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Hepatic Interferon-Stimulated Genes Are Differentially Regulated in the Liver of Chronic Hepatitis C Patients With Different Interleukin-28B Genotypes

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Pretreatment up-regulation of hepatic interferon (IFN)-stimulated genes (ISGs) has a stronger association with the treatment-resistant interleukin (IL)28B minor genotype (MI; TG/GG at rs8099917) than with the treatment-sensitive IL28B major genotype (MA; TT at rs8099917). We compared the expression of ISGs in the liver and blood of 146 patients with chronic hepatitis C who received pegylated IFN and ribavirin combination therapy. Gene expression profiles in the liver and blood of 85 patients were analyzed using an Affymetrix GeneChip (Affymetrix, Santa Clara, CA). ISG expression was correlated between the liver and blood of the MA patients, whereas no correlation was observed in the MI patients. This loss of correlation was the result of the impaired infiltration of immune cells into the liver lobules of MI patients, as demonstrated by regional gene expression analysis in liver lobules and portal areas using laser capture microdissection and immunohistochemical staining. Despite having lower levels of immune cells, hepatic ISGs were up-regulated in the liver of MI patients and they were found to be regulated by multiple factors, namely, IL28A/B, IFN-λ4, and wingless-related MMTV integration site 5A (WNT5A). Interestingly, WNT5A induced the expression of ISGs, but also increased hepatitis C virus replication by inducing the expression of the stress granule protein, GTPase-activating protein (SH3 domain)-binding protein 1 (G3BP1), in the Huh-7 cell line. In the liver, the expression of WNT5A and its receptor, frizzled family receptor 5, was significantly correlated with G3BP1. *Conclusions:* Immune cells were lost and induced the expression of other inflammatory mediators, such as WNT5A, in the liver of IL28B minor genotype patients. This might be related to the high level of hepatic ISG expression in these patients and the treatment-resistant phenotype of the IL28B minor genotype. (HEPATOLOGY 2014;00:000-000)

Interferon (IFN) and ribavirin (RBV) combination therapy has been a popular modality for treating patients with chronic hepatitis C (CHC); however, ~50% of patients usually relapse, particularly those with hepatitis C virus (HCV) genotype 1b and a high viral load.[1] The recently developed direct-acting antiviral drug, telaprevir, combined with pegylated (Peg)-IFN plus RBV, significantly improved sustained virologic response (SVR) rates; however, the SVR rate was not satisfactory (29%-33%) in patients who had no

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCL, CC chemokine ligand; CHC, chronic hepatitis C; CLLs, cells in liver lobules; CPAs, cells in portal areas; CXCL10/IP-10, chemokine (C-X-C motif) ligand 10/interferon-gamma-induced protein 10; CXCR3, chemokine (C-X-C motif) receptor 3; DCs, dendritic cells; DVL, disheveled; FZD5, frizzled family receptor 5; G3BP1, GTPase-activating protein (SH3 domain)-binding protein 1; GGT, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; IFN, interferon; IHC, immunohistochemical; IL, interleukin; ISGs, interferon-stimulated genes; JFH-1, Japanese fulminant hepatitis type 1; LCM, laser capture microdissection; MA, major genotype; MAd, major genotype, down-regulated; MAu, major genotype, up-regulated; MI, minor genotype; Mx, myxovirus (influenza virus) resistance; NK, natural killer; OAS2, 2′-5′-oligoadenylate synthetase 2; PALT, portal-tract-associated lymphoid tissue; Peg_IFN, pegylated IFN; RBV, ribavirin; RTD-PCR, real-time detection polymerase chain reaction; SG, stress granule; siRNA, small interfering RNA; SVR, sustained virologic response; WNT5A, wingless-related MMTV integration site 5A.

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response to previous therapy.[2] Therefore, IFN responsiveness is still an essential clinical determinant for treatment response to triple (Peg-IFN+RBV+DAA) therapy.

A recent landmark genome-wide association study identified a polymorphism in the interleukin (IL)28B, IFN-λ3) gene that was associated with either a sensitive (major genotype; MA) or resistant (minor genotype; MI) treatment response to Peg-IFN and RBV combination therapy and was characterized by either up- (-u) or down-regulation (-d) of interferon-stimulated genes (ISGs).[3-5] However, the underlying mechanism for the association of this polymorphism and treatment response has not been clarified. Previously, we showed that up-regulation of the pretreatment expression of hepatic ISGs was associated with an unfavorable treatment outcome and was closely related to the treatment-resistant IL28B genotype (TG or GG at rs8099917).[6] It could be speculated that the pretreatment activation of ISGs would repress additional induction of ISGs after treatment with exogenous IFN. However, it is unknown how hepatic ISGs are up-regulated in treatment-resistant CHC patients and why patients with high levels of ISG expression cannot eliminate HCV. Therefore, other mechanisms should be involved in the unfavorable treatment outcome of patients with the treatment-resistant IL28B genotype.

In the present study, we performed gene expression profiling in the liver and blood and compared the expression of ISGs between them. Furthermore, ISG expression in liver lobules and portal areas was analyzed separately using a laser capture microdissection (LCM) method. Finally, we identified an immune factor that is up-regulated in patients with the treatment-resistant IL28B genotype and mediates favorable signaling for HCV replication.

Materials and Methods

Patients. We analyzed 168 patients with CHC who had received Peg-IFN-α2b (Schering-Plough K.K., Tokyo, Japan) and RBV combination therapy for 48 weeks at the Graduate School of Medicine, Kanazawa University Hospital, Japan and its related hospitals, as reported previously (Table 1 and Supporting Table 1).[6]

Preparation of Liver Tissue and Blood Samples. A liver biopsy was performed on samples from 168 patients, and blood samples were obtained from 146 of these patients before starting therapy (Table 1 and Supporting Table 1). Detailed procedures are described in the Supporting Materials and Methods.

Affymetrix GeneChip Analysis. Liver tissue samples from 91 patients and blood samples from 85 patients were analyzed using an Affymetrix GeneChip (Affymetrix, Santa Clara, CA). LCM analysis was performed in 5 MAu, MAd, and MI patients. Affymetrix GeneChip analysis and LCM were performed, as described previously.[6,7] Detailed procedures are described in the Supporting Materials and Methods.

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), as described previously.[7] Pathway analysis was performed using MetaCore (Thomson Reuters, New York, NY). Detailed procedures are described in the Supporting Materials and Methods.

Quantitative Real-Time Detection Polymerase Chain Reaction, Cell Lines, Cell Migration Assay, Vector Preparation, HCV Replication Analysis, and Statistical Analysis. These procedures are described in detail in the Supplemental Material and Methods.

Results

Differential ISG Expression in Liver and Blood of Patients With Different IL28B Genotypes. Previously, we showed that pretreatment up-regulation of hepatic ISGs was associated with an unfavorable treatment outcome and was closely related to the treatment-resistant IL28B MI (TG or GG at rs8099917).[6] To examine whether expression of hepatic ISGs would reflect the expression of blood ISGs, we compared ISG expression between the liver and blood. We utilized three ISGs (interferon-induced protein 44 [IFI44], interferon-induced protein with

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Table 1. Clinical Characteristics of 146 Patients Whose Liver and Blood Samples Were Analyzed by RT-PCR

	Major (MA)				Minor (MI)		
Clinical Category	Major ISG Up (MAu)		Major ISG Down (MAd)				*P* Value
No. of patients	n = 42		n = 68		n = 36		NA
Age and sex							
Age (years)	55	(30-72)	56	(31-72)	55	(30-73)	NS
Sex (M vs. F)	27 vs. 15		34 vs. 34		19 vs. 17		NS
Treatment responses							
SVR/TR/NR	24/12/6		30/33/6		6/7/23*		MAu vs. MI < 0.0001, MAd vs. MI < 0.0001
IL28B genotype (TT vs. TG+GG)	TT		TT		TG/GG (31/5)		NA
Liver factors							
F stage (1/2/3/4)	14/13/11/4		30/20/11/7		14/8/10/4		NS
A grade (A0-1 vs. A2-3)	16 vs. 26		37 vs. 31		20 vs. 16		NS
ISGs (Mx1, IFI44, IFIT1)	3.83*	(2.14-9.48)	1.30*	(0.36-2.08)	5.52*	(0.86-17.3)	MAu vs. MAd < 0.0001, MAu vs. MI < 0.0001, MAd vs. MI < 0.0001
IL28A/B	41.3*	(4-151)	11.7*	(1-53)	22.7*	(3-93)	MAu vs. MAd < 0.0001, MAu vs. MI = 0.0004, MAd vs. MI = 0.031
Blood factors							
ISGs (Mx1, IFI44, IFIT1)	11.1*	(2.78-24.9)	4.76	(0.41-20.6)	5.64	(0.71-2.8)	MAu vs. MAd < 0.0001, MAu vs. MI < 0.0001
IL28A/B	1.6	(0.1-7.7)	1.3	(0.2-6.4)	1.3	(0.3-3.6)	NS
Laboratory parameters							
HCV-RNA (KIU/mL)	2,430	(160-5,000)	2,692	(140-5,000)	1,854*	(126-5,000)	MAd vs. MI = 0.017
BMI (kg/m2)	24	(18.7-31.9)	24	(16.3-34.7)	22.8	(19.1-30.5)	NS
AST (IU/L)	86*	(22-258)	54	(18-192)	64	(21-178)	MAu vs. MAd = 0.0008
ALT (IU/L)	112*	(17-376)	75	(16-345)	79	(18-236)	MAu vs. MAd = 0.023
γ-GTP (IU/L)	99*	(21-392)	47	(4-367)	74	(20-298)	MAu vs. MAd = 0.0003
WBC (/mm3)	4,761	(2,100-8,100)	4,982	(2,800-9,100)	4,823	(2,500-8,200)	NS
Hb (g/dL)	14.1	(11.4-16.7)	14.1	(9.3-16.9)	13.9	(11.2-16.4)	NS
PLT (× 104/mm3)	15.2	(9.2-27.8)	16.8	(7-39.4)	16.3	(9-27.8)	NS
TG (mg/dL)	112	(42-248)	102	(42-260)	136*	(30-323)	MAd vs. MI = 0.02
T-Chol (mg/dL)	162	(90-221)	169	(107-229)	167	(81-237)	NS
LDL-Chol (mg/dL)	77	(36-123)	83*	(42-134)	72	(29-107)	MAd vs. MI = 0.04
HDL-Chol (mg/dL)	40	(18-67)	43	(27-71)	47*	(27-82)	NS
Viral factors							
ISDR mutations ≦ 1 vs. ≧ 2	23 vs. 19*		51 vs. 17		26 vs. 10		MAu vs. MAd = 0.02
Core aa 70 (wild-type vs. mutant)	24 vs. 18		42 vs. 22		16 vs. 20*		MAd vs. MI = 0.02

*$P < 0.05$.

Abbreviations: BMI, body mass index; ALT, alanine aminotransferase; WBC, leukocytes; Hb, hemoglobin; PLT, platelets; TG, triglycerides; T-chol, total cholesterol; LDL-chol, low density lipoprotein cholesterol; HDL-chol, high density lipoprotein cholesterol; NA, not applicable; NS, not significant.

tetratricopeptide repeats 1 [IFIT1], and myxovirus (influenza virus) resistance [Mx1]) with a high dynamic range, comparable relative expression, and good predictive performance.[6] Mean values of the three ISGs detected by real-time detection polymerase chain reaction (RTD-PCR) in 168 liver tissue samples (Supporting Table 1) showed a significant up-regulation of their expression in nonresponder or treatment-resistant IL28B MI (TG/GG; rs8099917) patients, compared to responder (SVR+TR) or treatment-sensitive IL28B MA (TT; rs8099917) patients, as reported previously (Fig. 1A and Supporting Fig. 1A).[6] However, ISG expression in 146 blood samples (Table 1) showed no difference between responders and nonresponders or the IL28B major and minor genotypes (Fig. 1B and Supporting Fig. 1B). To explore these findings further, gene expression profiling using Affymetrix GeneChips was performed on liver and blood samples from 85 patients (Supporting Tables 2 and 3), and the expression of 37 representative ISGs[6] was compared (Fig. 1C-E). MA patients were divided into two groups according to their ISG expression pattern in the liver: MAu and MAd. MI patients expressed ISGs at a higher level than MAu patients. Interestingly, ISG expression in MA patients showed a similar expression pattern in liver and blood, and ISGs were up-regulated in MAu patients and down-regulated in the MAd patients. However, MI patients showed a different ISG expression pattern in liver and blood, where ISGs were up-regulated in the liver, but down-regulated in the blood (Fig. 1C). The correlation of the mean values of the three ISGs (IFI44, IFIT1, and Mx1) between liver and blood from 146 patients demonstrated a significant correlation between values in MA patients (Fig. 1D), whereas no correlation was observed in MI patients (Fig. 1E). Interestingly, ISG expression correlated significantly between liver and blood of responders, but not of nonresponders, in MA and MI patients (Supporting Fig. 1C-F). These results indicate that the correlation of ISG expression in the liver and blood is an important predictor of treatment response.

Clinical Characteristics of IL28B MA Patients With Up- and Down-Regulated ISGs and IL28B MI Patients. From the expression pattern of ISGs and mean values of the three ISGs (IFI44, IFIT1, and Mx1), we could use receiver operating characteristic curve analysis to set a threshold of 2.1-fold to differentiate MAu and MAd patients. Following this criterion, 42 MAu, 68 MAd, and 36 MI patients (total, 146) were grouped (Table 1). Hepatic ISG expression was highest in MI patients, whereas blood ISG expression was highest in MAu patients. Conversely, hepatic IL28A/B (IFN-λ2/3) expression was highest in MAu patients, whereas blood IL28A/B expression showed no difference among the three groups. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase (GGT) levels were significantly higher in MAu patients than in MAd patients. Interestingly, serum ALT levels were significantly correlated with ISG expression in MA patients, but not in MI patients (Supporting Fig. 2E,F).

Gene expression profiling in peripheral immune cells showed the presence of active inflammation in MAu patients, whereas the inactive or remissive phase of inflammation was observed in MAd patients. In contrast, monophasic and intermediate inflammation existed in MI patients (Supporting Fig. 3).

Reduced Number of Immune Cells in the Liver Lobules of IL28B MI Patients. To examine the discordant expression of ISGs in liver and blood of MI patients, we performed LCM to collect cells in liver lobules (CLLs) and cells in portal areas (CPAs) separately from each of five liver biopsied samples from MAu, MAd, and MI patients (Fig. 2A). Interestingly, the ISG expression pattern in CLLs from MA patients was similar to that of CPAs, and ISGs were up-regulated in MAu patients and down-regulated in MAd patients. ISG expression in CLLs from the MI patients was different to that in CPAs, and ISGs were up-regulated in CLLs, but down-regulated in CPAs (Fig. 2A). We hypothesized that the discordance of ISG expression between CLLs and CPAs in MI patients might be the result of the lower number of immune cells that infiltrated the liver lobules of these patients. To prove this hypothesis, immunohistochemical (IHC) staining was performed (Fig. 2B). IHC staining showed that IFI44 was strongly expressed in the cytoplasm and nucleus of CLLs from MI patients, whereas it was intermediately expressed in MAu patients and weakly expressed in MAd patients. Interestingly, IFI44 was strongly expressed in CPAs of MAu patients and weakly expressed in CPAs of MAd patients, showing a correlation between expression in CLLs and CPAs of MA patients, whereas ILI44 expression was relatively weak in CPAs, compared with CLLs, in MI patients (Fig. 2B). In the same section of the specimens, there were less CD163-positive monocytes and macrophages in MI patients than in MAu and MAd patients. Similarly, there were fewer CD8-positive T cells in MI patients than in MAu and MAd patients (Fig. 2B). Semiquantitative evaluation of CD163- and CD8-positive lymphocytes in liver lobules showed a significantly lower number of cells in

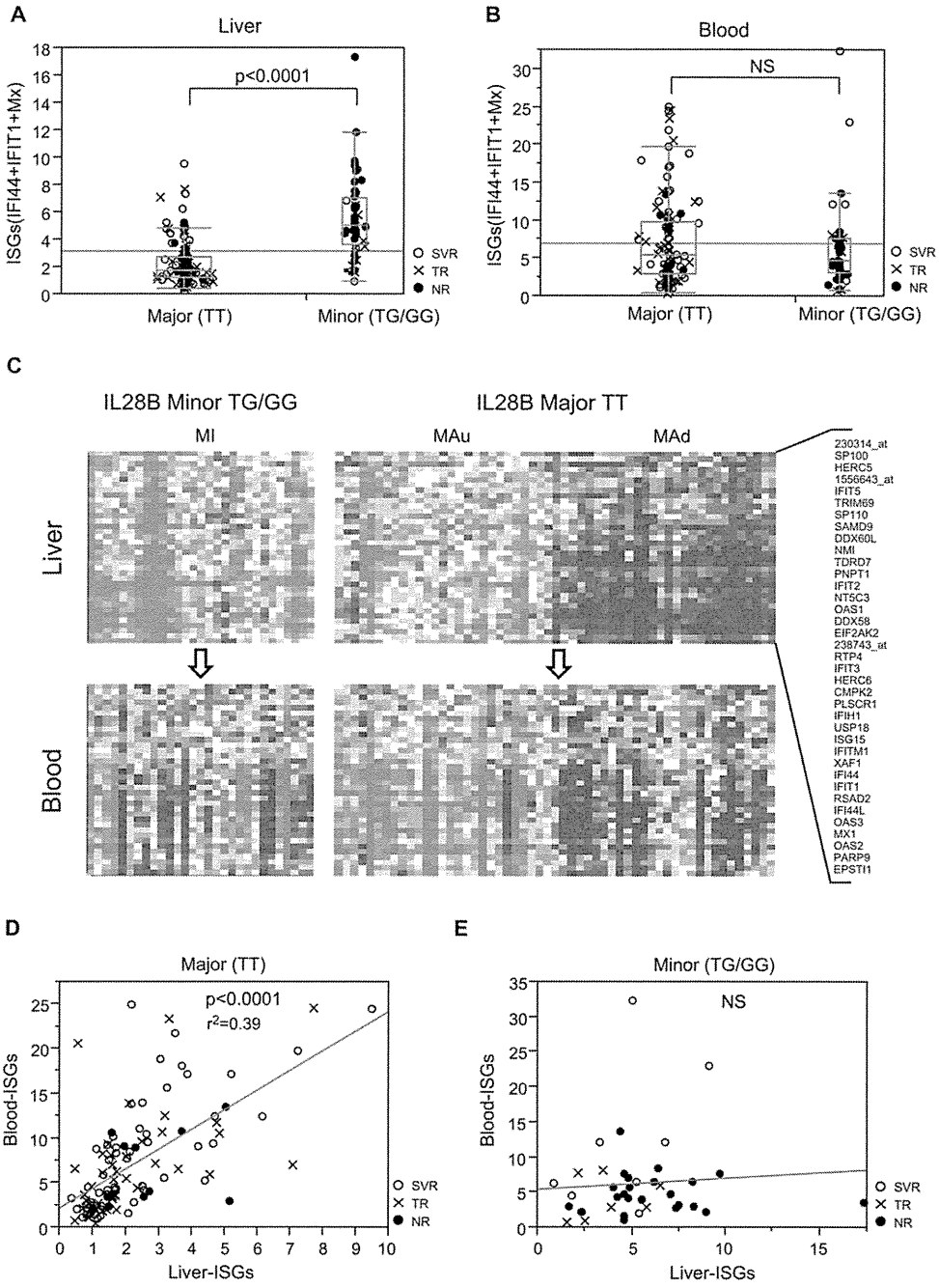

Fig. 1. Comparison of ISG expression in liver and blood of patients with different IL28B genotypes. (A and B) RTD-PCR results of mean ISG expression (IFI44-IFIT1+Mx1) in liver (A) and blood (B) of IL28B major (MAu/Mad) and minor (MI) genotype patients. (C) One-way hierarchical clustering analysis of 85 patients using 37 representative ISGs derived from liver (upper) and blood (lower). (D and E) Correlation of mean ISG expression (IFI44-IFIT1+Mx1) in liver and blood of IL28B major (MA; D) and minor (MI; E) genotype patients.

MI patients than in MAu and MAd patients (Supporting Fig. 4A,B). To support these findings, we examined the expression of 24 surface markers of immune cells in CLL, including dendritic cells (DCs), natural killer (NK) cells, macrophages, T cells, B cells, and granulocytes (Supporting Fig. 5A). The expression of immune cell-surface markers was repressed in MI patients, compared to MAu and MAd patients. Furthermore, whole-liver expression profiling in 85 patients showed the reduced expression of these surface markers in MI patients, compared to MAu and MAd patients (Supporting Fig. 5B). These results indicated that fewer immune cells had infiltrated the liver lobules of MI patients.

In addition to these findings, various chemokines, such as CC chemokine ligand (CCL)19, CCL21, CCL5, and chemokine (C-X-C motif) ligand (CXCL)13, which are important regulators for the recruitment of DCs, NK cells, T cells, and B cells in the liver, were significantly down-regulated in MI patients, compared to MAd and MAu patients (Supporting Fig. 4C-F).

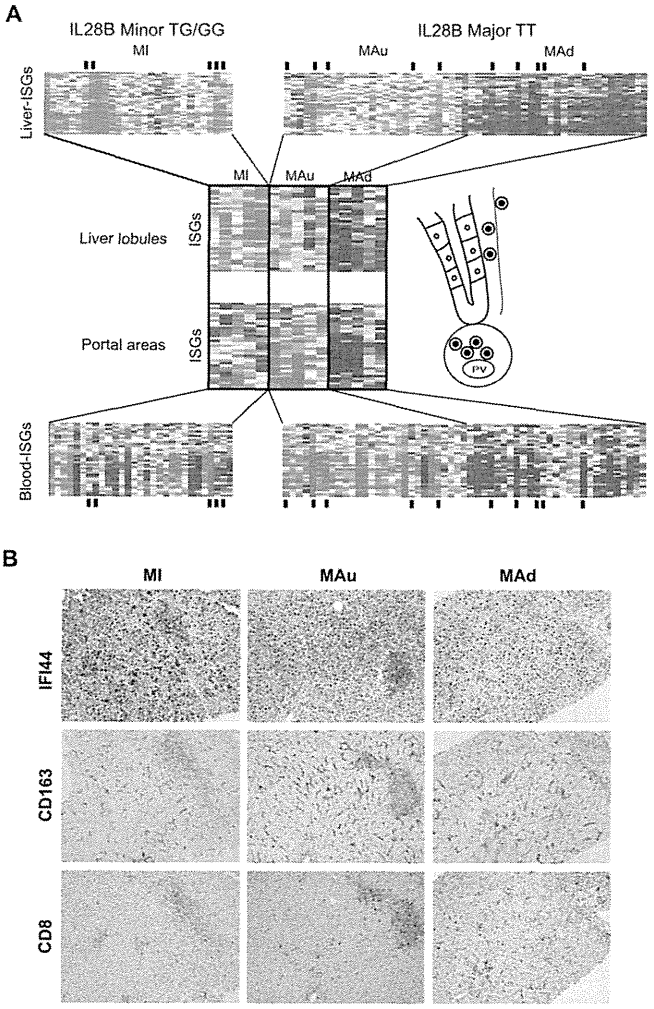

Fig. 2. LCM and IHC staining of biopsied liver specimens. (A) Comparison of the ISG expression pattern of whole liver (upper), CLLs (upper middle), CPAs (lower middle), and blood (bottom). CLLs and CPAs were obtained from 5 MI, MAu, and MAd patients, who are indicated by small black bars. (B) IHC staining of IFI44, CD163, and CD8 in MI, MAu, and MAd patients.

Hepatic ISG Expression Is Significantly Correlated With IL28A/B, but not IFN-α or IFN-β. The lower number of immune cells in the liver lobules of MI patients implies that reduced levels of IFN are produced from DCs, macrophages, and so on. These findings prompted us to examine the relationship between hepatic ISGs and IFN-α, IFN-β, IL29/IFN-λ1, and IL28A/B in CHC patients. Hepatic ISG expression was significantly correlated with IL28A/B, but not IFN-β (Fig. 3A-C) or IFN-α (data not shown) in MAu, MAd, and MI patients. Expression of IL29 was correlated with hepatic ISG expression only in MAu patients. These results indicate that hepatic ISGs would be mainly induced by IL28A/B in CHC patients. Interestingly, the correlation between hepatic ISGs and IL28A/B was strongest in MA patients ($P < 0.0001$ in MAu; $P = 0.0006$ in MAd), whereas rather a weak correlation was observed in MI patients ($P = 0.015$). Moreover, the ratio of hepatic ISGs to IL28A/B was larger in MI patients than in MA patients (S = 0.061 in MI; S = 0.028 in MAu; S = 0.020 in MAd), suggesting the presence of additional factors that can induce expression of ISGs in MI patients. Therefore, we evaluated the expression of the recently discovered IFN-λ4 in MI patients. Interestingly, there was a significant correlation between hepatic ISG and IFN-λ4 expression ($P = 0.0003$; Fig. 3C).

Wingless-Related MMTV Integration Site 5A and Its Receptor, Frizzled Receptor 5, Are Significantly Up-Regulated in the Liver of Patients With the IL28B MI. IFN-λ4 is a promising factor to induce ISG expression in MI patients,[8] and the functional relevance of IFN-λ4 for the pathogenesis of CHC is under investigation. We searched for other factors that could induce ISG expression in MI patients. A closer observation of gene expression profiling in CLLs obtained by LCM demonstrated that WNT signaling was specifically up-regulated in MI patients

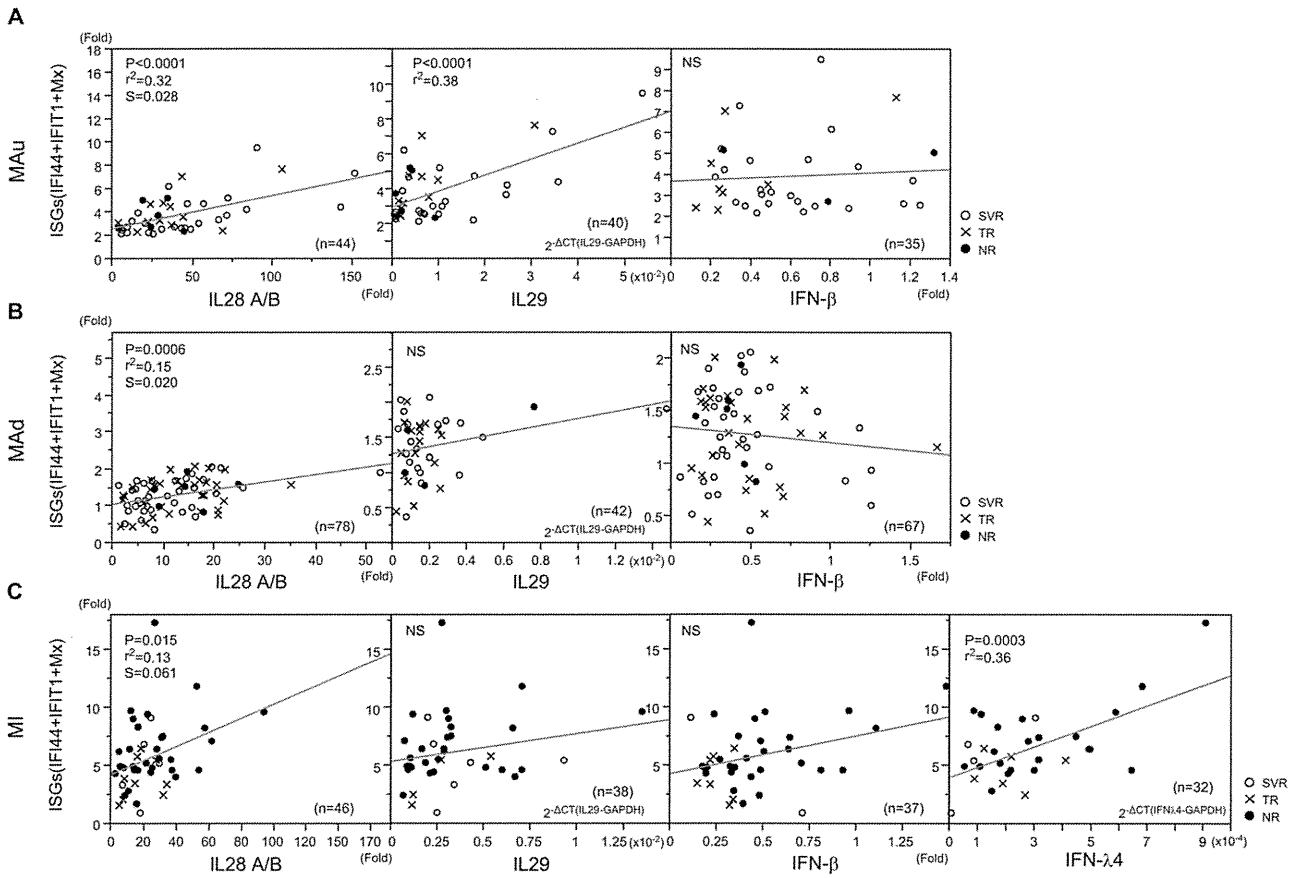

Fig. 3. Correlation analysis of hepatic ISGs and IL28A/B, IL29, IFN-β, and IFN-λ4. Correlation of mean ISG (IFI44+IFIT1+Mx1) and IL28A/B, IL29, IFN-β, and IFN-λ4 expression was evaluated in MAu (A), MAd (B), and MI (C) patients. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Supporting Fig. 6). Further observation enabled us to identify that the WNT ligand, wingless-related MMTV integration site 5A (WNT5A), and its receptor, frizzled receptor 5 (FZD5), were up-regulated in MI patients. RTD-PCR results on 168 liver-biopsied samples confirmed the significant up-regulation of WNT5A and FZD5 in MI patients, compared to MAu and MAd patients (Fig. 4A,B). Interestingly, WNT5A expression was negatively correlated with chemokine expression (Supporting Fig. 7). IHC staining showed up-regulation of FZD5 in liver lobules of MI patients, but not in MAu or MAd patients (Fig. 4C). WNT5A expression was significantly correlated with hepatic ISG expression in MI and MAd patients (Fig. 4D). Interestingly, we found a weak, but significant, correlation between WNT5A and IFN-λ4 expression in MI patients (Fig. 4E).

WNT5A Induces ISG Expression, but Stimulates HCV Replication in Huh-7 Cells. To examine the functional relevance of up-regulated expression of WNT5A in MI patients, we first evaluated expression levels of WNT5A and ISGs (2′-5′-oligoadenylate synthetase 2 [OAS2], Mx1, IFI44, and IFIT1) in two immortalized human hepatocyte cell lines, THLE-5b and TTNT cells (Supporting Materials and Methods), and one human hepatoma cell line, Huh-7 cells (Supporting Fig. 8A,B). WNT5A was moderately expressed in THLE-5b and TTNT cells, whereas its expression in Huh-7 cells was minimal. Interestingly, ISG expression in these cells correlated well with expression of WNT5A (Supporting Fig. 8B). Small interfering RNA (siRNA) to WNT5A efficiently repressed WNT5A expression to ~20% of the control in THLE-5b cells, and in this condition, ISG expression was significantly decreased to 30%-50% of the control (Supporting Fig. 8C). Conversely, transduction of WNT5A using a lentivirus expression system in Huh-7 cells significantly increased OAS2 expression (Supporting Fig. 8D), as well as Mx1 and IFIT1 expression (data not shown), in the presence and absence of HCV infection. Surprisingly, HCV replication, as determined using Gaussia luciferase activity, increased in WNT5A-transduced cells (Supporting Fig. 8E). Furthermore, WNT5A-transduced cells supported more HCV replication than

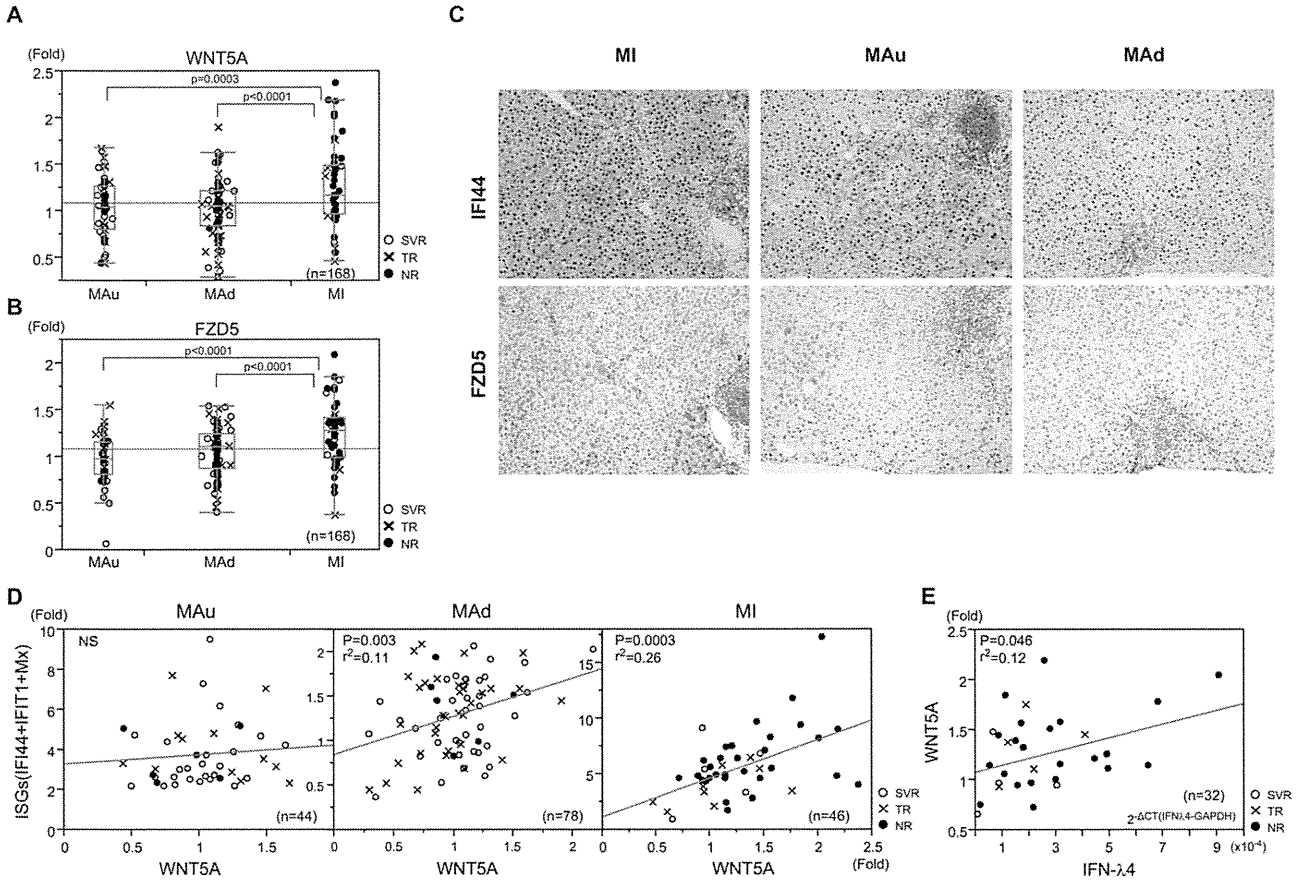

Fig. 4. WNT5A and FZD5 are up-regulated in IL28B MI patients. (A) RTD-PCR results of WNT5A expression in liver of MAu, MAd, and MI patients. (B) RTD-PCR results of FZD5 expression in liver of MAu, MAd, and MI patients. (C) IHC staining of IFI44 and FZD5 expression in liver of MAu, MAd, and MI patients. (D) Correlation of mean ISG (IFI44+IFIT1+Mx1) and WNT5A expression in liver of MAu, MAd, and MI patients. (E) Correlation of WNT5A and IFN-λ4 expression in liver of MI patients.

nontransduced cells under IFN treatment (Supporting Fig. 8F).

WNT5A-FZD5 Signaling Induces the Expression of the Stress Granule Protein, GTPase-Activating Protein (SH3 Domain)-Binding Protein 1, Which Supports HCV Replication. These findings were further confirmed by using Huh-7 cells that were continuously infected with Japanese fulminant hepatitis type 1 (JFH-1; Huh7-JFH1), which is a genotype 2a HCV isolate.[9] Interestingly, expression of WNT5A in Huh7-JFH1 cells was significantly up-regulated, compared with uninfected Huh-7 cells, and showed an equivalent expression level with THLE-5b cells (Fig. 5A). siRNA to WNT5A efficiently repressed WNT5A expression to ~20% of the control, and in this condition, ISG expression (IFI44 was not expressed in Huh-7 cells), HCV RNA, and infectivity were repressed to 25%-65%, 60%, and 40% of the control, respectively (Fig. 5B and Supporting Fig. 9A). Interestingly, CXCL13 expression was significantly increased in this condition. We evaluated the expression of GTPase-activating protein (SH3 domain)-binding protein 1 (G3BP1), a recently recognized stress granule (SG) protein that supports HCV infection and replication.[10] Expression of G3BP1 was repressed to 60% of the control by knocking down WNT5A. Conversely, overexpression of WNT5A in Huh7-JFH1 cells significantly decreased CXCL13 expression and increased HCV RNA, infectivity, and G3BP1 expression (Fig. 5C and Supporting Fig. 9B). A recent report demonstrated that G3BP1 is a disheveled (DVL)-associated protein that regulates WNT signaling downstream of the FZD receptor.[11] Knocking down FZD5 in Huh7-JFH1 cells significantly reduced the expression of DVL1-3, G3BP1, Mx1, and IFIT1 as well as HCV infectivity (Supporting Fig. 9C,D). Interestingly, G3BP1 expression was significantly up-regulated in liver of MI patients (Fig. 5D). Furthermore, G3BP1 expression was significantly correlated with WNT5A expression in liver of the CHC patients (Fig. 5E). More dramatically, a strong correlation was observed between expression of FZD5 and G3BP1 in liver of CHC patients (Fig. 5F).

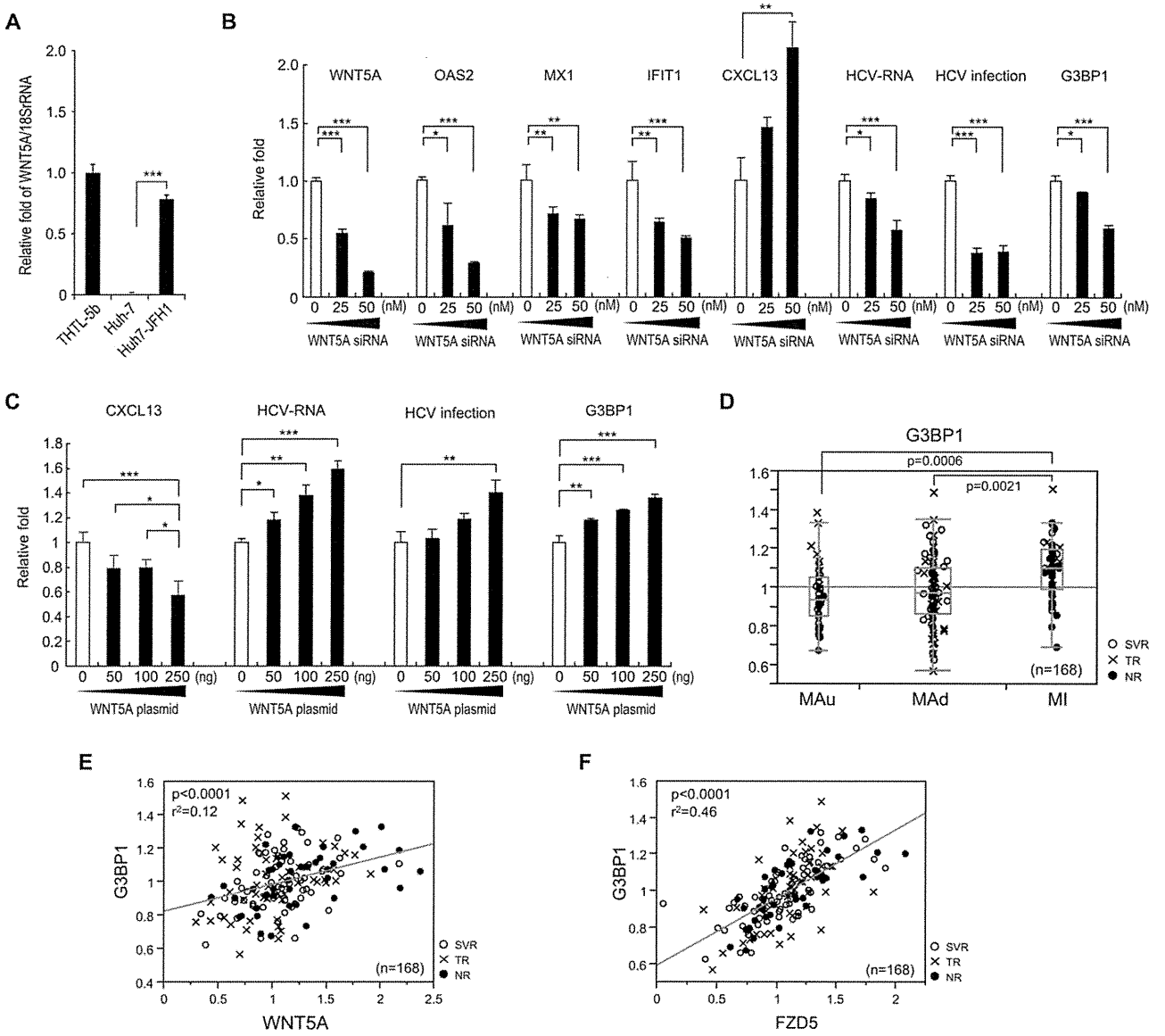

Fig. 5. Relationship between WNT5A and FZD5 signaling and the SG protein, G3BP1. (A) WNT5A expression in THLE-5b, Huh-7, and Huh7-JFH1 cells. (B) Knocking down WNT5A and changes of OAS2, Mx1, IFIT1, CXCL13, and G3BP1 expression, HCV RNA, and infectivity in Huh7-JFH1 cells. (C) Overexpression of WNT5A after transfection with pCMV-WNT5A and decrease in CXCL13 expression and increase in HCV RNA, infectivity, and G3BP1 expression. (A-C) Experiments were performed in duplicate and repeated three times (n = 6). Values are the means ± standard error. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$. (D) RTD-PCR results for G3BP1 expression in liver of MAu, MAd, and MI patients. (E) Correlation of WNT5A and G3BP1 expression in the liver. (F) Correlation of FZD5 and G3BP1 expression in the liver. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Discussion

The underlying mechanism for the association of the IL28B genotype with treatment responses to IFN-based therapy for HCV has not yet been clarified. We and others have shown that pretreatment up-regulation of hepatic ISGs was associated with an unfavorable treatment outcome[7,12,13] and was closely related to treatment-resistant MI IL28B, compared with treatment-sensitive MA IL28B.[6]

By comparing ISG expression in liver and blood, we found that their expression was correlated in MA patients, but not in MI patients. LCM analysis of ISG expression in CLLs and CPAs showed the loss of the correlation between CLLs and CPAs in MI patients (Fig. 2A). This might be the result of the impaired migration of immune cells into liver lobules that was demonstrated by decreased expression of immune cell-surface markers in CLLs by LCM (Supporting Fig. 5A) and IHC staining (Fig. 2B). Lymphocyte accumulation in the portal area (portal-tract-associated lymphoid tissue; PALT) might be involved in extravasation of lymphocytes from vessels in the portal area, but

others demonstrated that DCs appeared in the sinusoidal wall and passed through the space of Disse to PALT, where the draining lymphatic duct is located.[14] There should be an active movement of immune cells between liver lobules and PALT, as reflected by the correlation of ISG expression in CLLs and CPAs in the MA patients of this study.

ISGs were reportedly up-regulated in hepatocytes of treatment-resistant IL28B genotype patients, but were up-regulated in Kupffer cells of treatment-sensitive genotype patients.[15] Our results confirmed these findings; however, we also showed that expression of various immune cell-surface markers, such as those on DCs, NK cells, macrophages, T cells, B cells, and granulocytes, was lower in MI than in MA patients (Supporting Fig. 5). In addition, we showed that expression of various chemokines was also repressed in MI patients, compared to MA patients (Supporting Fig. 4C-F).

Up-regulation of pretreatment chemokine (C-X-C motif) ligand 10/interferon-gamma-induced protein 10 (CXCL10/IP-10) serum levels is also associated with an unfavorable treatment outcome.[16] CXCL10 expression in the liver was significantly correlated with hepatic ISG expression and was higher in nonresponders than in responders (Supporting Fig. 10). Our results support the usefulness of serum CXCL10 for prediction of treatment outcome. Chemokine (C-X-C motif) receptor 3 (CXCR3) expression, a receptor for CXCL10, was inversely correlated with hepatic ISG expression and was significantly lower in MI than in MA patients (Supporting Fig. 10).

The lower number of immune cells in the liver lobules of MI patients would imply the reduced production of IFN from DCs, macrophages, and so on. Correlation analysis showed that hepatic ISGs were mainly associated with type III IFNs (IL28A/B and IL29), but not type I IFNs (IFN-α or IFN-β), although a significant association with IL29 was only observed in MA patients with up-regulated ISGs. This might be related to the high serum ALT levels in MAu patients (Fig. 3). Closer examination of hepatic ISGs and IL28A/B suggested that factors other than IL28A/B might regulate ISG expression in MI patients. During the preparation of this study, IFN-λ4 was newly identified to be expressed in hepatocytes from treatment-resistant IL28B genotype patients.[8] Interestingly, we found a significant correlation between hepatic ISGs and IFN-λ4 in MI patients (Fig. 3C). Moreover, a closer examination of gene expression profiling in MI patients enabled us to detect up-regulation of the noncanonical WNT ligand, WNT5A. RTD-PCR analysis of 168 patients confirmed up-regulation of WNT5A and its receptor, FZD5, in MI patients. Importantly, WNT5A expression was significantly correlated with hepatic ISG expression in MI patients. A recent report showed that WNT5A induces expression of ISGs, increases sensitivity of keratinocytes to IFN-α,[17] and might be involved in the immune response to influenza virus infection.[18] Therefore, we examined the role of WNT5A in hepatocytes. Interestingly, expression of WNT5A and ISGs was well correlated, and knocking down WNT5A using siRNA reduced expression of ISGs in THLE-5b cells (Supporting Fig. 8). Conversely, transduction of Huh-7 cells with WNT5A using a lentivirus system increased expression of ISGs. Despite the increase in ISG expression, WNT5A did not suppress HCV replication, but rather increased it in Huh-7 cells (Supporting Fig. 8). These results were also confirmed by using Huh-7 cells continuously infected with JFH-1. By knocking down or overexpressing WNT5A in Huh7-JFH1 cells, we showed that HCV-RNA was positively regulated by WNT5A (Fig. 5B,C).

WNT5A and its receptor, FZD5, mediate noncanonical WNT signaling, such as planar cell polarity and the WNT-Ca^{2+}-signaling pathway through G proteins. WNT5A reportedly inhibits B- and T-cell development by counteracting canonical WNT signaling.[19] We found that G3BP1, an SG assembly factor, was up-regulated by WNT5A (Fig. 5C). SGs were reportedly formed by endoplasmic reticulum stress, followed by HCV infection, and localized around lipid droplets with HCV replication complexes.[10] G3BP1 contributes to SG formation and increases HCV replication and infection in Huh-7 cells.[10] Moreover, a recent report demonstrated that G3BP1 is a DVL-associated protein that regulates WNT signaling downstream of the FZD receptor.[11] In this study, repression of WNT5A or FZD5 significantly reduced expression of DVL1-3, G3BP1, Mx1, and IFIT1 as well as HCV infectivity in Huh7-JFH1 cells (Fig. 5 and Supporting Fig. 9).

Importantly, we found a significant correlation between WNT5A and G3BP1 expression in liver tissue samples (Fig. 5E). We also found a significant correlation between FZD5 and G3BP1 expression in liver tissue samples (Fig. 5F). Thus, up-regulated noncanonical WNT5A-FZD5 signaling participates in the induction of ISG expression, but preserves HCV replication and infection in hepatocytes by increasing levels of the SG protein, G3BP1. These findings may explain the pathophysiological state of the treatment-resistant phenotype in MI patients.

In this study, we demonstrated impaired immune cell infiltration of the liver in treatment-resistant IL28B genotype patients, and we also demonstrated

that up-regulation of hepatic ISGs in treatment-resistant IL28B genotype patients was mediated by multiple factors, including IL28A/B, IFN-λ4, and WNT5A. We found a significant negative correlation between WNT5A and various chemokines in liver of CHC patients (Supporting Fig. 7). Interestingly, WNT5A directly repressed one of these chemokines, CXCL13, a B-lymphocyte chemoattractant, in HCV-infected hepatocytes. These results indicate that loss of immune cells from the liver may be associated with the induction of other inflammatory factors, such as WNT5A, in MI patients, although we did not identify which cells express WNT5A. Further studies are needed to explore their functional relevance in the pathogenesis of CHC.

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Original Article

Feasibility and efficacy of hepatic arterial infusion chemotherapy for advanced hepatocellular carcinoma after sorafenib

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Aim: Sorafenib is the standard treatment for advanced hepatocellular carcinoma (HCC). However, although there is no proven therapeutic procedure following the termination of sorafenib, hepatic arterial infusion chemotherapy (HAIC) may be a treatment option in advanced HCC. The aim of this study was to evaluate feasibility and efficacy of HAIC for patients with advanced HCC as subsequent therapy.

Methods: We retrospectively evaluated 27 consecutive patients with advanced HCC who were treated with HAIC following sorafenib between June 2009 and December 2012 at our hospital. Cisplatin (20 mg/m^2 per day) was administered via the hepatic artery for 10 min, prior to the continuous administration of 5-fluorouracil (330 mg/m^2 per day) over 24 h from days 1–5 and 8–12 and the s.c. administration of pegylated interferon α-2b (1 μg/kg) on days 1, 8, 15, and 22. A treatment cycle consisted of 28 days of drug administration followed by 14 days of rest.

Results: The toxicity profile showed that hematological toxicities were common, and grade 3/4 neutropenia and thrombocytopenia were observed (51.9% and 48.1%, respectively). Five patients (18.5%) experienced device-related complications. No unexpected adverse reactions and no treatment-related deaths were observed. Partial response was obtained in eight patients (29.6%), and stable disease was noted in nine patients (33.3%). Median progression-free survival and median survival time from initiation of HAIC were 4.0 and 7.6 months, respectively.

Conclusions: Because HAIC was well tolerated and exhibited moderate antitumor activity, it is a potentially useful treatment procedure in patients with advanced HCC even after failure of sorafenib.

Key words: hepatic arterial infusion chemotherapy, hepatocellular carcinoma, sorafenib

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the sixth most common cancer and the third leading cause of cancer-related mortality worldwide.[1] A variety of new techniques of imaging modalities have enabled the detection of HCC at an early stage,[2] and advances in various therapeutic procedures have improved its curability.[3,4] However, the number of patients with HCC who can be treated curatively is limited because of impaired hepatic function and frequent recurrence even after curative therapy. The prognosis of patients with advanced HCC where tumor has spread over the liver or invaded major vessels remains extremely poor.[5]

Sorafenib, an oral multikinase inhibitor that blocks tumor cell proliferation and angiogenesis, is the only systemic therapy that has shown survival benefit for patients with advanced HCC,[6,7] and it is recognized worldwide as standard first-line therapy in advanced HCC.[8,9] Alternative systemic chemotherapies using cytotoxic agents or novel targeted drugs have been attempted in patients with advanced HCC;[10,11] however, to date none have proven effective, except sorafenib. Moreover, following sorafenib therapy most patients are not suitable candidates for subsequent therapy because of the progressive nature of their disease, poor general condition, and impaired hepatic function.

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Compared with systemic chemotherapy, hepatic arterial infusion chemotherapy (HAIC) is based on theoretical advantages such as higher concentrations of drugs delivered directly to tumors[12] and first-pass effect reducing systemic toxicity.[13] Although few reports have recorded the survival benefits of HAIC, HAIC in combination with interferon (IFN) has been reported to be a useful treatment procedure in patients with advanced HCC.[14,15] Although an optimal protocol of HAIC has not been established, the clinical benefits of HAIC regimen consisting of 5-fluorouracil (5-FU) and cisplatin with IFN were reported in a randomized phase II study.[15] However, it remains unclear whether HAIC is also safe and effective in patients with advanced HCC who were previously administered sorafenib.

The aim of the present study was to evaluate the feasibility and efficacy of HAIC in patients with advanced HCC after failure of sorafenib therapy. This approach provides useful information in determining treatment strategies for sorafenib-refractory patients with HCC.

METHODS

Patients

ALL OF 68 consecutive patients with unresectable advanced HCC who had received sorafenib monotherapy at Kanazawa University Hospital and for whom this therapy was subsequently stopped because of tumor progression or/and unacceptable adverse effects between June 2009 and December 2012 were considered for enrollment. HCC was diagnosed by either histological confirmation or typical radiological findings, which showed hyperattenuation in the early phase and hypoattenuation in the late phase on dynamic computed tomography (CT).[16] All patients underwent dynamic CT to assess the extent of the cancer, and their hepatic and major organ functions were evaluated by physical examination and laboratory findings. We reviewed patients' medical records and investigated their backgrounds, treatment courses, and outcomes.

Sorafenib

The following were the inclusion criteria for sorafenib at our institution: patients with advanced HCC involving macroscopic vascular invasion, extrahepatic lesions and/or intrahepatic multiple lesions considered unsuitable for surgical resection, locoregional therapy or transarterial chemoembolization; all patients with an Eastern Cooperative Oncology Group performance status score of 2 or less and with appropriate function of major organs, such as bone marrow, kidney and heart; and patients categorized as Child–Pugh A in terms of hepatic function.

HAIC

The inclusion criteria for HAIC at our institution is nearly same as that of sorafenib. Patients with extrahepatic lesions were also considered eligible if these lesions were mild, and intrahepatic lesions were considered as prognostic factors. With regard to hepatic function, patients categorized as Child–Pugh A or B were eligible.

The reservoir system implantation technique was the same as described previously.[15] Catheters were introduced through the right femoral artery, and angiography from the celiac artery was initially performed to localize the HCC and evaluate intra- and extrahepatic vascularization. We then inserted a catheter with a side vent into the gastroduodenal artery, positioning the vent in the common hepatic artery using an image-guided procedure. The gastroduodenal artery, right gastric artery and other arteries presumed to supply the gastroduodenal region were embolized as far as possible to prevent gastrointestinal mucositis. The other end of the catheter was connected to an injection port that was subcutaneously implanted in the right lower abdomen. Finally, blood flow redistribution was confirmed.

Hepatic arterial infusion chemotherapy was initiated approximately 5 days after implantation of the reservoir, and the following protocol was then implemented: 5-FU (330 mg/m^2 per day) was continuously administrated via the hepatic artery using an infuser pump over 24 h from days 1–5 and 8–12, and cisplatin (20 mg/m^2 per day) was also administrated via the hepatic artery for 10 min prior to 5-FU administration. Pegylated IFN-α-2b (1.0 μg/kg) was s.c. administrated on days 1, 8, 15, and 22. A treatment cycle consisted of 28 days of drug administration followed by 14 days of rest. The treatment protocol was approved by the Ethics Committee of Kanazawa University, and informed consent for participation in the study was obtained from each subject. The study conformed to the guidelines of the 1975 Declaration of Helsinki.

Evaluation

Tumor staging was assessed according to the criteria of the Liver Cancer Study Group of Japan.[17,18] The efficacies of HAIC and sorafenib were assessed every 4–6 weeks by dynamic CT, and response to chemotherapy was assessed according to the Response Evaluation Criteria in Solid Tumors ver. 1.1.[19] Response rate was defined as

the sum of complete and partial response rates. Similar to an approach adopted in a recent report, the causes of progression after sorafenib therapy (progression pattern) were classified as follows: intrahepatic growth, extrahepatic growth, new intrahepatic lesion or new extrahepatic lesion and/or vascular invasion.[20] Adverse effects, including both hematological and non-hematological toxicities, were assessed by the Common Terminology Criteria for Adverse Events version 4.0.

Statistical analysis

Progression-free survival (PFS) was calculated from the first day of HAIC until either the date of radiological progression, the date of death or the last day of the follow-up period. Overall survival (OS) was calculated from the first day of HAIC until either the date of death or the last day of the follow-up period. A χ^2-test was used to analyze the predictive factor for the response to HAIC. To compare prognosis according to response to chemotherapy and the progression pattern, cumulative survival was calculated using the Kaplan–Meier method[21] and any differences were evaluated using the log-rank test. $P<0.05$ were considered to be statistically significant, and all tests were two-sided. All statistical analyses were performed using the SPSS statistical software program package (version 11.0 for Windows; SPSS, Chicago, IL, USA).

RESULTS

Patients

OF 68 PATIENTS, 41 were not treated with HAIC because of either poor general condition ($n=12$), massive extrahepatic lesions ($n=9$), inadequate major organ function ($n=8$), treatment with HAIC prior to sorafenib therapy ($n=7$) or refusal to be treated with HAIC ($n=5$). Finally, 27 patients who had been treated with HAIC were analyzed in this study, all of whom had previously received sorafenib monotherapy. The response and tumor control rates for sorafenib therapy were 7.4% and 44.4%, respectively. In 22 patients (81.5%), sorafenib therapy was terminated because of tumor progression and in five (18.5%) because of unacceptable adverse effects. The median period of sorafenib therapy was 2.4 months (range, 0.1–18.0).

Patient characteristics at commencement of treatment with HAIC are summarized in Table 1. Because hepatic function was impaired in more than half of the patients in this study, 18 patients (66.7%) were classified as Child–Pugh class B or C. Macroscopic vascular invasion and extrahepatic metastasis were observed in 25.9% and 44.4% of the patients, respectively.

Table 1 Patient characteristics

	($n=27$)
Age, years	
Median, range	68, 44–84
Sex, *n* (%)	
Male	23 (85.2)
ECOG PS†, *n* (%)	
0	24 (88.9)
1	3 (11.1)
HBs antigen‡, *n* (%)	
Positive	9 (33.3)
HCV antibody§, *n* (%)	
Positive	15 (55.6)
Child-Pugh class at start of HAIC, *n* (%)	
A	9 (33.3)
B	16 (59.3)
C‡‡	2 (7.4)
Child-Pugh class at start of sorafenib, *n* (%)	
A	21 (77.8)
B§§	6 (22.2)
Ascites, *n* (%)	
Presence	18 (66.7)
Albumin, g/dL	
Median, range	3.2, 2.1–3.9
Prothrombin consumption test, %	
Median, range	82, 37–112
LCSGJ¶ tumor stage, *n* (%)	
II, III	12 (44.4)
IVA	4 (14.8)
IVB	11 (40.7)
Macroscopic vascular invasion, *n* (%)	
Yes	7 (25.9)
Extrahepatic spread, *n* (%)	
Yes	12 (44.4)
AFP††, ng/mL	
Median, range	404, <10–175560

†ECOG PS: Eastern Cooperative Oncology Group performance status.
‡HBs antigen: hepatitis B surface antigen.
§HCV antibody: hepatitis C virus antibody.
¶LCSGJ: Liver Cancer Study Group of Japan.
††AFP: α-fetoprotein.
‡‡Child-Pugh class B at decision making of HAIC.
§§Child-Pugh class A at decision making of sorafenib.

Treatment

A total of 60 courses were administered to 27 patients, with a median number of 2 (range, 0–5). All but two patients completed at least one course of HAIC. The

median duration between cessation of sorafenib therapy and commencement of HAIC was 1.2 months (range, 0–9.0). The median observation period from commencement of HAIC was 7.0 months (range, 0.8–48.0). Treatment with HAIC was terminated in 25 patients due to radiological tumor progression (20 patients), symptomatic tumor progression (one patient) or change in the treatment procedure (four patients); however, there were no patients in whom HAIC was terminated because of adverse effects. HAIC was continued in the remaining two patients until the last day of the follow-up period.

Safety

All 27 patients were assessed for adverse effects, and the toxicity profile of HAIC is summarized in Table 2. Hematological toxicities were common, particularly grade 3/4 neutropenia and grade 3/4 thrombocytopenia, which were observed in 14 (51.9%) and 12 (48.1%) patients, respectively, even though no serious complication such as sepsis or bleeding were observed and all toxicities were tolerable and reversible. Mild and low-frequency nonhematological toxicities were observed, except in one patient who had grade 3 diarrhea. Although 5 patients (18.5%) had device-related complications (3 catheter obstruction, 1 hepatic artery occlusion, and 1 hepatic arteritis), all issues were satisfactorily resolved by either exchanging the reservoir or conservative therapy. No unexpected adverse reactions were noted, and no treatment-related deaths were observed.

Table 2 Hepatic arterial infusion chemotherapy toxicities

	All grade *n* (%)	Grade 3 *n* (%)	Grade 4 *n* (%)
Hematological toxicities			
Leukocytopenia	20 (74.1)	10 (37.0)	0 (0)
Neutropenia	21 (77.8)	10 (37.0)	4 (14.8)
Anemia	12 (44.4)	1 (3.7)	1 (3.7)
Thrombocytopenia	22 (88.9)	13 (48.1)	0 (0)
Nonhematological toxicities			
Anorexia	7 (25.9)	0 (0)	0 (0)
Fever	5 (18.5)	0 (0)	0 (0)
Diarrhea	4 (14.8)	1 (3.7)	0 (0)
Fatigue	4 (14.8)	0 (0)	0 (0)
Hiccoughs	3 (11.1)	0 (0)	0 (0)
Gastric ulcer	3 (11.1)	0 (0)	0 (0)
Creatinine increased	2 (7.4)	0 (0)	0 (0)
Mucositis oral	2 (7.4)	0 (0)	0 (0)
Nausea	1 (3.7)	0 (0)	0 (0)
Ascites	1 (3.7)	0 (0)	0 (0)
Edema	1 (3.7)	0 (0)	0 (0)
Abdominal pain	1 (3.7)	0 (0)	0 (0)
Hypokalemia	1 (3.7)	0 (0)	0 (0)
Encephalopathy	1 (3.7)	0 (0)	0 (0)
Device-related complications			
Catheter obstruction	3 (11.1)	0 (0)	0 (0)
Hepatic artery occlusion	1 (3.7)	0 (0)	0 (0)
Vasculitis	1 (3.7)	0 (0)	0 (0)

Response to treatment and patient outcomes

Of the 27 patients, one died due to tumor progression and hepatic failure before radiological assessment could be performed; however, the remaining 26 were assessable for response to treatment. Tumor responses to HAIC are shown in Table 3. Although no patient achieved complete response, eight patients (29.6%) achieved partial response (PR) and nine (33.3%) achieved stable disease (SD); therefore, the response rate to HAIC was 29.6%. These results were independent of the Child–Pugh class, the response to previous sorafenib therapy and the progression pattern (Table 3), and none of the tested factors were found to be a significant predictive factor for response to HAIC (Table S1).

The median PFS of patients from commencement of HAIC was 4.0 months (Fig. 1). The median survival time (MST) of all patients was 7.6 months, with a 1-, 2-, and 3-year survival rate of 29.4%, 24.5% and 16.4%, respectively (Fig. 2a). The MST of patients who achieved PR were 36.7 months, which was significantly better than that of patients who achieved SD/progressive disease/not evaluable, namely, 6.6 months ($P < 0.01$; Fig. 2b). Patient prognosis did not differ according to the progression pattern (Fig.S1).

DISCUSSION

THE DEVELOPMENT OF a safe and effective alternative therapy is essential because sorafenib, which represented a breakthrough in the treatment of advanced HCC, had a low response rate and frequent adverse effects, often leading to a cessation of treatment.[22,23] An increasing number of emerging agents, including novel molecular targeted drugs, have been attempted in sorafenib refractory HCC. Nevertheless, their efficacy was found to be limited (response rate, 0–4.3%; time to progression, 1.6–2.7 months).[24–26]

The first aim of this study was to investigate the feasibility of HAIC in advanced HCC after the failure of sorafenib therapy. In this study, the frequency of

Table 3 Tumor response

Response to HAIC†	All, *n* (%)	Child-Pugh class[b]		Response to sorafenib				Progression pattern[c]		
		A	B or C	PR	SD	PD	NE	IHG§§	NIH¶¶	NEH[a]
CR‡	0 (0)	0	0	0	0	0	0	0	0	0
PR§	8 (29.6)	1	7	0	4	3	1	3	0	1
SD¶	9 (33.3)	5	4	1	5	3	0	7	2	0
PD††	9 (33.3)	3	6	0	4	4	1	6	2	0
NE‡‡	1 (3.7)	0	1	1	0	0	0	1	0	0
Total	27 (100)	9	18	2	13	10	2	17	4	1

†HAIC: hepatic arterial infusion chemotherapy.†, ‡, §, ¶, ††, ‡‡, §§ and ¶¶.
‡CR: complete response.
§PR: partial response.
¶SD: stable disease.
††PD: progressive disease.
‡‡NE: not evaluable.
§§IHG: intrahepatic growth.
¶¶NIH: new intrahepatic lesion.
[a]NEH: new extrahepatic lesion.
[b]At decision-making of HAIC.
[c]At termination of sorafenib therapy.

hematological toxicity, particularly neutropenia and thrombocytopenia, was high. One of the possible causes of these toxicities was pre-existing pancytopenia derived from liver cirrhosis in most patients, and another was the concurrent administration of IFN added to 5-FU and CDDP.[15] All of the patients recovered immediately after the end of treatment and no additional complications were noted. Moreover, the frequencies of leukocytopenia, neutropenia and thrombocytopenia observed in this study (74.1%, 77.8% and 88.9%, respectively) were very similar to those of patients who were not pretreated by sorafenib and underwent HAIC with the same protocol, including 5-FU/cisplatin/IFN (75.4%, 77.2% and 89.5%, respectively),[15] which suggested that prior administration of sorafenib did not have an additional impact on hematological toxicities. With regard to non-hematological toxicities, most of them were less frequent than those in a previous report,[15] and there were no unexpected adverse reactions. These favorable results may be derived from newly available drugs such as a second-generation 5-hydroxytryptamine 3 receptor antagonist and neurokinin-1-receptor antagonist or active supportive therapy. These findings suggested that HAIC was considered tolerable even for those patients who were previously administered sorafenib.

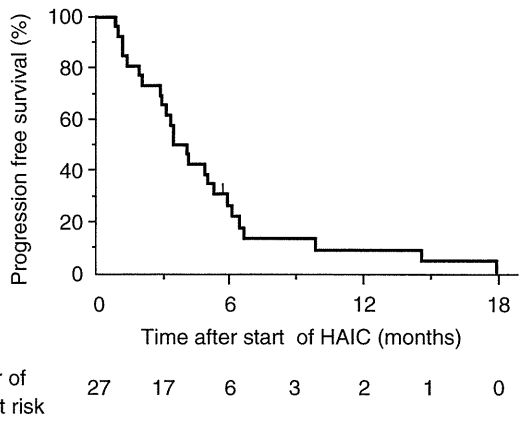

Figure 1 Kaplan–Meier plot of progression-free survival (PFS) since commencement of hepatic arterial infusion chemotherapy (HAIC). Median PFS was 4.0 months.

The response rate obtained in the present study (29.6%) appears to be low compared with that of previous reports.[14,15] Although it is difficult to compare the response rates among studies, possible reasons include variation in patients' hepatic function, the criteria used to evaluate responses, the effect of previous administration of sorafenib, and the relatively small number of patients. In addition, the proportion of patients with extrahepatic lesions may have been a meaningful factor because it was higher (44.4%) in this study than that of the previous study (0–14%)[14,15] and the response rate was reported to be lower in patients with HCC having extrahepatic metastases than in those without.[27] We could not identify any significant

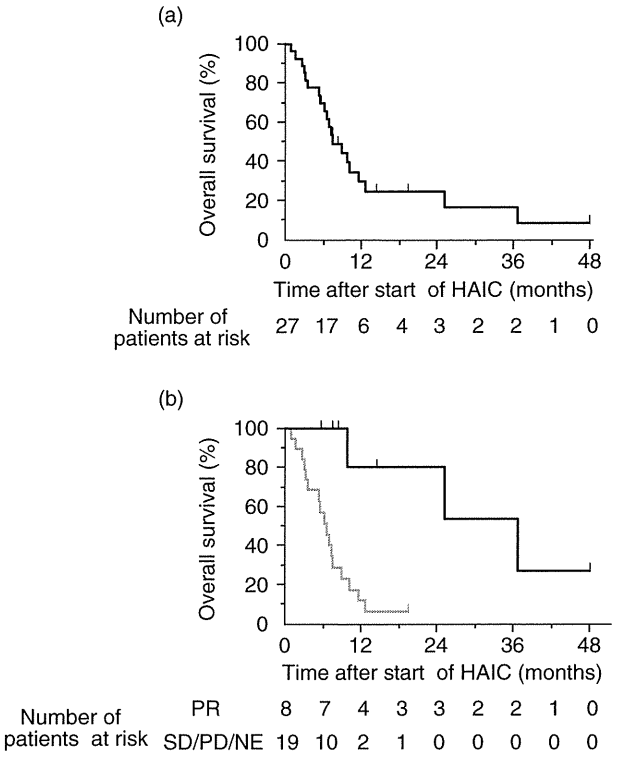

Figure 2 Kaplan–Meier plot of overall survival since commencement of hepatic arterial infusion chemotherapy (HAIC): (a) all patients and (b) according to response to HAIC. The median survival time (MST) of all patients was 7.6 months, and the MST of patients who achieved partial response (PR) were 36.7 months (black line), which was significantly better than that of the patients with stable disease (SD)/progressive disease (PD)/ not evaluable (NE), namely, 6.6 months (gray line) ($P < 0.01$).

predictive markers for the response to HAIC in this study, and further investigation is needed to examine the factors affecting the response rate of HAIC, and to select the appropriate population to receive HAIC after sorafenib therapy.

Another interesting finding of the present study was that half of our patients were categorized as Child–Pugh class B, and no correlation was observed between the response to HAIC and Child–Pugh classification. Although certain molecular targeted agents are currently being tested for sorafenib-refractory patients with HCC, the objectives in most of these trials are restricted to patients with good hepatic function. Other reports have described systemic chemotherapy by combination of gemcitabine and oxaliplatin is potentially safe for patients with Child–Pugh class B[28] and useful in sorafenib-refractory patients with HCC.[29] The results of the present study suggest that HAIC may be also considered as one of treatment procedures for patients with Child–Pugh class B after sorafenib therapy.

The present study has several limitations, including its retrospective nature, the small number of patients, the lack of controls, and single-institution subsets. A prospective trial with a larger number of patients in proper design is needed to confirm our findings.

In conclusion, HAIC has good feasibility and moderate antitumor activity and is a useful treatment option for patients with advanced HCC after failure of sorafenib therapy.

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NONE.

CONFLICTS OF INTEREST

NONE TO DECLARE.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's web-site:

Figure S1. Kaplan–Meier plot of overall survival since commencement of hepatic arterial infusion chemotherapy according to progression pattern. Patient prognosis did not differ among intrahepatic growth (IHG) group (black line), new intrahepatic lesion (NIH) group (gray line), and new extrahepatic lesion and/or vascular invasion (NEH) group (dashed line).
Table S1. Predictive marker for response to hepatic arterial infusion chemotherapy.

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BMC Cancer

RESEARCH ARTICLE Open Access

Peretinoin, an acyclic retinoid, improves the hepatic gene signature of chronic hepatitis C following curative therapy of hepatocellular carcinoma

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Abstract

Background: The acyclic retinoid, peretinoin, has been shown to be effective for suppressing hepatocellular carcinoma (HCC) recurrence after definitive treatment in a small-scale randomized clinical trial. However, little has been documented about the mechanism by which peretinoin exerts its inhibitory effects against recurrent HCC in humans *in vivo*.

Methods: Twelve hepatitis C virus-positive patients whose HCC had been eradicated through curative resection or ablation underwent liver biopsy at baseline and week 8 of treatment with either a daily dose of 300 or 600 mg peretinoin. RNA isolated from biopsy samples was subjected to gene expression profile analysis.

Results: Peretinoin treatment elevated the expression levels of *IGFBP6*, *RBP1*, *PRB4*, *CEBPA*, *G0S2*, *TGM2*, *GPRC5A*, *CYP26B1*, and many other retinoid target genes. Elevated expression was also observed for interferon-, Wnt-, and tumor suppressor-related genes. By contrast, decreased expression levels were found for mTOR- and tumor progression-related genes. Interestingly, gene expression profiles for week 8 of peretinoin treatment could be classified into two groups of recurrence and non-recurrence with a prediction accuracy rate of 79.6% ($P<0.05$). In the liver of patients with non-recurrence, expression of *PDGFC* and other angiogenesis genes, cancer stem cell marker genes, and genes related to tumor progression was down-regulated, while expression of genes related to hepatocyte differentiation, tumor suppression genes, and other genes related to apoptosis induction was up-regulated.

Conclusions: Gene expression profiling at week 8 of peretinoin treatment could successfully predict HCC recurrence within 2 years. This study is the first to show the effect of peretinoin in suppressing HCC recurrence *in vivo* based on gene expression profiles and provides a molecular basis for understanding the efficacy of peretinoin.

Keywords: Acyclic retinoid, Gene expression, Hepatocellular carcinoma

Background

Hepatocellular carcinoma (HCC) is the sixth most common form of cancer worldwide, and it is estimated that there are more than 740,000 new cases each year [1]. Early-stage HCC is indicated for definitive treatment by surgical resection or local therapy [2-4]; however, the prognosis of HCC is typically poor, and around 50% of patients experience recurrence within 3 years of definitive therapy [5-7]. Indeed, some researchers estimate that the 3-year recurrence rate is higher than 70% for hepatitis C virus (HCV)-positive patients [8], and past clinical experience with interferon-based therapy, systemic chemotherapy, and other treatment modalities has shown the lack of effective standard therapy for suppressing tumor recurrence after definitive treatment for HCC [9-11].

Peretinoin (NIK-333) has only been reported to suppress HCC recurrence in a small-scale randomized controlled

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