAM$^+$ or CK19$^+$ cells and increased CYP3A4$^+$ cells *in vivo* (Supplementary Fig. S3A and B). FACS analysis of xenografted tumors further confirmed the decrease of EpCAM$^+$ cell population by OSM treatment *in vivo* (Supplementary Fig. S3C). Immunohistochemical analysis revealed that the combination of OSM with 5-FU strongly induced the activation of caspase 3 compared with PBS control, OSM, or 5-FU (Fig. 5B). Taken together, these data suggest that hepatocytic differentiation of EpCAM$^+$ HCC cells induced by OSM was the most effective for inhibition of tumor growth *in vivo* when the conventional chemotherapeutic agent 5-FU was coadministered.

Discussion

A growing body of evidence suggests that there are similarities between normal stem cells and CSCs in terms of self-renewal programs (29). We have recently reported that Wnt/β-catenin signaling augments self-renewal and inhibits the differentiation of EpCAM$^+$ liver CSCs (18). In the present study, we have shown that the OSM-OSMR signaling pathway is maintained in HCCs with stem/progenitor cell features. OSM induces hepatocytic differentiation and activates cell division in dormant EpCAM$^+$ liver CSCs (Fig. 5C). Furthermore, we have shown that the combination of OSM and 5-FU effectively inhibits tumor cell growth, revealing the importance of targeting both CSCs and non-CSCs for eradication of the tumor.

OSM is a pleiotropic cytokine that belongs to the IL-6 family which includes IL-6, IL-11, and leukemia-inhibitory factor. These cytokines share the gp130 receptor subunit as a common signal transducer, and activate Janus tyrosine kinases and the STAT3 pathway. However, gp130 forms a heterodimer with a unique partner such as the IL-6 receptor, leukemia-inhibitory factor receptor, or OSMR, thus transducing a certain signaling uniquely induced by each cytokine (30). Of note, OSM is known to activate hepatocytic differentiation programs in hepatoblasts in an OSMR-specific manner (27), and our data showed that OSM could induce hepatocytic differentiation and active cell proliferation in EpCAM$^+$ HCC through OSMR signaling.

OSMR is expressed in hepatoblasts in the fetal liver (26). We have found that OSMR is frequently expressed in normal hepatic progenitors but is rarely detected in hepatocytes in adult livers. Interestingly, OSMR$^+$ HCC was characterized by

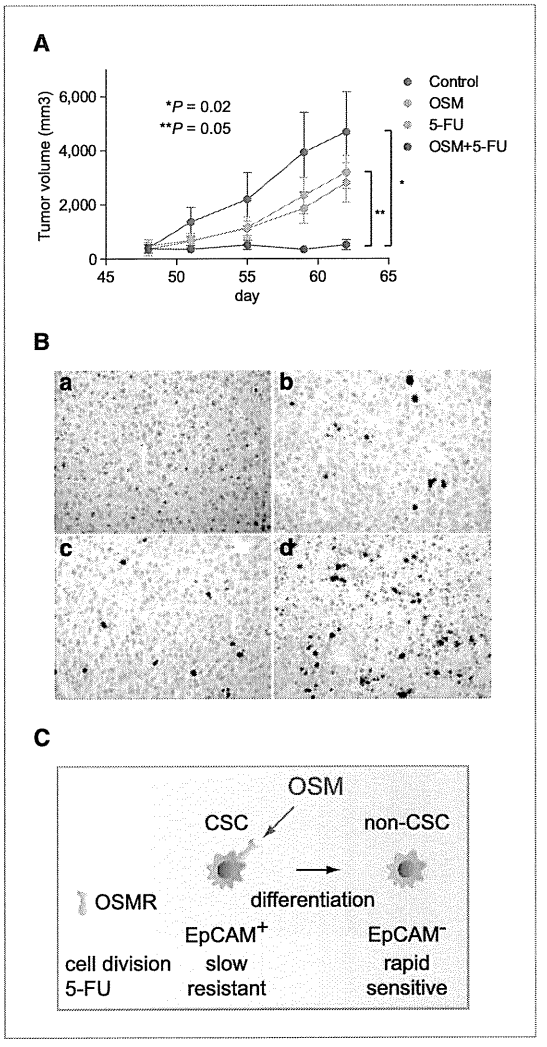

Figure 5. A, effect of PBS, OSM, 5-FU, and OSM plus 5-FU injections on the growth of primary EpCAM$^+$ AFP$^+$ HCC xenograft tumors in NOD/SCID mice (n = 4 in each group). Intratumoral injection of 50 μL of PBS, OSM (2 μg/tumor), 5-FU (250 μg/tumor), or OSM (2 μg/tumor) and 5-FU (250 μg/tumor) was initiated 48 days after transplantation, twice per week. B, representative images of activated caspase 3 staining of xenograft tumors in each treatment group (a, PBS; b, OSM; c, 5-FU; and d, OSM and 5-FU). C, a schematic diagram of the effect of OSM on EpCAM$^+$ liver CSCs. Dormant EpCAM$^+$ liver CSCs with OSMR expression respond to OSM and differentiate into rapidly dividing EpCAM$^-$ non-CSCs that are highly sensitive to 5-FU.

Published OnlineFirst May 18, 2010; DOI:10.1158/0008-5472.CAN-09-4210

high serum AFP, frequent EpCAM positivity, and poorly differentiated morphology, suggesting that OSMR is more likely expressed in HCC with stem/progenitor cell features (16). Although the regulatory mechanisms of OSMR are still unclear, it is plausible that OSMR expression is regulated by a signaling pathway activated during the process of hepatogenesis. Because gp130 is known to be ubiquitously expressed, regulation of OSM signaling might be largely dependent on the expression status of OSMR in normal and tumor tissues. Recent studies have shown the potential role of methylation of CpG islands located in OSMR promoter in colorectal cancer (31, 32). Clarification of OSMR promoter activity regulation, including CpG methylation, might provide clues for better understanding of hepatocytic differentiation signaling in both normal hepatic stem cells and CSCs.

It has been postulated that both normal stem cells and CSCs are dormant and show slow cell cycles. Consistent with this, CSCs are considered to be more resistant to chemotherapeutic agents than non-CSCs, possibly due to slow cell cycles as well as an increased expression of ATP-binding cassette transporters, robust DNA damage responses, and activated antiapoptotic signaling (20, 33, 34). Therefore, development of an effective strategy by targeting CSC pools together with conventional chemotherapies is essential to eradicate a tumor mass. Two strategies have been investigated to reduce the CSCs population in the tumor; that is, inhibition of self-renewal programs and activation of differentiation programs. We have shown that hepatocytic differentiation of liver CSCs by OSM results in enhanced cell proliferation *in vitro*. We have further shown here that OSM-mediated hepatocytic differentiation of liver CSCs in combination with conventional chemotherapy effectively suppresses HCC growth. It is possible that OSM may boost antitumor activity of 5-FU by "exhausting dormant CSCs" through hepatocytic differentiation and active cell division. It is encouraging that similar success with differentiation therapy has recently been reported in several cancers (24, 35, 36). In addition, HNF4-α–mediated differentiation of HCC cells has recently been reported to be effective for the eradication of HCC (37). However, although the combination of OSM and 5-FU effectively inhibited tumor growth in our model, we could not observe the shrinkage of the tumor. Thus, induction of CSC's differentiation with eradication of non-CSCs might not be enough for the eradication of the tumor, which might suggest the importance of inhibiting self-renewal as well as stimulating differentiation of CSCs. Because we induced the hepatocytic differentiation of the subcutaneous tumor by local injection of OSM, further rigorous studies are clearly required to assess the effect of OSM on liver CSCs and its utility for differentiation therapy in HCC.

CSCs may acquire resistance against differentiation therapy by additional genetic/epigenetic changes during treatment by clonal evolution, as observed in conventional chemotherapy. Indeed, it has recently been suggested that bone morphogenetic protein–mediated brain CSC differentiation failed in a subset of brain tumors in which bone morphogenetic protein receptor promoters were methylated and silenced (23). Similarly, OSMR silencing by promoter methylation might result in the development of OSM-resistant clones in HCC.

In conclusion, OSMR is expressed in certain types of HCC with stem/progenitor cell features, and OSM induces hepatocytic differentiation and active cell division of $OSMR^+$ liver CSCs to enhance chemosensitivity to 5-FU. The clinical safety and utility of OSM should be evaluated in the near future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Masayo Baba and Nami Nishiyama for excellent technical assistance.

Grant Support

Ministry of Education, Culture, Sports, Science and Technology, Japan grant-in-aid (no. 20599005).

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Received 11/17/2009; revised 03/12/2010; accepted 03/31/2010; published OnlineFirst 05/18/2010.

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Hepatic ISG Expression Is Associated With Genetic Variation in Interleukin 28B and the Outcome of IFN Therapy for Chronic Hepatitis C

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See related article, Younossi and Stepanova, on page 718 in *CGH*.

BACKGROUND & AIMS: Multiple viral and host factors are related to the treatment response to pegylated-interferon and ribavirin combination therapy; however, the clinical relevance and relationship of these factors have not yet been fully evaluated. **METHODS:** We studied 168 patients with chronic hepatitis C who received pegylated-interferon and ribavirin combination therapy. Gene expression profiles in the livers of 91 patients were analyzed using an Affymetrix genechip (Affymetrix, Santa Clara, CA). The expression of interferon-stimulated genes (ISGs) was evaluated in all samples by real-time polymerase chain reaction. Genetic variation in interleukin 28B (IL28B; rs8099917) was determined in 91 patients. **RESULTS:** Gene expression profiling of the liver differentiated patients into 2 groups: patients with up-regulated ISGs and patients with down-regulated ISGs. A high proportion of patients with no response to treatment was found in the up-regulated ISGs group (P = .002). Multivariate logistic regression analysis showed that ISGs (<3.5) (odds ratio [OR], 16.2; $P < .001$), fibrosis stage (F1-F2) (OR, 4.18; $P = .003$), and ISDR mutation (≥2) (OR, 5.09; $P = .003$) were strongly associated with the viral response. The IL28B polymorphism of 91 patients showed that 66% were major homozygotes (TT), 30% were heterozygotes (TG), and 4% were minor homozygotes (GG). Interestingly, hepatic ISGs were associated with the IL28B polymorphism (OR, 18.1; $P < .001$), and its expression was significantly higher in patients with the minor genotype (TG or GG) than in those with the major genotype (TT). **CONCLUSIONS: The expression of hepatic ISGs is strongly associated with treatment response and genetic variation of IL28B. The differential role of host and viral factors as predicting factors may also be present.**

Keywords: Pegylated Interferon, Ribavirin; Gene Expression; Single Nucleotide Polymorphism.

A human liver infected with hepatitis C virus (HCV) develops chronic hepatitis, cirrhosis, and, in some instances, hepatocellular carcinoma.[1] Interferon (IFN) and ribavirin (RBV) combination therapy is a popular modality for treating patients with chronic hepatitis C (CH-C); approximately 50% of patients usually relapse, particularly those with HCV genotype 1b and a high viral load.[2,3] Therefore, it is beneficial to predict the response of patients with the 1b genotype and a high viral load to pegylated-IFN (Peg-IFN) and RBV combination therapy before starting treatment because therapy can be long, costly, and have many adverse effects. Amino acid (aa) substitutions in the interferon sensitivity determining region (ISDR), located in the HCV nonstructural region 5A, are useful for predicting the response of patients with genotype 1b to IFN therapy.[4] However, viral factors alone do not sufficiently predict the outcome of treatment in every case.[5]

In addition to viral factors, hepatic gene expression before and during IFN treatment has been examined to determine host factors associated with the response to treatment.[6,7] Interferon-stimulated genes (ISGs) up-regulated in the liver prior to treatment might be related to the poor induction of ISGs and the impaired eradication of HCV during treatment.[6–9] This may be because the ISGs have already been maximally induced before treat-

Abbreviations used in this paper: aa, amino acid; AST, aspartate aminotransferase; cDNA, complementary DNA; CH-C, chronic hepatitis C; Down-ISGs, down-regulated ISGs; EVR, early virologic response; GWAS, genome-wide association studies; HCV, hepatitis C virus; IFN, interferon; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; IL, interleukin; IL28B, interleukin 28B; ISDR, interferon sensitivity determining region; ISGs, interferon stimulated genes; Mx1, myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse); NR, no response; Peg, pegylated; RBV, ribavirin; ROC, receiver operating characteristic; RTD, real-time detection; PCR, polymerase chain reaction; RTD-PCR, real-time detection-polymerase chain reaction; SNP, single nucleotide polymorphism; SVR, sustained viral response; TR, transient response; Up-ISGs, up-regulated ISGs.

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0016-5085/$36.00
doi:10.1053/j.gastro.2010.04.049

ment. However, the clinical relevance of the expression of ISGs as predictive factors for the outcome of treatment has not yet been fully evaluated.

In parallel to gene expression analysis, genome-wide association studies (GWAS) have been used to identify loci associated with the response to treatment; genetic variation in interleukin 28B (IL28B) was found to predict hepatitis C treatment-induced viral clearance.[10-12]

In this study, with a relatively large cohort of CH-C patients treated with Peg-IFN and RBV, we validated the clinical relevance of the expression of hepatic ISGs as predictive factors for the outcome of treatment. In addition, we demonstrated that the expression of hepatic ISGs was closely related to genetic variation in IL28B.

Materials and Methods

Patients

We enrolled 168 patients with CH-C at the Graduate School of Medicine, Kanazawa University Hospital and its related hospitals, Japan (Table 1, Supplementary Table 1). The cohort included 92 men and 76 women, ranging from 21 to 73 years of age, who were registered prospectively in 2005 and 2007. All patients had HCV

Table 1. Comparison of Clinical Factors Between Patients With and Without NR

Clinical category	SVR+TR		NR		Univariate *P* value	Multivariate odds (95% CI)	Multivariate *P* value
No. of patients	n = 125		n = 43			—	
Age and sex							
Age, *y*	57	(30–72)	56	(30–73)	.927	—	
Sex (M vs F)	68 vs 57		24 vs 19		.872	—	
Liver factors							
F stage (F1-2 vs F3-4)	95 vs 30		20 vs 23		.001	4.18 (1.61–11.5)	.003
A grade (A0-1 vs A2-3)	68 vs 57		19 vs 24		.248	—	
ISGs (Mx, IFI44, IFIT1)							
(<3.5 vs ≥3.5)	103 vs 22		12 vs 31		<.001	16.2 (6.21–47.8)	<.001
Laboratory parameters							
HCV-RNA (*KIU/mL*)	2300	(126–5000)	1930	(140–5000)	.725	—	
BMI (kg/m^2)	23.2	(16.3–34.7)	23.4	(19.5–40.6)	.439	—	.107
AST (*IU/L*)	46	(18–258)	64	(21–283)	.017	—	
ALT (*IU/L*)	60	(16–376)	82	(18–345)	.052	—	
γ-GTP (*IU/L*)	36	(4–367)	75	(26–392)	<.001	—	
WBC ($/mm^3$)	4800	(2100–11,100)	4800	(2500–8200)	.551	—	
Hb (*g/dL*)	14	(9.3–16.6)	14.4	(11.2–17.2)	.099	—	
PLT ($\times 10^4/mm^3$)	15.7	(7–39.4)	15.2	(7.6–27.8)	.378	—	
TG (*mg/dL*)	98	(30–323)	116	(45–276)	.058	—	
T-Chol (*mg/dL*)	167	(90–237)	160	(81–214)	.680	—	
LDL-Chol (*mg/dL*)	82	(36–134)	73	(29–123)	.019	—	
HDL-Chol (*mg/dL*)	42	(20–71)	47	(18–82)	.098	—	
FBS (*mg/dL*)	94	(60–291)	96	(67–196)	.139	—	
Insulin (*μU/mL*)	6.6	(0.7–23.7)	6.8	(2–23.7)	.039	—	
HOMA-IR	1.2	(0.3–11.7)	1.2	(0.4–7.2)	.697	—	
Viral factors							
ISDR mutations ≤1 vs ≥2	80 vs 44		34 vs 9		.070	5.09 (1.69–17.8)	.003
Treatment factors							
Total dose administered							
Peg-IFN (*μg*)	3840	(960–7200)	3840	(1920–2880)	.916	—	
RBV (*g*)	202	(134–336)	202	(36–336)	.531	—	
Achieved administration rate							
Peg-IFN (%)							
≥80%	84		28		.975	—	
<80%	42		14				
RBV (%)							
≥80%	76		24		.745	—	
<80%	50		18				
Achievement of EVR	101/125 (81%)		0/43 (0%)		<.001	—	

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; EVR, early virologic response; γ-GTP, γ-glutamyl transpeptidase; ISDR, interferon sensitivity determining region; Mx1, myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse); WBC, leukocytes; HOMA-IR, homeostasis model assessment of insulin resistance; Hb, hemoglobin; RBV, ribavirin; PLT, platelets; TG, triglycerides; TR, transient response; T-chol, total cholesterol; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol; FBS, fasting blood sugar; CI, confidence interval.

genotype 1b and high viral loads (≥100K IU/mL) measured by quantitative Cobas Amplicor assays (Roche Diagnostics Co Ltd, Tokyo, Japan). All patients had undergone liver biopsy before combination therapy. Exclusion criteria for patients not eligible for Peg-IFN and RBV combination therapy were as follows: (1) pregnant women or women of childbearing potential, nursing mothers, or male patients whose partner might become pregnant; (2) patients with hepatocellular carcinoma; (3) patients with serious complications in the heart, kidneys, or lungs; (4) patients with autoimmune diseases, such as autoimmune hepatitis, and primary biliary cirrhosis; and (5) patients infected with the hepatitis B virus. Informed consent was obtained from all patients, and ethics approval for the study was obtained from the Ethics Committee for Human Genome/Gene Analysis Research at Kanazawa University Graduate School of Medical Science.

All patients were administered Peg-IFN-α 2b (Schering-Plough KK, Tokyo, Japan) and RBV combination therapy for 48 weeks. Peg-IFN was given in weekly doses and adjusted to body weight according to the manufacturer's instructions (45 kg or less, 60 μg/dose; 46–60 kg, 80 μg/dose; 61–75 kg, 100 μg/dose; 76–90 kg, 120 μg/dose; and 91 kg or more, 150 μg/dose). Similarly, RBV (Schering-Plough KK) was administered in daily doses adjusted to body weight according to the manufacturer's instructions (60 kg or less, 600 mg/day; 61–80 kg, 800 mg/day; and 81 kg or more, 1000 mg/day).

The final outcome of treatment was assessed 24 weeks after the cessation of combination therapy. We defined treatment outcomes according to the decrease in viremia as follows: sustained viral response (SVR), clearance of HCV viremia 24 weeks after the cessation of therapy; transient response (TR), no detectable HCV viremia at the cessation of therapy but relapsed during the follow-up period; and no response (NR), HCV viremia detected at the cessation of therapy. An early virologic response (EVR) (complete EVR) was defined as undetectable HCV-RNA in the serum by 12 weeks. HCV genotypes were determined according to the method of Okamoto et al. Serum HCV RNA was determined using qualitative and quantitative COBAS Amplicor assays (Roche Diagnostics Co, Ltd, Tokyo, Japan). The grading and staging of chronic hepatitis were histologically assessed according to the method of Desmet et al (Table 1).[13]

Preparation of Liver Tissue Samples

Liver biopsy samples were taken from all patients before treatment. The biopsy samples were divided into 2 parts: the first part was immersed in formalin for histologic assessment, and the second was immediately immersed in RNAlater (QIAGEN, Valencia, CA) for RNA isolation. Liver tissue RNA was isolated using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. Isolated RNA was stored at −70°C until use.

Affymetrix Genechip Analysis

The quality of the isolated RNA was estimated after electrophoresis using an Agilent 2001 Bioanalyzer (Agilent, Santa Clara, CA). Aliquots of total RNA (50 ng) isolated from the liver biopsy specimens were subjected to amplification using the WT-Ovation Pico RNA Amplification System (NuGen, San Carlos, CA) according to the manufacturer's instructions. Approximately 10 μg of complementary DNA (cDNA) was amplified from 50 ng of total RNA, and 5 μg of cDNA was used for fragmentation and biotin labeling using the FL-Ovation cDNA Biotin Module V2 (NuGen) according to the manufacturer's instructions. Biotin-labeled cDNA was suspended in 220 μL of hybridization cocktail (NuGen), and 200 μL was used for hybridization to the Affymetrix Human 133U Plus 2.0 GeneChip (Affymetrix, Santa Clara, CA) containing 54,675 probes. After stringent washing, the microarray chips were stained with streptavidin-phycoerythrin, and probe hybridization was determined using a GeneChip Scanner 3000 (Affymetrix). Data files (CEL) were obtained using the GeneChip Operating Software 1.4 (Affymetrix).

Hierarchical Clustering and Pathway Analysis of Genechip Data

Genechip data analysis was performed using BRB-Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.htm). The data were log transferred, normalized, centered, and applied to the average linkage hierarchical clustering with centered correlation.

For genechip analysis, we selected 37 representative ISGs. Hepatic gene expression profiling was obtained from 30 CH-C patients before and 1 week after the initiation of IFN and RBV combination therapy and the 100 most up-regulated genes were selected (submitted for publication). ISGs were suppressed in patients with a rapid viral response and up-regulated in patients with a slow viral response before treatment. Using the 100 treatment-induced genes, we evaluated hepatic gene expression in 30 patients before treatment. Hierarchical clustering analysis showed that a cluster of 37 ISGs was up-regulated in patients with a slow viral response.

Pathway analysis was performed using MetaCore (GeneGo, St. Joseph, MI). Functional ontology enrichment analysis was performed to compare the gene ontology process distribution of differentially expressed genes ($P < .01$).

Quantitative Real-time Detection-Polymerase Chain Reaction

We performed quantitative real-time detection (RTD)-polymerase chain reaction (PCR) (RTD-PCR) using TaqMan Universal Master Mix (PE Applied Biosystems, Carlsbad, CA). Primer pairs and probes for myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse) (Mx1), 2′-5′-oligoadenylate synthelase 3 (OAS3), interferon-induced protein 44 (IFI44),

interferon-induced protein 44-like (IFI44L), 2′-5′-oligoadenylate synthetase 2 (OAS2), ubiquitin specific peptidase 18 (USP18), radical S-adenosyl methionine domain containing 2 (RSAD2), interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), interferon induced with helicase C domain 1 (IFIH1), XIAP associated factor 1 (XAF1), cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial (CMPK2), epithelial stromal interaction 1 (EPSTI1), hect domain and RLD 6 (HERC6), poly (ADP-ribose) polymerase family, member 9 (PARP9), phospholipid scramblase 1 (PLSCR1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from the TaqMan assay reagents library. Primer pairs and probes for IL28B were designed as previously described.[12] The standard curve was obtained in every assay using the RNA obtained from a normal liver.[14,15] The expression values were normalized by GAPDH, and normalized values indicate the relative fold expression to a normal liver.

Amino Acid Substitutions of ISDR in the Nonstructural 5A Region

The nucleotide sequence of ISDR in the nonstructural 5A region was determined by direct sequencing of PCR amplified materials.[4] Mutant-type ISDR was defined as containing 2 or more aa substitutions.

Genetic Variation of IL28B Polymorphism

A single nucleotide polymorphism (SNP) of IL28B was evaluated in 91 patients whose hepatic gene expression profiling was obtained. We genotyped 32 patients using Affymetrix Genome-Wide Human SNP Array 6.0 as previously described.[12] The results for rs8105790, rs11881222, rs8099917, and rs7248668 were retrieved from a database to evaluate the association of these SNPs. rs12979860 was determined by direct sequencing, and rs8099917 was determined using TaqMan Pre-Designed SNP Genotyping Assays (PE Applied Biosystems) as recommended by the manufacturer.

Statistical Analysis

The Mann–Whitney *U* test was used to analyze continuous variables. Fisher exact test and χ^2 test were used for the analysis of categorical data. The overall plausibility of the treatment response groups was assessed using Fisher C statistic (Supplementary Table 2).[16,17] C is defined by $C = -2 \Sigma \ln(p_i)$, where p_i is the probability (*P* value) of each independent statement (clinical factors). C follows a χ^2 distribution with 2k degrees of freedom, k being the number of independent statements (clinical factors).[16] A nonsignificant C value means that the treatment response in the 2 groups was not statistically independent.

Multivariate analysis was performed using a stepwise logistic regression model. Each cut-off point for the continuous variables was decided by analysis of the receiver operating characteristic (ROC) curve. A *P* value of less than .05 was considered significant. Statistical analyses were performed using JMP7 for Windows (SAS Institute, Cary, NC).

Results

Response Rate and Clinical Characteristics

The clinical characteristics of the patients are shown in Table 1 and Supplementary Table 1. All of the patients were infected with HCV genotype 1b and had a high viral load (>100K IU/mL). No patients were coinfected with the hepatitis B virus (HBV). The intention-to-treat analysis showed that SVR, TR, and NR were observed in 70 (42%), 55 (33%), and 43 (25%) patients, respectively (Supplementary Table 1). Before comparing patients with 3 different responses, the overall plausibility of the treatment response groups was assessed using Fisher C statistic. Fisher C statistic utilizes the *P* values obtained by comparing pretreatment factors including age, gender, liver factors, laboratory parameters, and viral factors. Because the SVR and TR groups could not be defined as different, they were grouped together and compared with NR (Table 1, Supplementary Table 2).

Eleven patients with NR discontinued the therapy after 24 weeks because of an insufficient effect, namely, serum HCV-RNA was still detectable at this time. The remaining patients completed 48 weeks of Peg-IFN and RBV combination therapy. The administration rate of Peg-IFN with 80% or more was achieved in 67% of patients, and the administration rate of RBV with 80% or more was achieved in 60% of patients (Table 1).

Analysis of Hepatic Gene Expression

Prior to treatment, 91 of 168 patients (Supplementary Table 3) were randomly selected, and their hepatic gene expression was determined using Affymetrix genechip analysis.

Hierarchical clustering using 37 representative ISGs (see Materials and Methods) demonstrated 2 clear clusters of patients: one was a group composed of patients with up-regulated ISGs (Up-ISGs), and the other was a group consisting of patients with down-regulated ISGs (Down-ISGs) (Figure 1). In patients with Up-ISGs, 21 (49%) showed NR, whereas 8 (17%) patients with Down-ISGs showed NR ($P = .002$). In contrast, 14 (33%) patients with Up-ISGs showed SVR, whereas 27 (56%) patients with Down-ISGs showed SVR ($P = .03$). There were no significant differences in the frequency of advanced stages of liver fibrosis (F3-F4) between patients with Up-ISGs and patients with Down-ISGs (18 [42%] and 17 [35%], respectively, $P = .664$). These data indicated that the up-regulation of ISGs in the liver before treatment was strongly associated with resistance to IFN treatment.

Host and Viral Factors Associated With the Response to Combination Therapy

To evaluate the multiple host and viral factors associated with the response to Peg-IFN and RBV combination therapy in all patients, univariate and multivariate analyses were performed. To assess the expression of hepatic ISGs, 15 genes (Mx1, OAS3, IFI44, IFI44L, OAS2,

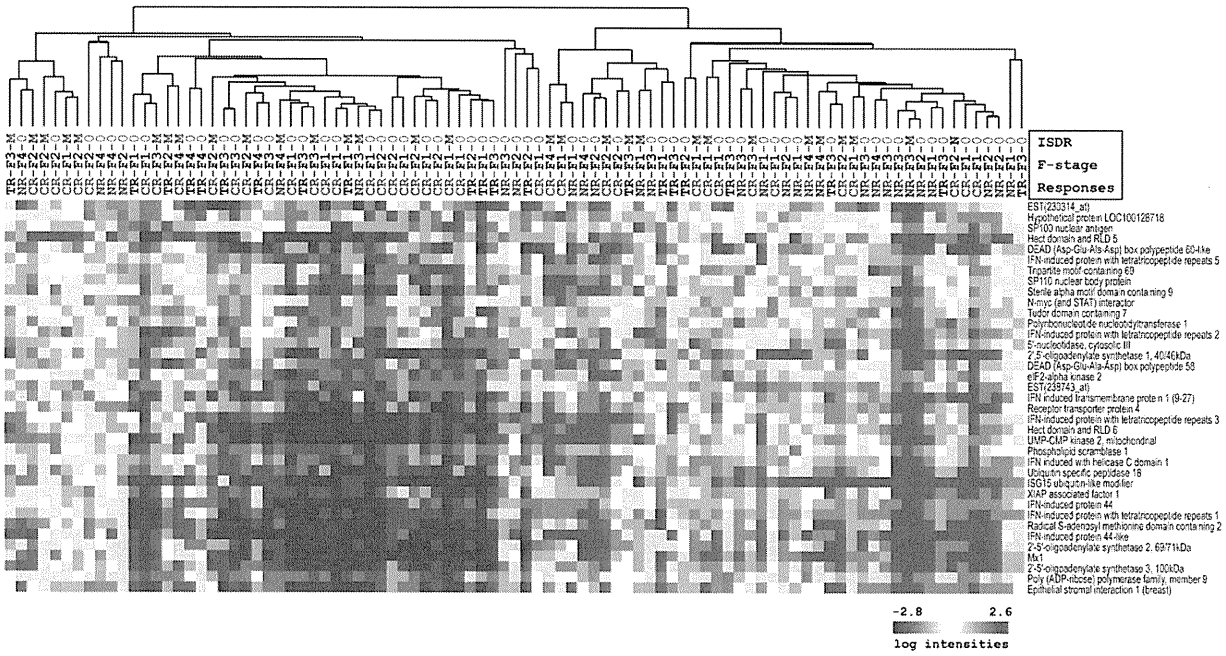

Figure 1. Hierarchical clustering analysis of 91 patients using 37 representative ISGs. Responses to therapy (SVR, TR, and NR), fibrosis stage (F1–F4), and status; ISDR mutations are also shown. ISDR mutation ≥2 = M, ≤1 = 0.

USP18, RSAD2, IFIT1, IFIH1, XAF1, CMPK2, EPSTI1, HERC6, PARP9, and PLSCR1) out of 37 representative ISGs were selected for their expression values of probe intensity, and their expression was confirmed in liver tissue obtained from 168 patients by RTD-PCR. Although there were significant correlations of their expression with each other, except RARP9 and PLSCR1 (Supplementary Table 4), the dynamic range of gene expression was high for 3 genes, namely, Mx1, IFI44, and IFIT1 (Supplementary Figure 1*A*). We averaged the expression values of Mx1, IFI44, and IFIT1 and used them for further study.

When we compared patients with SVR+TR and NR, the fibrosis stage of the liver ($P = .001$), expression of hepatic ISGs ($P < .001$), aspartate aminotransferase (AST) serum level ($P = .017$), γ-glutamyl transpeptidase (γ-GTP) ($P < .001$), low-density lipoprotein cholestrol (LDL-Chol) ($P = .019$), and insulin (μU/mL) ($P = .039$) were significantly different prior to treatment (Table 1). For treatment factors, the total dose and administration of IFN and RBV were not significantly different between these 2 groups. EVR was observed in 101 (81%) patients, and the proportion was significantly different ($P < .001$) between patients with SVR+TR and NR (Table 1).

Regression analysis of pretreatment factors showed a strong correlation among γ-GTP, alanine aminotransferase (ALT), and aspartate aminotransferase (AST); and homeostasis model assessment-insulin resistance (HOMA-IR), fasting blood sugar, and insulin; and total cholesterol (T-Chol), high-density lipoprotein cholestrol (HDL-Chol), and LDL-Chol (data not shown). We selected fibrosis stage, ISGs, HCV-RNA, ISDR mutation, and body mass index (BMI) as factors for multivariate analysis. Stepwise multivariate logistic regression analysis was performed using the selected factors. From the ROC curve, we set the cut-off value for the expression of ISGs as 3.5 (Supplementary Figure 1*B*). The results showed that expression of hepatic ISGs (<3.5), fibrosis stage (F1-F2), and ISDR mutation (≥2) were significant pretreatment factors contributing to SVR+TR (Table 1).

Clinical Parameters Associated With the Expression of Hepatic ISGs

Univariate and multivariate analyses revealed that the expression of hepatic ISGs was a strong predictor of the treatment outcome for SVR+TR patients. We next examined which clinical parameters were associated with the expression of hepatic ISGs (Table 2). Univariate analysis showed that the expression of ISGs was strongly correlated with the serum levels of γ-GTP ($P < .001$) and AST ($P < .001$) and weakly correlated with HCV-RNA, fasting blood sugar, insulin, HOMA-IR, triglyceride (TG), and LDL-Chol. Multivariate analysis showed that γ-GTP ($P < .001$), HCV-RNA ($P < .001$), and LDL-Chol ($P = .048$) were significantly associated with hepatic ISGs. Noticeably, the expression of ISGs was negatively correlated with HCV-RNA in SVR+TR patients ($P = .009$), whereas this correlation was not evident in NR patients ($P = .298$) (Table 2, Supplementary Figure 2). These results may indicate that endogenous ISGs suppress HCV in SVR+TR patients, whereas they are not active in NR patients.

Table 2. Clinical Factors Associated With Expression of Hepatic Interferon-Stimulated Genes

	Univariate analysis				Multivariate analysis			
Clinical factor	β	95% CI		P value	β	95% CI		P value
AST (*IU/L*)	0.274	0.13	0.42	<.001	—	—	—	
γ-GTP (*IU/L*)	0.326	0.18	0.47	<.001	0.288	0.14	0.43	<.001
HCV-RNA (*KIU/mL*)	−0.170	−3.19	−0.02	.025	−0.255	−0.40	−0.11	<.001
SVR+TR	−0.237	−0.32	−0.05	.009	—	—	—	
NR	−0.168	−0.57	0.18	.298	—	—	—	
FBS (*mg/dL*)	0.182	0.03	0.35	.021	—	—	—	
Insulin (*μU/mL*)	0.190	0.03	0.34	.016	—	—	—	
HOMA-IR	0.181	0.03	0.33	.017	—	—	—	.073
TG (*mg/dL*)	0.201	0.05	0.35	.011	—	—	—	.089
LDL-Chol (*mg/dL*)	−0.177	−0.33	−0.02	.025	−0.143	−0.28	0.00	.048

γ-GTP, γ-glutamyl transpeptidase; AST, aspartate aminotransferase; FBS, fasting blood sugar; TG, triglycerides; TR, transient response; NR, no response; SVR, sustained viral response; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-chol, low-density lipoprotein cholesterol; CI, confidence interval; β, β coefficient; CI, confidence interval.

Expression of Hepatic ISGs Before Treatment Is Associated With Genetic Variation of IL28B

Recently, a GWAS successfully identified the genomic locus associated with the treatment response to Peg-IFN and RVB combination therapy for CH-C. Genetic variation in IL28B predicts HCV treatment-induced viral clearance.[11,12] We determined the genetic variation in IL28B of 32 patients[12] (Table 3). The SNPs rs8105790, rs11881222, rs8099917, and rs7248668 had a significant association with treatment response (odds ratio: 24.7–27.1, $P = 1.84 \times 10^{-30}$–2.68×10^{-32}). These SNPs are located in block 2 of the IL28B haplotype and show significant linkage disequilibrium in the HapMap data.[12] Ge et al[11] reported a different SNP (rs12979860) that was located between rs11881222 and rs8099917. The nucleotide sequence of rs12979860 was determined by direct sequencing, and the results are shown in Table 3. There was a strong association of rs12979860 and the other 4 SNPs indicating that this SNP was located within the same haplotype block. We confirmed these findings in multiple samples from Japanese patients (data not shown).

We selected rs8099917 for further study and evaluated it using TaqMan Pre-Designed SNP Genotyping Assays. The G nucleotide of rs8099917 was associated with a poor response to treatment (minor allele), whereas the T was associated with a fair response to treatment (major allele).[12] Out of 91 patients (Supplementary Table 3), the proportion of major homozygotes (TT), heterozygotes (TG), and minor homozygotes (GG) were 66% (60/91), 30% (27/91), and 4% (4/91), respectively (Table 4); 86% (51/60) of the major genotype (TT) patients had SVR or TR, whereas 65% (20/31) with the minor genotypes (TG or GG) had NR ($P < .001$).

Interestingly, hepatic gene expression profiles revealed that patients with the minor genotype showed higher expression of hepatic ISGs, whereas patients with the major genotype showed lower expression of hepatic ISGs (Figures 2 and 3). To examine further the relationship of the genetic variation in IL28B and its expression levels, we evaluated the expression of IL28B in the liver by RTD-PCR (Figure 3). IL28B expression was approximately 10-fold less than the expression of ISGs. Although IL28B expression tended to be higher in some patients with the major genotype, there was no significant difference in IL28B expression in the liver between the major and minor genotypes (Figure 3*A*). Nevertheless, the expression of ISGs was clearly high in patients with the minor genotype ($P < .0001$) (Figure 3*B*). IL28 activates signal tranducers and activators of transcription 1 (STAT1) through downstream signaling from a heterodimeric class II cytokine receptor that consists of IL-10 receptor β (IL-10Rβ) and IL-28 receptor α (IL-28Rα).[18,19] Therefore, we examined the correlation between the expression of IL28B and ISGs. IL28B expression correlated with the expression of ISGs ($r = 0.44$, $P < .001$); however, the correlation was different according to the SNP genotype. We observed a steep-slope correlation for the minor genotype and a slow-slope correlation for the major genotype (Figure 3*C* and *D*). Interestingly, 4 minor homozygotic (GG) patients showed a steeper correlation than the heterozygotes (TG) (Figure 3*D*). Thus, the IL28B polymorphism might differentially regulate the expression of ISGs in the liver, leading to the different treatment outcomes.

We performed univariate and multivariate analyses to identify the clinical factors associated with the major and minor genotypes (Table 4). Univariate analysis showed that higher hepatic ISGs and lower body mass index were significantly associated with the minor genotype; however, multivariate analysis showed that only hepatic ISGs (≥3.5) were associated with the minor genotype ($P < .001$; OR, 18.1; 95% confidence interval: 3.95–113). We further compared the predictive capacity of multivariate models using the expression of hepatic ISGs (<3.5 vs ≥3.5) or the IL28B genotype (major vs minor) (Supplementary Table 6). The predictive performance and fitness of the multivariate model using the IL28B genotype was superior to that using the expression of hepatic ISGs. However, when these factors were included in the same model, the expression of hepatic ISGs was still useful for the predictive model independent of the IL28B genotype (Supplementary Table 6).

Table 3. Clinical Characteristics of 32 Patients Genotyped by GWAS and 5 SNPs in Strong Linkage Disequilibrium With IL28B,[11] Including rs12979860

Patient No.	Response	Age, y	Sex	F stage	ISGs	IL28B	RefSNP (chr pos) Minor allele	rs8105790 (44424341) C	rs11881222 (44426763) G	rs12979860 (44430627) T	rs8099917 (44435005) G	rs7248668 (44435661) A
1	SVR	42	M	1	4.20	83.8		TT	AA	CC	TT	GG
2	SVR	59	M	1	2.62	45.5		TT	AA	CC	TT	GG
3	SVR	41	F	1	1.54	1.3		TT	AA	CC	TT	GG
4	TR	57	M	1	3.18	21.7		TT	AA	CC	TT	GG
5	TR	68	F	1	1.43	20.3		TT	AA	CC	TT	GG
6	SVR	44	M	1	0.97	4.6		TT	AA	CC	TT	GG
7	SVR	61	M	2	2.15	6.1		TT	AA	CC	TT	GG
8	SVR	50	M	2	3.25	66.4		TT	AA	CC	TT	GG
9	SVR	49	M	2	1.25	ND		TT	AA	CC	TT	GG
10	TR	59	F	2	1.29	17.4		TT	AA	CC	TT	GG
11	SVR	48	F	2	1.00	90.2		TT	AA	CC	TT	GG
12	TR	65	F	2	2.86	36.4		TT	AA	CC	TT	GG
13	NR	34	M	3	0.82	17.8		TT	AA	CC	TT	GG
14	SVR	55	M	3	0.83	13.8		TT	AA	CC	TT	GG
15	TR	68	M	3	0.75	20.6		TT	AA	CC	TT	GG
16	SVR	64	M	3	0.94	15.7		TT	AA	CC	TT	GG
17	SVR	67	F	3	1.50	25.7		TT	AA	CC	TT	GG
18	SVR	48	M	4	1.69	7.9		TT	AA	CC	TT	GG
19	NR	66	F	1	4.57	16.5		TC	AG	CT	TG	GA
20	SVR	52	F	1	5.23	29.3		TC	AG	CT	TG	GA
21	NR	55	F	1	8.25	57.2		TC	AG	CT	TG	GA
22	SVR	49	F	1	5.36	ND		TC	AG	CT	TG	GA
23	TR	44	M	1	2.08	7.0		TC	AG	CT	TG	GA
24	NR	63	M	1	2.77	10.5		TC	AG	CT	TG	GA
25	NR	61	F	2	3.98	39.1		TC	AG	CT	TG	GA
26	NR	42	M	2	4.89	5.9		TC	AG	CT	TG	GA
27	SVR	49	M	3	3.31	6.9		TC	AG	CT	TG	GA
28	TR	71	F	3	5.53	27.3		TC	AG	CT	TG	GA
29	TR	63	M	3	3.40	33.5		TC	AG	CT	TG	GA
30	NR	70	F	3	4.78	8.1		TC	AG	CT	TG	GA
31	TR	62	F	3	3.53	14.0		TC	AG	CT	TG	GA
32	NR	56	M	4	7.37	30.8		CC	GG	TT	GG	AA

NOTE. The Pearson correlation of the r^2 estimates for adjacent pairs; rs8099917 vs rs8105790, rs8099917 vs rs11881222, rs8099917 vs rs12979860, and rs8099917 vs rs7248668 = 0.99, 0.99, 0.98, and 0.97, respectively.
IL28B, interleukin 28B; GWAS, genome-wide association studies; ISGs, interferon stimulated genes; SNP, single nucleotide polymorphism; SVR, sustained viral response; TR, transient response; NR, no response; M, male; F, female.

To examine further the different hepatic gene expression of patients with the major or minor genotypes, pathway analysis of differentially expressed genes between the 2 groups was performed. By comparing the expression of hepatic genes between patients with the major and minor genotypes, 1359 differentially expressed genes were identified ($P < .01$; 711 genes were up-regulated with the minor genotype, and 648 genes were up-regulated with the major genotype). Pathway analysis of these genes demonstrated that signaling pathways related to interferon action, apoptosis, and Wnt signaling were up-regulated in the liver of patients with the minor genotype, whereas B-cell-, dendritic cell-, and natural killer cell-related genes were up-regulated in the liver of patients with the major genotype (Supplementary Figure 3). These results suggest that IL28B may be involved in innate and adaptive immune responses and that different antiviral signaling pathways might be involved in the liver of patients with different SNPs.

Discussion

Multiple viral and host factors may be related to the treatment response to Peg-IFN and RBV combination therapy. For the viral factors, a higher number of aa substitutions in the ISDR of nonstructural 5A region was strongly associated with a favorable response to IFN-α monotherapy in patients with genotype-1 HCV.[4]

Besides viral factors, host factors such as age, gender, fibrotic stage of the liver, and the presence of steatosis and insulin resistance were associated with the treatment outcome.[20] Analysis of hepatic gene expression demonstrated that the up-regulation of ISGs in the liver before treatment may be related to a poor treatment response.[6-9] To reveal the underlying mechanism of treatment resistance, 2 reports compared gene expression profiling in the liver before and during therapy and showed that patients with up-regulated ISGs in the liver prior to treatment failed to further induce ISGs following the ad-

Table 4. Comparison of Clinical Factors Between Patients With Major (TT) and Minor (TG+GG) Alleles

Clinical category	TT		TG+GG		Univariate *P* value	Multivariate odds (95% CI)	Multivariate *P* value
No. of patients	n = 60		n = 31			—	
Treatment response							
SVR+TR vs NR	51 vs 9		11 vs 20		<.001	—	
Age and gender							
Age, *y*	56	(30–69)	56	(30–71)	.843	—	
Sex (M vs F)	39 vs 21		19 vs 12		.518	—	
Liver factors							
F stage (F1-2 vs F3-4)	36 vs 24		23 vs 17		.905	—	
A grade (A0-1 vs A2-3)	27 vs 33		20 vs 11		.075	—	
ISGs (Mx, IFI44, IFIT1) (<3.5 vs ≥3.5)	46 vs 14		5 vs 26		<.001	18.1 (3.95–113)	<.001
Laboratory parameters							
HCV-RNA (*KIU/mL*)	2055	(160–5000)	1970	(126–5000)	.602	—	
BMI (kg/m^2)	24.5	(16.3–40.5)	22.9	(19.1–26.6)	.006	—	.077
AST (*IU/L*)	59	(22–258)	54	(21–283)	.227	—	
ALT (*IU/L*)	75	(24–376)	60	(18–236)	.077	—	
γ-GTP (*IU/L*)	61	(4–392)	53	(20–229)	.517	—	.167
WBC ($/mm^3$)	4450	(2100–11,100)	4600	(2500–8200)	.947	—	
Hb (*g/dL*)	14.2	(11.4–16.7)	14.5	(11.2–17.2)	.606	—	
PLT ($\times 10^4/mm^3$)	15.4	(7–39.4)	16.2	(9.2–27.7)	.832	—	
TG (*mg/dL*)	98	(58–248)	131	(30–303)	.053	—	.055
T-Chol (*mg/dL*)	172	(115–222)	168	(129–237)	.910	—	
LDL-Chol (*mg/dL*)	84	(42–123)	69	(51–107)	.052	—	.055
HDL-Chol (*mg/dL*)	44	(18–72)	45	(29–77)	.218	—	
FBS (*mg/dL*)	95	(59–291)	96	(66–206)	.849	—	
Insulin (*μU/mL*)	7.5	(0.7–23.2)	9.2	(2–23.2)	.195	—	
HOMA-IR	1.3	(0.3–11.7)	1.2	(0.4–9.6)	.339	—	
Viral factors							
ISDR mutations (≤1 vs ≥2)	38 vs 22		23 vs 7		.194	—	.083
Treatment factors							
Total dose administrated							
Peg-IFN (*μg*)	3960	(1500–7200)	3840	(1920–5760)	.377	—	
RBV (*g*)	203	(26–336)	201	(106–268)	.777	—	
Achieved administration rate							
Peg-IFN (%)							
≥80%	41		17		.207	—	
<80%	19		14				
RBV (%)							
≥80%	34		19		.671	—	
<80%	26		12				
Achievement of EVR	40/60 (62%)		9/31 (29%)		<.001	—	

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; EVR, early virologic response; γ-GTP, γ-glutamyl transpeptidase; ISDR, interferon sensitivity determining region; Mx1, myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse); WBC, leukocytes; HOMA-IR, homeostasis model assessment of insulin resistance; Hb, hemoglobin; RBV, ribavirin; PLT, platelets; TG, triglycerides; TR, transient response; T-chol, total cholesterol; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol; FBS, fasting blood sugar; CI, confidence interval.

ministration of IFN and could not eliminate HCV.[6,7] We performed a similar analysis and observed that these findings were more evident in liver lobular cells than in infiltrating lymphocytes in the portal area (submitted for publication). Thus, both viral and host factors might be closely related to the treatment response to Peg-IFN and RBV combination therapy. However, the clinical relevance and relationships of these factors have not been fully evaluated. In this study, we validated the clinical significance of the expression of hepatic ISGs on treatment outcome using a relatively large cohort of patients and compared its significance with other viral and host factors. To compare the patients with SVR, TR, and NR, we assessed the overall plausibility of each group using Fisher C statistic,[16] and patients with SVR and TR were grouped together for further analysis.

We examined hepatic gene expression in 91 of 168 patients using the Affymetrix genechip. Expression profiling using 37 representative ISGs (see Materials and Methods), which were selected from gene expression profiling comparing pretreatment and under treatment liver, differentiated 2 groups of

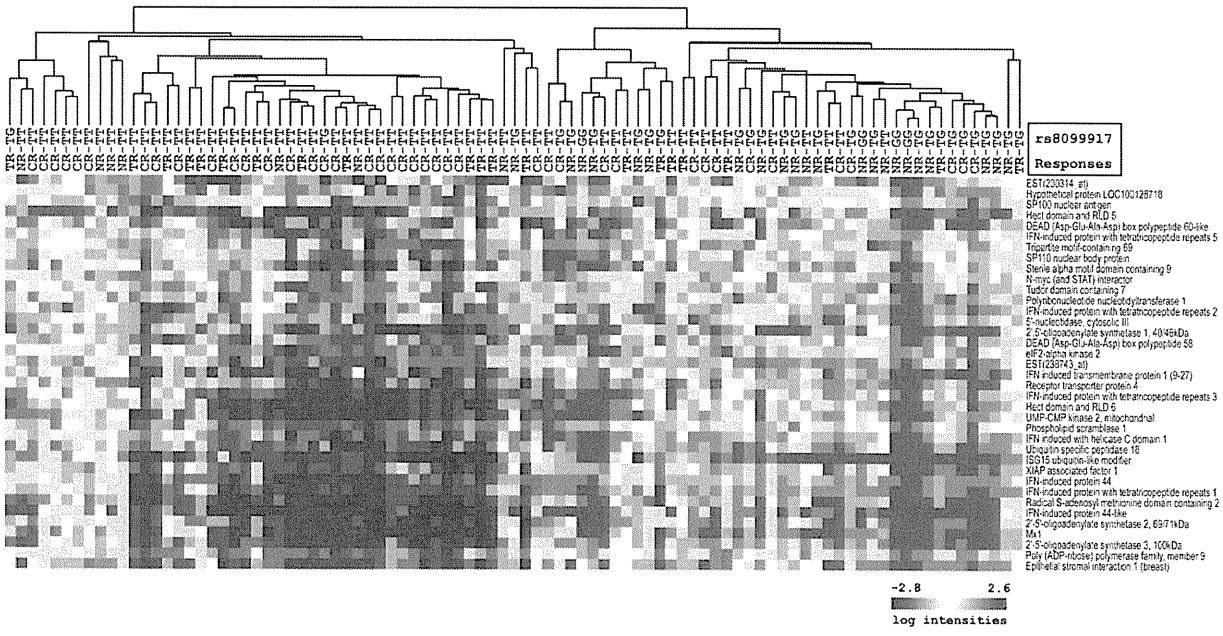

Figure 2. Hierarchical clustering analysis of 91 patients with the defined genotype of IL28B. Responses to therapy (SVR, TR, and NR) and IL28B genotype (TT, TG, or GG) are shown. The structure of the dendrogram and heat map is the same as in Figure 1.

patients: the Up-ISG and Down-ISG groups (Figure 1). The proportion of patients with NR to treatment was significantly higher in the Up-ISGs group.

Multivariate analysis showed that hepatic ISGs (<3.5), fibrosis stage (F1-F2), and ISDR mutations (≥2) significantly contributed to the outcome for the SVR+TR group (Table 1). Discriminate analysis using variables selected by multivariable analysis predicted the SVR+TR patients with 82% accuracy and NR patients with 79% accuracy. However, the accuracy decreased to 67% for SVR+TR patients and 53% for NR patients when the expression of hepatic ISGs was removed from the variables (data not shown). Interestingly, the expression of hepatic ISGs was strongly correlated with γ-GTP and weakly correlated with insulin resistance. A recent study describing the association between insulin resistance and poor treatment outcome might be partially explained by this observation.[20]

In this study, we utilized 3 ISGs (Mx1, IFI44, and IFIT1) out of 15 validated by RTD-PCR. The expression values of these ISGs were higher than those of other ISGs (Supplementary Figure 1*A*). We averaged these ISGs and set the cut-off value as 3.5 from the ROC curve (Supplementary Figure 1*B*). The sensitivity, specificity, and positive and negative predictive values on the likelihood of achieving SVR+TR using this cut-off value were 82% (103/125), 72% (31/43), 90% (103/115), and 58% (31/53), respectively. The results were compared with those observed for the 15 ISGs (Supplementary Table 5). These results showed that the 3.5 cut-off value for Mx1, IFI44, and IFIT1 would be valuable for clinical use.

Despite the importance of the expression of hepatic ISGs, viral factors may also allow us to predict the outcome of treatment. Multivariate analysis showed that ISDR mutations (≥2) independently contributed to the treatment outcome, although univariate analysis did not show significance (P = .07); therefore, ISDR might be uniquely and differentially involved in treatment resistance.

What causes the differences in the expression of hepatic ISGs? In parallel to the gene expression analysis, a GWAS was applied to identify genomic loci associated with treatment response, and a polymorphism in IL28B was found to predict hepatitis C treatment-induced viral clearance.[10-12] To examine the relationship between the genetic variation of IL28B and hepatic gene expression, we determined the IL28B polymorphism in 91 patents (Table 3). The patients with the minor genotype (TG or GG) had an increased expression of hepatic ISGs compared with the patients with major genotype (TT) (Figures 2 and 3). In European-Americans, the proportion of major homozygotes is 39% (CC at rs1297986), 49% for heterozygotes (TC), and 12% for minor homozygotes (TT).[11] Although the proportion of minor homozygotes was much less in this study (GG, 4%), as reported in a previous study in Japan,[12] more patients are required for proper evaluation. It is interesting that the expression of hepatic ISGs in minor homozygotes (GG) was higher than in heterozygotes (TG) in this study.

The results clearly showed that the differences in the expression of hepatic ISGs before treatment are associated with the IL28B polymorphism and results in different treatment outcomes. Although we could not detect significant differences in the expression levels of IL28B depending on the different SNP, some patients with the major genotype showed a higher expression of IL28B. Because IL28B expression was approximately 10-fold less than the expression of ISGs, the lower

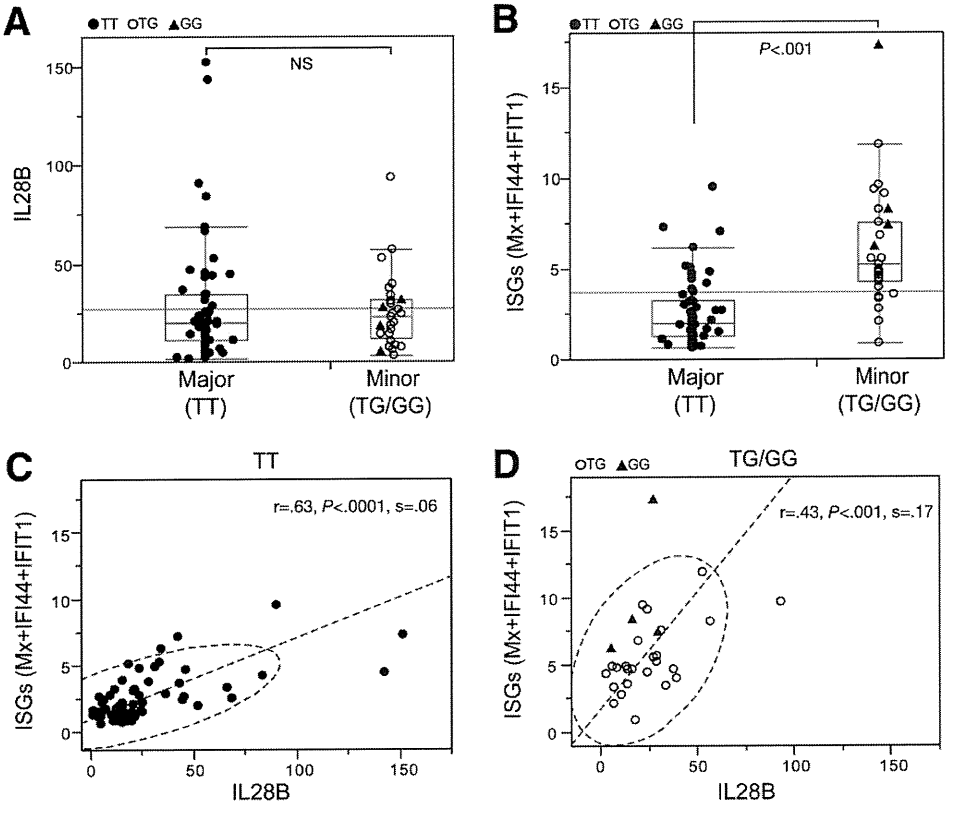

Figure 3. (*A*) IL28 expression in the liver of 91 patients with the major (TT) or minor (TG or GG) genotype (rs8099917). (*B*) Expression of ISGs in the liver of patients with the major (TT) or minor (TG or GG) genotype (rs8099917). (*C*) Relationship between IL28 and ISGs in the liver of patients with the major (TT) genotype (rs8099917). (*D*) Relationship between IL28 and ISGs in the liver of patients with the minor (TG or GG) genotype (rs8099917).

expression of IL28B may be a reason for the decreased ability to distinguish differences in its expression. Another possibility may be the specificity of the IL28B primers used in this study; because IL28B shares a 98.2% nucleotide sequence homology with IL28A, IL28B specific primers are not available.[21] When the expression of IL28B and hepatic ISGs were compared, a significant correlation was observed, and, interestingly, IL28B and ISGs derived from different SNPs were correlated in a different way (Figure 3*C* and *D*). It appeared that hepatic ISGs were more induced by the reduced amounts of IL28B in patients with the minor genotype. The mechanism behind these findings has yet to be determined; however, IL28B interacts with a heterodimeric class II cytokine receptor that consists of IL-10 receptor β (IL-10Rβ) and IL-28 receptor α (IL-28Rα).[18,19] It is possible that IL28B could mediate antiviral signaling through IL-10 signaling as well as STAT1 activation. The Th 2 dominant signaling of IL28B may modulate signaling pathways in livers with CH-C and contributes to the different expression of ISGs. Another possibility may be that the cell origin of hepatic ISGs is different. A recent study revealed cell-type specific ISG expression in macrophages and hepatocytes, which could be related to the IFN response.[22] As more of the B-cell-, dendritic cell-, and natural killer cell-related genes were up-regulated in the liver of patients with the major genotype, ISGs could be expressed by these cells, whereas they are expressed by hepatocytes in the liver of patients with the minor genotype. It is known that the induction of ISGs in lymphocytes is lower than that in hepatocytes. The precise mechanism should be investigated further as a different regulatory mechanism for the expression of ISGs may be present.

In conclusion, we presented the clinical relevance of the expression of hepatic ISGs for the treatment outcome of Peg-IFN and RBV combination therapy. The different expressions of hepatic ISGs before treatment might be due to polymorphisms in IL28B. Further studies are required to clarify the detailed pathways of IL28B and hepatic gene expression through molecular biologic and immunologic aspects.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.04.049.

Appendix 1. The Hokuriku Liver Study Group (HLSG) is Composed of the Following Members:

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Received October 9, 2009. Accepted April 14, 2010.

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Acknowledgments

The authors thank Nami Nishiyama and Yuki Hatayama for excellent technical assistance.

Participating investigators are listed in Appendix 1.

Conflicts of interest

The authors disclose no conflicts.

Funding

This work was supported in part by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan.