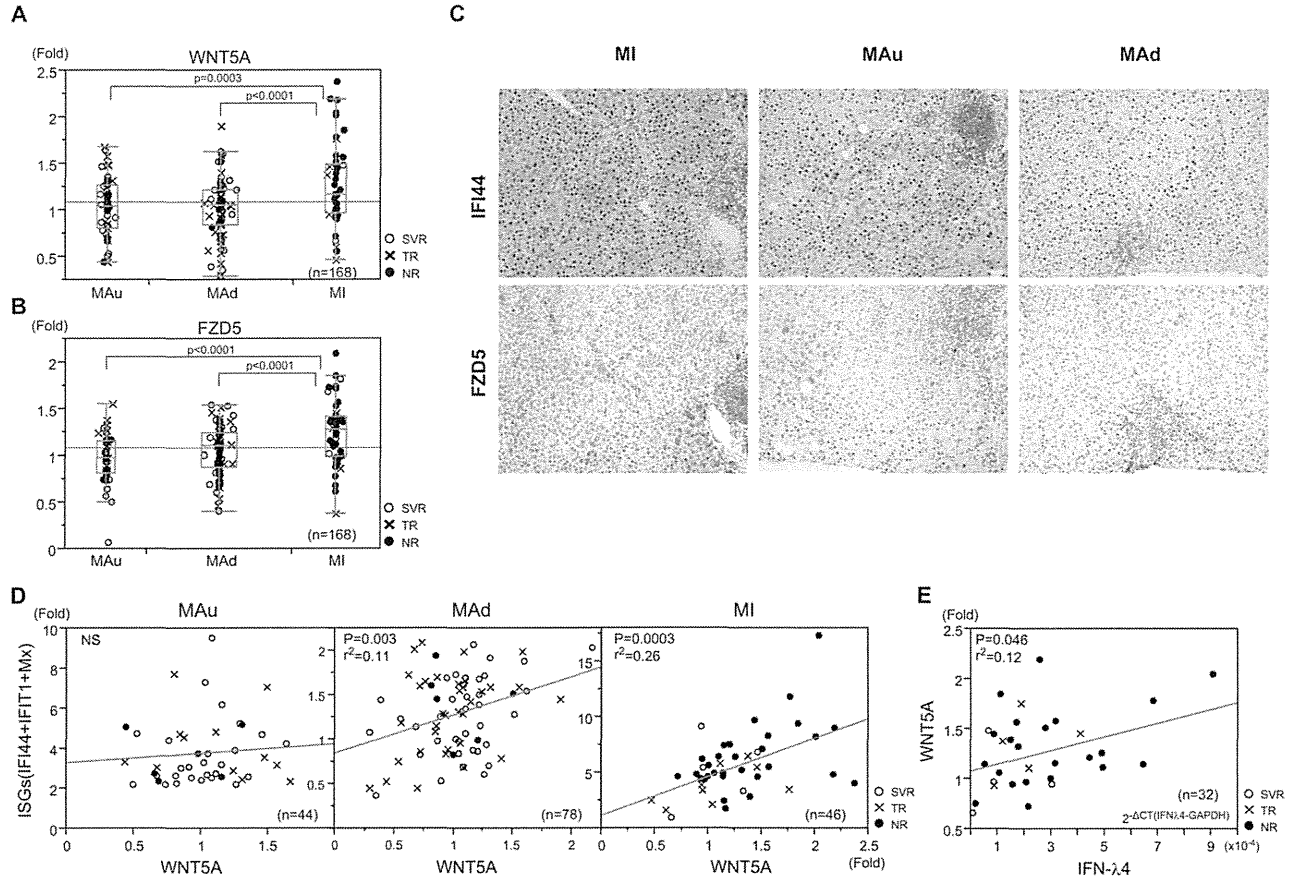

Fig. 4. WNT5A and FZD5 are up-regulated in IL28B MI patients. (A) RTD-PCR results of WNT5A expression in liver of MAu, MAd, and MI patients. (B) RTD-PCR results of FZD5 expression in liver of MAu, MAd, and MI patients. (C) IHC staining of IFI44 and FZD5 expression in liver of MAu, MAd, and MI patients. (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).

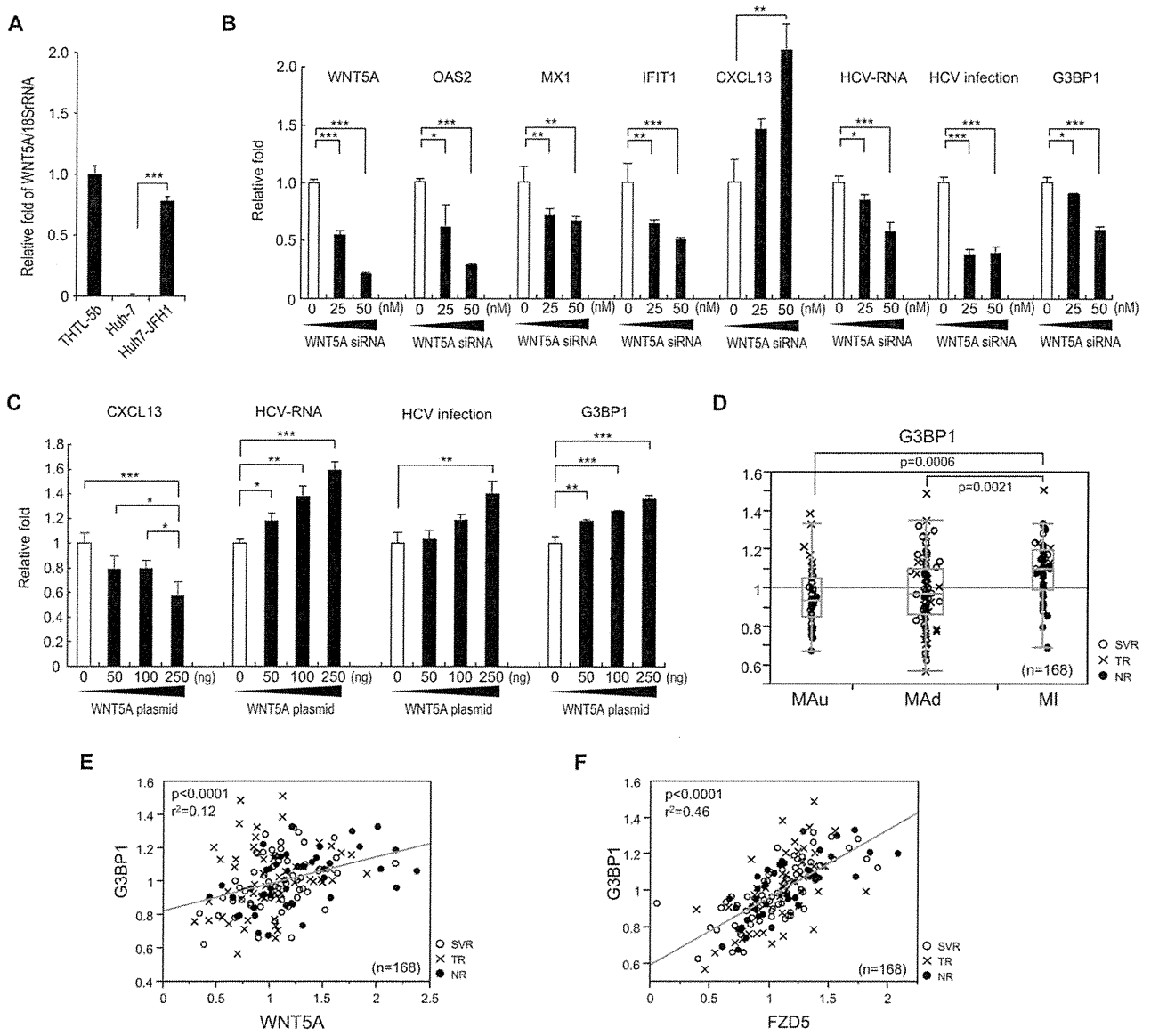

Fig. 5. Relationship between WNT5A and FZD5 signaling and the SG protein, G3BP1. (A) WNT5A expression in THLE-5b, Huh-7, and Huh7-JFH1 cells. (B) Knocking down WNT5A and changes of OAS2, Mx1, IFIT1, CXCL13, and G3BP1 expression, HCV RNA, and infectivity in Huh7-JFH1 cells. (C) Overexpression of WNT5A 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.

Acknowledgment: The authors thank Mina Nishiyama for her technical assistance.

References

1. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL, Jr., et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975-982.
2. Zeuzem S, Andreone P, Pol S, Lawitz E, Diago M, Roberts S, et al. Telaprevir for retreatment of HCV infection. N Engl J Med 2011;364:2417-2428.
3. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.
4. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100-1104.
5. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105-1109.
6. Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, Sakai Y, et al. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology 2010;139:499-509.
7. Honda M, Nakamura M, Tateno M, Sakai A, Shimakami T, Shirasaki T, et al. Differential interferon signaling in liver lobule and portal area cells under treatment for chronic hepatitis C. J Hepatol 2010;53:817-826.
8. Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H, et al. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat Genet 2013;45:164-171.
9. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
10. Garaigorta U, Heim MH, Boyd B, Wieland S, Chisari FV. Hepatitis C virus (HCV) induces formation of stress granules whose proteins regulate HCV RNA replication and virus assembly and egress. J Virol 2012;86:11043-11056.
11. Bikkavilli RK, Malbon CC. Arginine methylation of G3BP1 in response to Wnt3a regulates beta-catenin mRNA. J Cell Sci 2011;124:2310-2320.
12. Sarasin-Filipowicz M, Oakeley EJ, Duong FH, Christen V, Terracciano L, Filipowicz W, Heim MH. Interferon signaling and treatment outcome in chronic hepatitis C. Proc Natl Acad Sci U S A 2008;105:7034-7039.
13. Chen L, Borozan I, Feld J, Sun J, Tannis LL, Coltescu C, et al. Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. Gastroenterology 2005;128:1437-1444.
14. Kudo S, Matsuno K, Ezaki T, Ogawa M. A novel migration pathway for rat dendritic cells from the blood: hepatic sinusoids-lymph translocation. J Exp Med 1997;185:777-784.
15. Chen L, Borozan I, Sun J, Guindi M, Fischer S, Feld J, et al. Cell-type specific gene expression signature in liver underlies response to interferon therapy in chronic hepatitis C infection. Gastroenterology 2010;138:1123-1133.e1-3.
16. Askarieh G, Alsio A, Pugnale P, Negro F, Ferrari C, Neumann AU, et al. Systemic and intrahepatic interferon-gamma-inducible protein 10 kDa predicts the first-phase decline in hepatitis C virus RNA and overall viral response to therapy in chronic hepatitis C. HEPATOLOGY 2010;51:1523-1530.
17. Romanowska M, Evans A, Kellock D, Bray SE, McLean K, Donandt S, Foerster J. Wnt5a exhibits layer-specific expression in adult skin, is upregulated in psoriasis, and synergizes with type 1 interferon. PLoS One 2009;4:e5354.
18. Shapira SD, Gat-Viks I, Shum BO, Dricot A, de Grace MM, Wu L, et al. A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. Cell 2009;139:1255-1267.
19. Staal FJ, Luis TC, Tiemessen MM. WNT signalling in the immune system: WNT is spreading its wings. Nat Rev Immunol 2008;8:581-593.

Hepatology Research

Hepatology Research 2014 doi: 10.1111/hepr.12266

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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Received 23 July 2013; revision 22 October 2013; accepted 23 October 2013.

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.

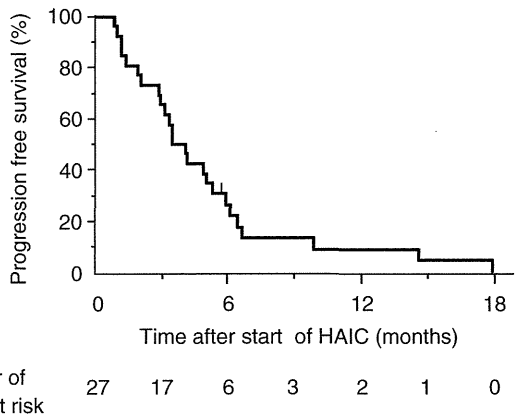

Figure 1 Kaplan–Meier plot of progression-free survival (PFS) since commencement of hepatic arterial infusion chemotherapy (HAIC). Median PFS was 4.0 months.

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

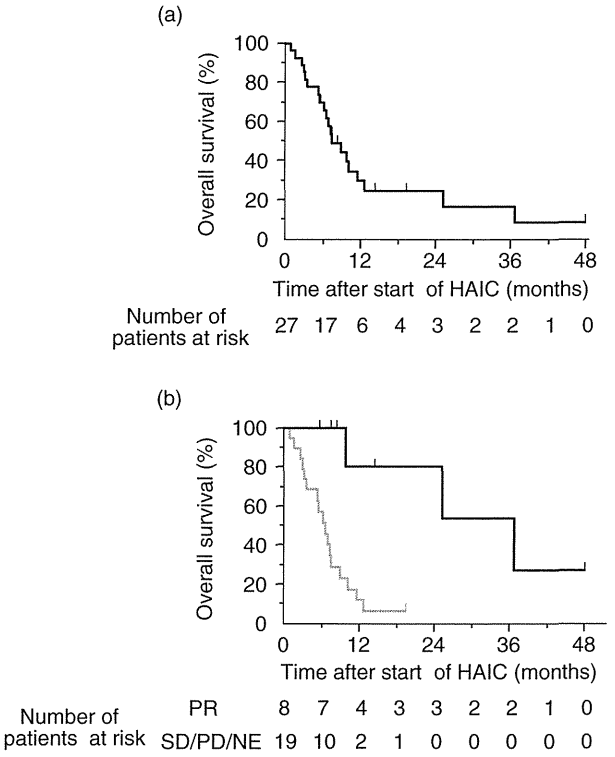

Figure 2 Kaplan–Meier plot of overall survival since commencement of hepatic arterial infusion chemotherapy (HAIC): (a) all patients and (b) according to response to HAIC. The median survival time (MST) of all patients was 7.6 months, and the MST of patients who achieved partial response (PR) were 36.7 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.

ACKNOWLEDGMENTS

NONE.

CONFLICTS OF INTEREST

NONE TO DECLARE.

REFERENCES

1 Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893–917.
2 Lee JM, Yoon JH, Kim KW. Diagnosis of hepatocellular carcinoma: newer radiological tools. *Semin Oncol* 2012; **39**: 399–409.
3 Song MJ, Chun HJ, Song S *et al.* Comparative study between doxorubicin-eluting beads and conventional transarterial chemoembolization for treatment of hepatocellular carcinoma. *J Hepatol* 2012; **57**: 1244–50.
4 Tateishi R, Shiina S, Teratani T *et al.* Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1000 cases. *Cancer* 2005; **103**: 1201–9.
5 Takizawa D, Kakizaki S, Sohara N *et al.* Hepatocellular carcinoma with portal vein tumor thrombosis: clinical characteristics, prognosis, and patient survival analysis. *Dig Dis Sci* 2007; **52**: 3290–5.
6 Cheng AL, Kang YK, Chen Z *et al.* Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009; **10**: 25–34.
7 Llovet JM, Ricci S, Mazzaferro V *et al.* Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378–90.
8 EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2012; **56**: 908–43.

9 Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53:** 1020–2.
10 Llovet JM, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. *Hepatology* 2003; **37:** 429–42.
11 Villanueva A, Llovet JM. Targeted therapies for hepatocellular carcinoma. *Gastroenterology* 2011; **140:** 1410–26.
12 Reed ML, Vaitkevicius VK, Al-Sarraf M *et al.* The practicality of chronic hepatic artery infusion therapy of primary and metastatic hepatic malignancies: ten-year results of 124 patients in a prospective protocol. *Cancer* 1981; **47:** 402–9.
13 Chang AE, Schneider PD, Sugarbaker PH, Simpson C, Culnane M, Steinberg SM. A prospective randomized trial of regional versus systemic continuous 5-fluorodeoxyuridine chemotherapy in the treatment of colorectal liver metastases. *Ann Surg* 1987; **206:** 685–93.
14 Obi S, Yoshida H, Toune R *et al.* Combination therapy of intraarterial 5-fluorouracil and systemic interferon-alpha for advanced hepatocellular carcinoma with portal venous invasion. *Cancer* 2006; **106:** 1990–7.
15 Yamashita T, Arai K, Sunagozaka H *et al.* Randomized, phase II study comparing interferon combined with hepatic arterial infusion of fluorouracil plus cisplatin and fluorouracil alone in patients with advanced hepatocellular carcinoma. *Oncology* 2011; **81:** 281–90.
16 Araki T, Itai Y, Furui S, Tasaka A. Dynamic CT densitometry of hepatic tumors. *AJR Am J Roentgenol* 1980; **135:** 1037–43.
17 Liver Cancer Study Group of Japan. *General Rules for the Clinical and Pathological Study of Primary Liver Cancer*, 4th Japanese edn. Tokyo: Kanehara, 2000.
18 Liver Cancer Study Group of Japan. *General Rules for the Clinical and Pathological Study of Primary Liver Cancer*, 2nd English edn. Tokyo: Kanehara, 2003.
19 Eisenhauer EA, Therasse P, Bogaerts J *et al.* New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009; **45:** 228–47.
20 Reig M, Rimola J, Torres F *et al.* Post-progression survival of patients with advanced hepatocellular carcinoma. Rationale for second line trial design. *Hepatology*, 2013. doi: 10.1002/hep.26586
21 Kaplan E, Meier P. Nonparametric estimation from incomplete observation. *J Am Stat Assoc* 1958; **53:** 457–81.
22 Kaneko S, Furuse J, Kudo M *et al.* Guideline on the use of new anticancer drugs for the treatment of Hepatocellular Carcioma 2010 update. *Hepatol Res* 2012; **42:** 523–42.
23 Morimoto M, Numata K, Kondo M *et al.* Higher discontinuation and lower survival rates are likely in elderly Japanese patients with advanced hepatocellular carcinoma receiving sorafenib. *Hepatol Res* 2011; **41:** 296–302.
24 Finn RS, Kang YK, Mulcahy M *et al.* Phase II, open-label study of brivanib as second-line therapy in patients with advanced hepatocellular carcinoma. *Clin Cancer Res* 2012; **18:** 2090–8.
25 Santoro A, Rimassa L, Borbath I *et al.* Tivantinib for second-line treatment of advanced hepatocellular carcinoma: a randomised, placebo-controlled phase 2 study. *Lancet Oncol* 2013; **14:** 55–63.
26 Yau T, Wong H, Chan P *et al.* Phase II study of bevacizumab and erlotinib in the treatment of advanced hepatocellular carcinoma patients with sorafenib-refractory disease. *Invest New Drugs* 2012; **30:** 2384–90.
27 Katamura Y, Aikata H, Kimura Y *et al.* Intra-arterial 5-fluorouracil/interferon combination therapy for hepatocellular carcinoma with portal vein tumor thrombosis and extrahepatic metastases. *J Gastroenterol Hepatol* 2010; **25:** 1117–22.
28 Dhooge M, Coriat R, Mir O *et al.* Feasibility of gemcitabine plus oxaliplatin in advanced hepatocellular carcinoma patients with Child–Pugh B cirrhosis. *Oncology* 2013; **84:** 6–13.
29 Mir O, Coriat R, Boudou-Rouquette P *et al.* Gemcitabine and oxaliplatin as second-line treatment in patients with hepatocellular carcinoma pre-treated with sorafenib. *Med Oncol* 2012; **29:** 2793–9.

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.

Honda et al. BMC Cancer 2013, 13:191
http://www.biomedcentral.com/1471-2407/13/191

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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trial [12] in which patients who were disease-free after definitive treatment received oral administration of 600 mg peretinoin daily for one year. The results showed that peretinoin significantly reduced the incidence of recurrent or new HCC [12] and improved patient survival rates [13]. Based on the results of rat pharmacological studies [14,15] and a phase I clinical study of peretinoin [16], a phase II/III clinical study of peretinoin was conducted in which the doses were set at 300 and 600 mg daily. The study demonstrated that, in the Child-Pugh A subgroup, 600 mg/day peretinoin (n=100) reduced the risk of HCC recurrence or death by approximately 40% compared to placebo (n=106) [hazard ratio (HR)=0.60; 95% confidence interval (CI): 0.40–0.89)] [17]. On the other hand, 300 mg daily doses of peretinoin were insufficient for tumor control and showed no substantial difference from the placebo [17]. A large-scale clinical study including several countries is now planned to confirm the clinical efficacy of peretinoin.

Little is known about the mechanism by which peretinoin exerts its inhibitory effects against recurrent HCC in humans *in vivo*. In order to investigate this mechanism, we conducted here a comparative study recruiting HCV-positive patients who successfully completed definitive treatment for HCC (similar to the phase II/III clinical study mentioned above). Patients underwent liver biopsy before and after 8 weeks of treatment with repeated doses of peretinoin, and the collected samples were analyzed for gene expression profiling using the remnant liver after eradication of HCC. We found that changes in the gene expression signature observed in this study help us to understand the means by which peretinoin suppresses HCC, in particular its inhibition against *de novo* carcinogenesis.

Methods

Patients

We enrolled 12 HCV-positive patients who were cured of their primary and first recurrent HCC by surgical hepatectomy or radiofrequency ablation therapy and other non-surgical local treatments (Table 1). Complete tumor removal was confirmed by dynamic computed tomography (CT) scans. Inclusion criteria were as follows: positive presence of HCV-RNA in the serum; Child-Pugh class A or B liver function; platelet counts ≥50,000/μL; and age ≥20 years. Exclusion criteria included the following: positive for hepatitis B surface antigen; tumor infiltration into the portal vein; use of transarterial embolization or transarterial chemoembolization (TAE/TACE) for definitive therapy; postoperative use of investigational medicinal products, antitumor agents, interferon, or vitamin K2 formulations; blood pressure unmanageable even with medication (systolic pressure ≥160 mmHg or diastolic pressure ≥100 mmHg); complication with renal impairment, cardiovascular disease, diabetes mellitus, autoimmune disease, asthma, or other severe disease; presence of neoplasm; allergy to CT contrast media; allergy to retinoids; history of total gastrectomy; possible pregnancy during study; and lactating mothers.

Study design

This trial was a randomized, parallel-group, open-label study. Twelve eligible patients signed the informed consent form for registration. They were randomized to receive one of the two peretinoin doses: 600 or 300 mg per day. Each dose group consisted of 6 patients. After randomization, patients underwent liver biopsy before the start of peretinoin treatment, then orally received peretinoin twice daily for 8 weeks. At the end of the 8-week therapy, they underwent a second liver biopsy (Figure 1A). The collected biopsy samples were kept in RNAlater® solution (Ambion Inc., Austin, TX) at 4°C overnight or longer. Within 3 days, the biopsy samples were removed from the RNAlater solution and partially subjected to RNA extraction and purification. The purified RNA samples were stored at −80°C until required for gene expression profiling. The remaining part of the biopsy samples was used to determine the intrahepatic peretinoin concentration. Samples were placed in polypropylene bottles containing 99.5% ethanol, and the air in the bottle was purged with argon. The bottles were tightly closed and stored at −80°C protected from light. Peripheral blood samples were also collected for the analysis of gene expression signatures and to determine plasma peretinoin levels.

After the second biopsy, patients were orally administered peretinoin twice daily for 88 weeks. During the treatment period, patients visited the hospital every 4 weeks for check-ups, drug compliance, and protocol-specified medical examinations. Drug compliance was assessed by pill counts. During the study, use of anticancer agents, interferon, vitamins K and A, and antiviral drugs (e.g., rivabirin) was prohibited. The study was registered at the Japan Pharmaceutical Information Center (JapicCTI-121757). This protocol was approved by the Institutional Review Board of Kanazawa University for clinical investigation following the provisions of Helsinki, Good Clinical Practice guidelines, local laws, and regulations. Written informed consent was obtained from all patients involved in this study. The detail protocol of this study is presented in Additional file 1: Study protocol.

Plasma peretinoin concentration

A 5-mL blood sample was drawn into an EDTA-2Na tube, immediately mixed, and centrifuged to obtain a plasma sample. The air in the sample tubes was replaced with argon, and the tubes were stored at −80°C protected

Table 1 Patient characteristics and prognosis

Patient	Dose	Age	Sex	P/R	Curative	MTD	Tumor no.	Tumor	Background liver		CP	ALT	PLT	Prognosis	
no.					treatment			histology	F	A				2 yrs	4.5 yrs
1	300	70	F	P	RFA	15	2	m-p	4	2	A	112	7.9	Rec	Rec
2	600	72	F	R	RFA	20	2	w	4	2	A	40	7.9	Rec	Rec λ
3	300	58	M	P	resection	25	1	m-p	2	1	A	16	19.2	nonRec	nonRec
4	600	54	M	P	resection	25	1	m-p	3	2	A	57	16.4	nonRec	Rec
5	600	60	F	P	RFA	23	1	m-p	4	2	B	23	6.4	nonRec	nonRec
6	300	73	F	P	RFA	20	2	m-p	3	2	A	31	14.2	Rec	Rec λ
7	300	69	F	P	RFA	11	3	w-m	4	1	A	38	11.5	Rec	Rec λ
8	600	74	F	P	RFA	16	2	m-p	4	1	A	45	5.1	nonRec	Rec
9	600	65	M	R	RFA	10	1	m-p	2	1	A	29	16.5	nonRec	nonRec
10	600	59	M	P	resection	34	1	m-p	4	2	B	60	9.4	nonRec	nonRec
11	300	70	F	R	RFA	15	1	w-m	4	2	B	98	7	nonRec	nonRec
12	300	66	M	P	RFA	15	1	m-p	4	1	A	90	10.6	nonRec	nonRec

Dose (mg/day), ALT(U/L), PLT($\times 10^4/\mu L$), MTD (mm).
F; female, M; male, P; primary HCC, R; (first) recurrent HCC, MTD; maximum tumor diameter, w; well-differentiated, m; moderately differentiated, p; poorly differentiated, F; fibrosis stage, A; activity grade, CP; Child-Pugh classification, ALT; alanine aminotransferase, PLT; platelet.
Rec; recurrence, nonRec; non-recurrence, λ; death.

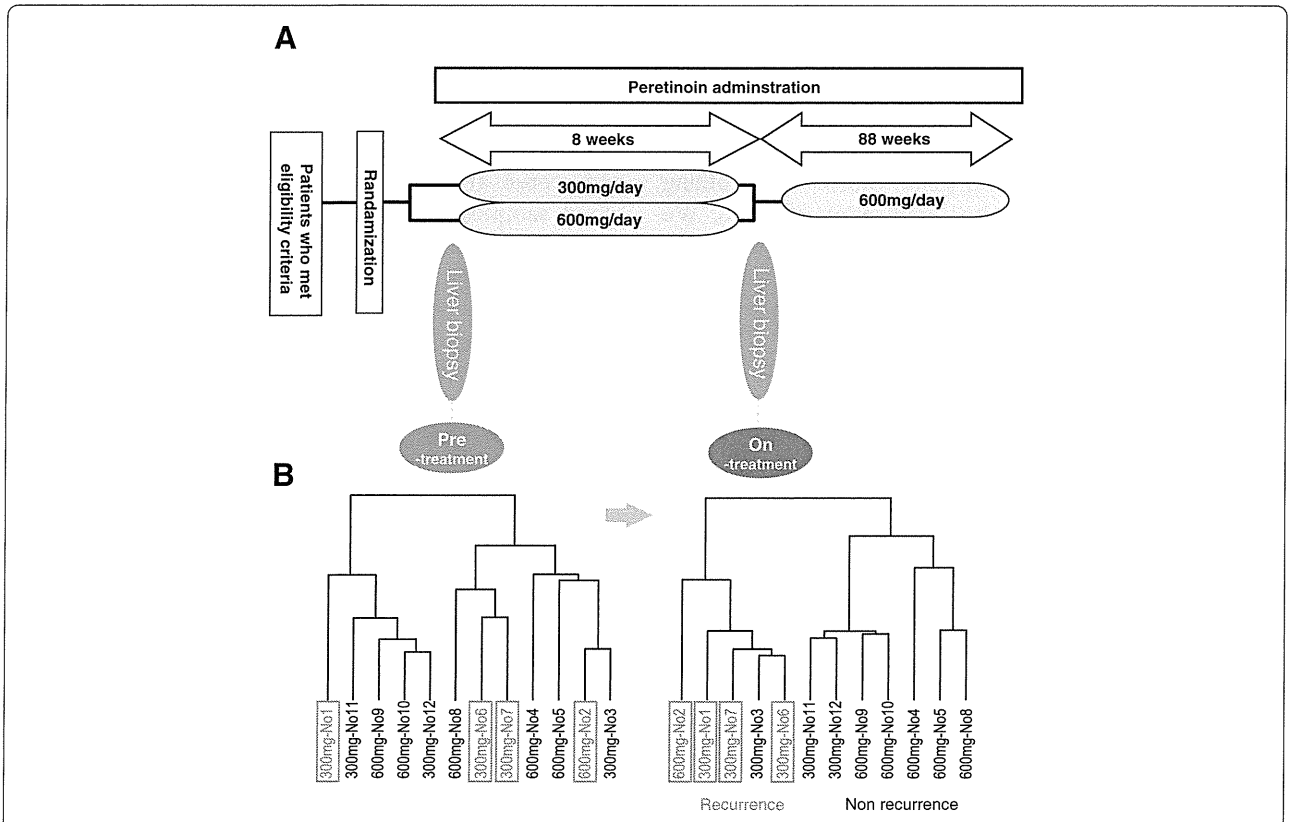

Figure 1 Peretinoin pharmacokinetics study design and change of gene expression profiling. A: Peretinoin pharmacokinetics study design. Twelve patients were enrolled in the study and two groups of 6 patients were randomly administered one of two doses of peretinoin (600 or 300 mg per day) for 8 weeks. A liver biopsy was obtained before the start of peretinoin administration and 8 weeks into the treatment. After the second liver biopsy, oral administration of peretinoin twice daily was resumed for 88 weeks. **B**: Hierarchical clustering analysis of patients using all expressed genes. Patient numbers (Table 1) and peretinoin dose are listed. Patients with HCC recurrence are shown in red and boxed. There was no significant association between hepatic gene expression and HCC recurrence before starting peretinoin treatment, while distinct clusters of patients were observed after week 8 depending on HCC recurrence.

from light. The plasma concentrations of the unchanged form of peretinoin and its lipid-bound form were determined as follows: first, the peretinoin-containing fractions were extracted from the plasma samples, then subjected to derivatization of peretinoin, and the concentration of the derivative was measured by liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry.

Liver peretinoin concentration

Collected liver tissue samples were immersed in 99.5% ethanol in containers, and the internal air was replaced with argon. The samples were stored at −80°C protected from light. The liver concentrations of the unchanged form of peretinoin and its lipid-bound form were determined as for the plasma concentrations above.

Microarray analysis

For gene expression profiling of the liver, in-house cDNA microarrays containing a representative panel of 10,000 liver-specific genes (Kanazawa liver chip 10K ver. 2.0) were used. RNA isolation, amplification of antisense RNA, labeling, and hybridization were conducted as previously described [18].

To identify genetic variants, paired *t*-tests were performed using BRB-Array Tools software (http://linus.nci.nih.gov/BRB-ArrayTools.html) to define *P*-values <0.05 as gene variants. Hierarchical cluster analysis, exploration of significantly expressed genes, and class prediction were also performed using the BRB-Array Tools.

Hierarchical clustering was carried out using centered correlation and average linkage. The class comparison tool in the BRB-Array Tools was used to extract significantly expressed genes. Genes whose expression levels were significantly different between two groups were located by the *t*-test at the $P<0.002$ significance level. Univariate permutation tests were repeated 1,000–2,000 times to control for errors. Class prediction was performed using the above-mentioned significantly differentiated genes as discriminators, and the results were cross-validated using seven algorithms: compound-covariate predictor, diagonal linear discriminant analysis, 1-nearest neighbor, 3-nearest neighbors, nearest centroid, support vector machine, and Bayesian compound covariate. The mean value of the seven success rates for class prediction was defined as the prediction accuracy rate [18].

Pathway analysis was performed using MetaCore™ (Thomson Reuters, New York, NY) and functional ontology enrichment analysis was performed to find differentially expressed pathway using differentially expressed genes [18,19].

The microarray data have been submitted to the Gene Expression Omnibus (GEO) public database at NCBI (Accession No. GSE29302).

Quantitative real-time detection polymerase chain reaction

Quantitative real-time detection polymerase chain reaction (RTD-PCR) was performed using the TaqMan Universal Master Mix (PE Applied Biosystems, Foster City, CA). Primer pairs and probes were purchased from the TaqMan assay reagents library. Standard curves were generated for each assay using RNA derived from normal human liver tissue. Expression data were normalized by GAPDH, and the results are shown as the relative fold expression to the normal liver.

Statistical analysis

Results are expressed as means ± S.D. Significance was tested by one-way ANOVA with Bonferroni's method, and differences were considered statistically significant at $P<0.05$.

Results

Safety

In this study, 88 adverse events were recorded in 12 patients (100%). Major adverse events included rhinopharyngitis (n=7), blood pressure elevation (n=5), peripheral edema (n=3), and enteritis (n=3). Most of these adverse events were mild or moderate, and were adequately controlled. Nine serious adverse events were documented in 5 patients, including hyperglycemia (n=2) and coronary stenosis (n=1). However, all reported serious adverse events were alleviated with appropriate treatment, and there was no substantial concern identified regarding the safety of peretinoin.

Plasma peretinoin concentration

Plasma peretinoin concentrations were determined at week 8 of treatment. The mean (± SD) plasma concentrations of the unchanged form of peretinoin were 82.3 (± 90.0) and 201.2 (± 111.4) ng/mL at 4 h post-dose and 35.8 (± 49.2) and 29.0 (±17.9) ng/mL at 8 h post-dose for the 300 and 600 mg per day groups, respectively. The plasma concentrations of the unchanged peretinoin measured at 4 h post-dose (≈ t_{max}) were dose-dependent. The mean (± SD) plasma concentrations of the lipid-bound form of peretinoin were 1478.8 (± 853.7) and 2789.8 (± 1630.0) ng/mL at 4 h post-dose and 1227.8 (± 942.7) and 2213.2 (± 1156.1) ng/mL at 8 h post-dose for the 300 and 600 mg per day groups, respectively. The plasma concentrations of the lipid-bound form of peretinoin were dose-dependent at 4 and 8 h post-dose.

Liver peretinoin concentration

Liver peretinoin concentrations were determined at week 8 of treatment. The measurements of the liver concentration of the unchanged form of peretinoin were all below the lower limit of quantitation at 4 h post-dose for all 6 patients in the 300 mg per day group. For the

600 mg per day group, 2 patients yielded measurements of 0.052 and 0.059 μg/g, while the remaining 4 patients produced results under the lower limit of quantitation (0.050 μg/g). The mean (± SD) concentrations of the lipid-bound form of peretinoin were 13.7508 (± 11.1097) and 12.8345 (± 8.7048) μg/g for the 300 and 600 mg per day groups, respectively.

Gene expression analysis

To analyze the gene expression signature of the liver tissue, we identified genes whose expression levels were significantly different before and after the start of the peretinoin treatment (Figure 1A). The identified genes were candidates for peretinoin-responsive genes. The phase II/III clinical study showed that a daily dose of 600 mg peretinoin reduced the risk of HCC recurrence, while a 300 mg dose was not significantly different from the placebo [17]. Therefore, gene expression patterns were compared before and after the start of the 600 mg peretinoin therapy (n=6). Consequently, 424 hepatic genes showed significantly different expression levels from baseline at week 8 (enhancement and suppression seen for 190 and 234 genes, respectively). Typical examples of these genes are represented in Table 2 where fold changes of gene expression for the 300 mg and 600 mg doses are shown respectively. In addition to the retinoid-induced genes, genes related to interferon, tumor suppressors, negative regulators of Wnt signaling, insulin-like growth factor (IGF) signaling, and hepatocyte differentiation were significantly up-regulated by peretinoin. By contrast, genes related to the mammalian target of rapamycin (mTOR), tumor progression, cell cycle, and metastasis/angiogenesis were down-regulated. Serial changes in peretinoin-responsive gene expression are shown in Additional file 2: Figure S1. Significant changes in expression were observed in response to 600 mg of peretinoin, while changes in expression were minimal with 300 mg of peretinoin.

Hierarchical clustering of patients using hepatic gene expression prior to administering peretinoin revealed no significant association with clinical outcome, but a significant association became clearly apparent 8 weeks after peretinoin treatment (Figure 1B). The patients were clustered into two groups: one containing patients with HCC recurrence (4 of 5 patients had recurrence) and the other containing those without recurrence (all 6 patients were recurrence free) within 2 years. Supervised learning methods using seven different algorithms showed that the patients receiving treatment could be differentiated into two groups with or without recurrence by 224 gene predictors ($P<0.002$) at 79.6% accuracy ($P<0.05$) (Table 3). Interestingly, 44 of 224 (20%) genes were peretinoin induced.

Although peretinoin-responsive genes were more induced in patients treated with the 600 mg dosage, gene expression profiling 8 weeks after peretinoin treatment could not be classified according to the dosage (Table 3). This might be because two patients treated with the 300 mg dosage (No. 11 and No. 12) had already expressed high levels of peretinoin-response genes before starting peretinoin treatment (Additional file 2: Figure S1). Interestingly, patients with high levels of peretinoin-response genes before treatment (No. 9–12) did not show HCC recurrence during the entire observation period (4.5 years; Table 1).

Hierarchical clustering of all 12 patients using 224 gene predictors is shown in Figure 2A. Clear gene clusters were observed according to patients with recurrence and those without, with the exception of one patient (No. 3, Table 1). Interestingly, in the liver of patients with non-recurrence, genes related to angiogenesis, cancer stem cells, Wnt signaling, and tumor progression were repressed, while genes inducing differentiation, tumor suppression, and apoptosis were up-regulated (Figure 2B, Table 4). Interestingly, PDGF-C was the most significant predictor to differentiate patients who will experience recurrence within 2 years (Table 4).

Consistent with these results, hierarchical clustering using pre-defined curated gene sets based on the NCBI's Cancer Genome Anatomy Project similarly differentiated patients into two groups with or without HCC recurrence (Figure 3). Among angiogenesis-related genes, PDGF-C, PDGF-B, vascular endothelial growth factor (VEGF)-B, VEGF-D, and fibroblast growth factor-basic (FGF-2) were repressed in patients without recurrence. As for cell signaling-related genes, MYC, SRC, and RAS-related genes were also repressed; retinoid X receptor alpha (RXRA) and CCAAT/enhancer binding protein (C/EBP), alpha were up-regulated in patients without recurrence. Some cytokines (IL-7, IL-13, and IL-18) and chemokines (e.g. CXCL7) were repressed, while major histocompatibility complex molecules and interferon-related molecules (e.g. IFNAR2) were up-regulated in patients without recurrence (Figure 3).

cDNA microarray analysis revealed that among these predictors, the mRNA level of PDGF-C was the most significant predictor for differentiating patients who will experience recurrence within 2 years (Table 4). This observation was also assessed by RTD-PCR (Figure 4). The expression of the catalytic enzyme of retinoic acid, CYP26B1, was significantly up-regulated at around 200 fold by peretinoin treatment, but its expression was equally induced in patients with or without recurrence. However, the expression of RAR-β, a retinoid receptor, was significantly up-regulated by peretinoin in patients without HCC recurrence (Figure 4).

Patients were followed up for a further 3 years (mean: 2.5 ± 0.5 years) after the cessation of peretinoin treatment. Other two patients experienced recurrence during

Table 2 Representative genes significantly up-regulated or down-regulated in response to peretinoin treatment

Parametric p-value	Ratios (Under/Pre)		Description	Symbol	GB acc
600 mg	600 mg	300 mg			
Up-regulated genes in response to peretinoin treatment					
Retinoid target genes					
0.0002	1.85	1.25	Cytochrome P450, family 26, subfamily B, polypeptide 1	CYP26B1	NM_019885
0.004	1.75	1.33	Insulin-like growth factor binding protein 6	IGFBP6	NM_002178
0.005	1.42	1.16	Regulatory factor X-associated ankyrin-containing protein	RFXANK	NM_134440
0.006	1.33	1.30	Putative lymphocyte G0/G1 switch gene	G0S2	NM_015714
0.013	1.54	0.90	Retinol binding protein 1	RBP1	NM_002899
0.014	1.56	0.87	Retinol binding protein 4	RBP4	NM_006744
0.034	1.27	1.07	Retinoic acid induced 3	GPRC5A	AI923823
0.040	1.22	1.19	Transglutaminase 2	TGM2	AI962033
0.044	1.23	1.14	CCAAT/enhancer binding protein (C/EBP), alpha	CEBPA	NM_004364
Interferon-related genes					
0.029	1.45	0.93	Guanylate binding protein 1, interferon-inducible, 67kDa	GBP1	NM_002053
0.047	1.39	0.94	Interferon-induced protein 44	IFI44	NM_006417
0.048	1.28	1.05	Chemokine (C-X-C motif) ligand 9	CXCL9	NM_002416
Negative regulator of Wnt and TGF-β signaling					
0.004	1.54	1.06	BMP and activin membrane-bound inhibitor homolog	BAMBI	NM_012342
0.008	1.45	1.11	Secreted frizzled-related protein 5	SFRP5	NM_003015
Anti-angiogenesis					
0.021	1.37	0.98	Thrombomodulin	THBD	NM_000361
0.038	1.28	0.99	Protein C receptor, endothelial (EPCR)	PROCR	NM_006404
Tumor suppressor related					
0.029	1.35	0.96	Jumonji domain containing 3	JMJD3	XM_043272
0.029	1.39	0.91	Jumping translocation breakpoint	JTB	NM_006694
0.034	1.39	1.32	Protein kinase, AMP-activated, alpha 2 catalytic subunit	PRKAA2	NM_006252
Down-regurated genes in response to peretinoin treatment					
mTOR-related-gene					
0.045	0.78	0.94	FK506 binding protein 12-rapamycin associated protein 1	FRAP1	NM_004958
Cytokine and growth factor					
0.019	0.77	1.25	Interleukin 13	IL13	NM_002188
0.031	0.74	1.00	Hepatocyte growth factor	HGF	NM_000601
Tumor progression related					
0.011	0.73	0.94	Junctional adhesion molecule 3	JAM3	NM_032801
0.013	0.70	1.00	V-myc myelocytomatosis viral oncogene homolog	Myc	NM_002467
0.017	0.73	1.12	Src-like-adaptor	SLA	NM_006748
0.028	0.78	1.10	Cell division cycle 2, G1 to S and G2 to M	CDC2	NM_001786
0.030	0.66	0.95	BCL2-associated athanogene	BAG1	NM_004323
0.039	0.64	0.93	Chemokine (C-C motif) receptor 9	CCR9	NM_031200
0.043	0.76	1.13	Pre-B-cell leukemia transcription factor 1	PBX1	H08835

The peretinoin-response genes were identified by comparing hepatic gene expression in the pre and under treatment of 6 patients who were treated with 600 mg dose of peretinoin. The fold changes of gene expression are shown in 300 mg and 600 mg dosage respectively.

Table 3 Supervised learning methods

Class		No. of predictors (p<0.002)	Prediction (%)	p-value
Pre-treatment	Recurrence vs non-recurrence	6	47.1	N.S.
On-treatment	Recurrence vs non-recurrence	224	79.6	< 0.05
On-treatment	300 mg vs 600 mg	38	72.7	N.S.

Seven algorithms of Compound-Covariate Predictor, Diagonal Linear Discriminant Analysis 1-Nearest Neighbor, 3-Nearest Neighbors, Nearest Centroid, Support Vector Machine, and Bayesian Compound Covariate were used for class prediction. Prediction % was calculated as the average of these seven algorithms.

further follow up period (No. 4 and No. 8 in Figure 2A, Table 1). Three patients with recurrence died at 0.3, 1.9, and 2.5 years after the cessation of peretinoin treatment. The Kaplan-Meier estimation of the recurrence-free ratio deduced from 224 gene predictors showed significant differences in HCC recurrence between patients with the recurrence expression pattern and those with non-recurrence expression (P=0.04). Moreover, Kaplan-Meier estimation of the survival ratio deduced from the same gene predictors showed a trend for improved survival of patients with non-recurrence expression patterns compared with those with the recurrence expression pattern (P=0.12) (Figure 2C, D).

With the exception of the number of tumors at the time of curative therapy, none of the other clinical parameters (e.g. peretinoin dose, tumor, background liver histology, or background liver function) were associated with the recurrence-free or survival ratio. Thus, the peretinoin response during the early period of administration deduced from the hepatic gene expression pattern can successfully predict HCC recurrence and, potentially, patient survival.

Discussion

Peretinoin [(2E,4E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,4,6,10,14-pentaenoic acid] is expected to be a powerful agent against HCC recurrence. This synthetic retinoid induces the transcriptional activation of the retinoic acid receptor (RAR) and retinoid X receptor (RXR), which are both members of the retinoid receptor family. One primary pathway of HCC development involves sustained hepatitis virus infection, which causes repeated cycles of hepatocellular necrosis and proliferation. During increased cell proliferation, mutations occur that lead to the development of HCC unless the dedifferentiated tumor cells are eliminated by apoptosis. The anti-HCC mechanism of action of peretinoin has previously been suggested to be a result of induction of cell apoptosis [20,21], enhancement of cell differentiation [21,22], suppression of cell proliferation by elevation of P21 protein expression and suppression of cyclin D1 expression [23,24]. The first route of action is independent of retinoid receptors, while the others are retinoid receptor-dependent, although all mechanisms remain largely speculative.

Peretinoin was previously shown to suppress *in vivo* hepatocarcinogenesis in 3′-methyl-4-dimethylaminoazobenzene- and *N*-diethylnitrosamine-induced rats [14,15,25], and in hepatoma-bearing mice and transgenic mice expressing a dominant-negative retinoic acid receptor [25,26]. Recently, we revealed that peretinoin effectively inhibits hepatic fibrosis and HCC development in *Pdgf-c Tg* mice. This demonstrated that PDGF signaling is a target of peretinoin in preventing the development of hepatic fibrosis and HCC [27]. The purpose of this study was to investigate how peretinoin exerts its therapeutic potential by analyzing its effects on the gene expression patterns in clinical samples.

Gene expression profiling in patients without HCC recurrence demonstrated the promotion of *RAR-β* expression, the most common retinoid target gene identified by basic research. Moreover, the expression of other retinoid target genes such as *C/EBP-α*, *IGFBP6*, *TGM2*, *G0S2*, *RBP1*, *RBP4*, and *GPRC5A* was also enhanced. Of these, *C/EBP-α*, *IGFBP6*, and *TGM2* have been shown to inhibit HCC proliferation when co-expressed with *RAR-β* by all-trans-retinoic acid [28,29]. In addition, the RXR-selective agonist (rexinoid)-induced expression of *IGFBP6*, which occurs following *RAR-β*-mediated transcriptional activation of *RAR/RXR*, has been shown to suppress tumor growth [30]. Moreover, *G0S2* and *GPRC5A* have been reported to possess tumor suppressive or apoptosis-inducing effects [31,32]. These primary response retinoid target genes are presumably retinoid-responsive genes. In addition to enhancing retinoid target gene expression, peretinoin induced changes in the expression levels of a variety of genes involved in hepatocarcinogenesis, such as those related to Wnt signaling, IGF signaling, interferon, mTOR, and cell cycle regulation. These results suggest that peretinoin modulates multiple signaling cascades involved in carcinogenesis, either directly or indirectly. Abnormalities in the genes regulating Wnt signaling, IGF signaling, interferon, mTOR, and the cell cycle have been indicated to play a crucial role in the development of HCC [33,34]. We argue that peretinoin suppresses HCC cell proliferation by improving the expression of these genes, thereby preventing HCC recurrence.

The cluster analysis performed in this study successfully differentiated patients with recurrence within 2 years and those without it. Supervised learning methods identified 224 genes as predictors for HCC recurrence (p<0.002). Importantly, 44 (20%) of these were peretinoin-responsive genes, suggesting that recurrence-related genes might be regulated by peretinoin-responsive genes.

A comparison of these groups of patients revealed that the non-recurrence group was associated with the

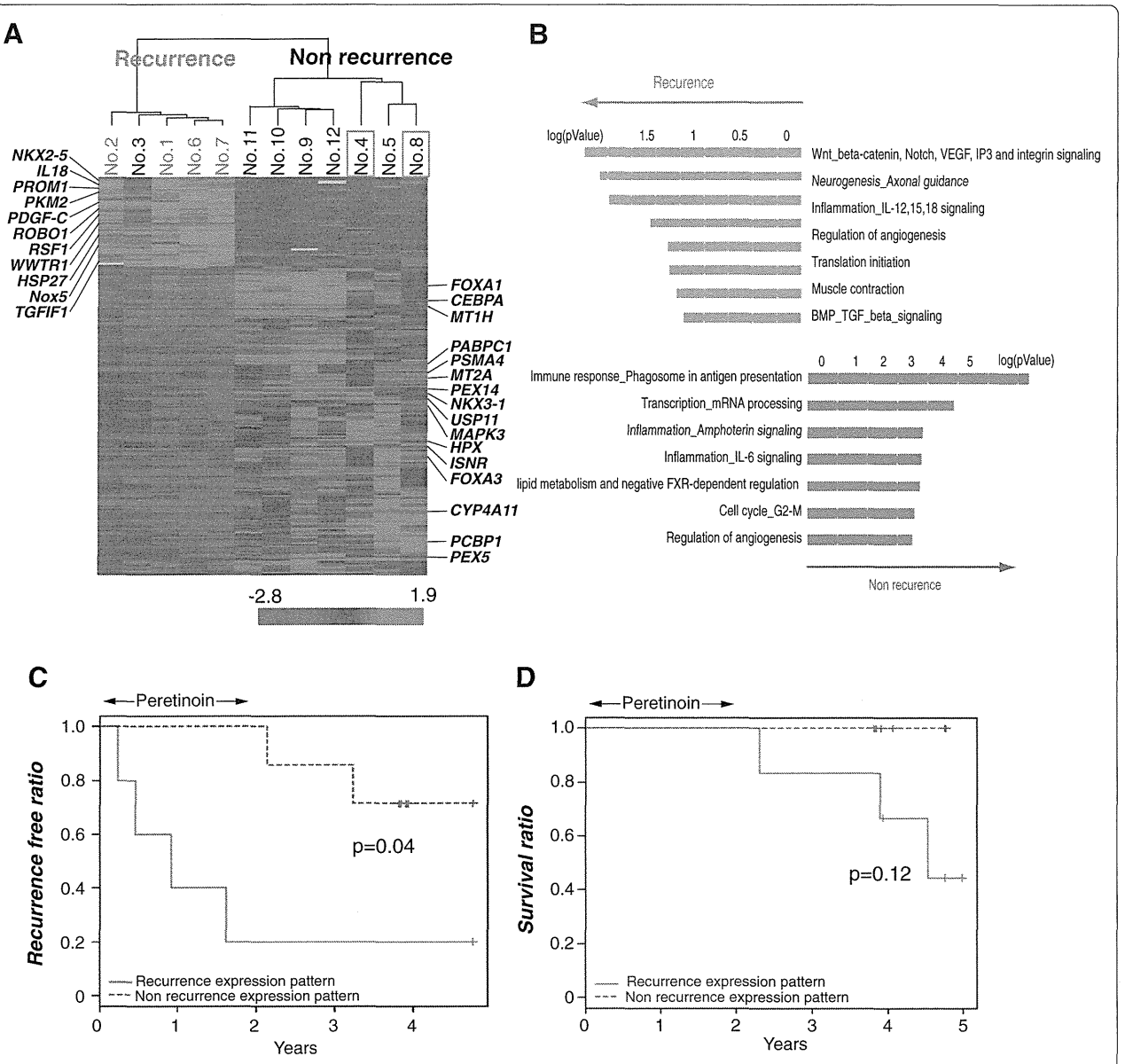

Figure 2 Expression profiling of 224 gene predictors and the prognosis of patients. A: Hierarchical clustering using 224 gene predictors of patients with or without HCC recurrence. Patients with HCC recurrence within 2 years are shown in red and patients with HCC recurrence after the cessation of peretinoin are boxed in red. **B**: Pathway analysis of differentially expressed genes using MetaCore (GeneGo). Functional ontology enrichment analysis was performed to find differentially expressed pathway maps or map folders using 224 differentially expressed genes ($p<0.002$) between patients with and without HCC recurrence. **C**, **D**: Kaplan-Meier estimation of recurrence-free ratio (**C**) and survival ratio (**D**) of patients with recurrence expression patterns (red) and those with non-recurrence expression (blue).

enhanced expression of genes related to hepatocellular differentiation and tumor suppression. The non-recurrence group also showed reduced expression of the genes promoting liver fibrosis and steatosis and the liver cancer stem cell marker genes. The genes related to hepatocellular differentiation, *MT1H*, *MT2A*, *FOXA1* (*HNF3α*), and *FOXA3* (*HNF3γ*), may be secondary response genes regulated by *C/EBP-α* [35,36]. Indeed, *C/EBP-α* manifested a significant shift in expression level before and during treatment with peretinoin, and could also differentiate between recurrence and non-recurrence within 2 years. Even after the cessation of peretinoin treatment, the expression of these genes was still significantly related to HCC recurrence (Figure 2C, D). Thus, we speculate that the differences in expression levels of peretinoin-response genes would determine the expression of recurrence-related genes (Additional file 3: Figure S2).

Interestingly, PDGF-C was the most significant predictor to differentiate those patients who will experience recurrence. Using a mouse model of PDGF-C over-

Table 4 Representative genes differentially expressed between HCC recurrence and non-recurrence groups

Parametric p-value	t-values	Description	Symbol	GB acc
Up-regulated genes in the recurrence group				
Angiogenesis related				
0.0001	−5.19	Platelet derived growth factor C	PDGFC	AI446155
0.0006	−4.37	Sperm equatorial segment protein 1	NOX5	NM_145658
0.0010	−4.13	Interleukin 18	IL18	AI800476
Cancer stem cell related				
0.0004	−4.63	Prominin 1	PROM1	NM_006017
0.0018	−3.83	Pyruvate kinase, muscle	PKM2	NM_002654
Positive regulator of Wnt				
0.0018	−3.84	TGFB-induced factor (TALE family homeobox)	TGIF1	AI866302
0.0018	−3.84	NK2 transcription factor related, locus 5	NKX2-5	NM_004387
Tumor progression related				
0.0005	−4.47	Transcriptional co-activator with PDZ-binding motif	WWTR1	AK025216
0.0017	−3.87	Roundabout, axon guidance receptor, homolog 1	ROBO1	NM_133631
0.0018	−3.84	Hepatitis B virus x associated protein	RSF1	NM_016578
0.0019	−3.79	Heat shock 27kDa protein 2	HSPB2	NM_001541
Up-regulated genes in the non-recurrence group				
Liver function and hepatocytes differenti related				
0.0002	4.88	Metallothionein 2A	MT2A	NM_005953
0.0002	4.08	CCAAT/enhancer binding protein (C/EBP), alpha	CEBPA	NM_004364
0.0003	4.72	Forkhead box A3	FOXA3	NM_004497
0.0006	4.42	Hemopexin	HPX	NM_000613
0.0006	4.35	Metallothionein 1H	MT1H	NM_005951
0.0013	4.01	Forkhead box A1	FOXA1	NM_004496
0.0014	3.97	FK506 binding protein 8, 38kDa	FKBP8	NM_012181
Tumor suppressor related				
0.0005	4.51	Deleted in colorectal carcinoma	DCC	X76132
0.0018	3.84	NK3 transcription factor related, locus 1	NKX3-1	NM_006167
Apoptosis inducing				
0.0015	3.93	BH3 interacting domain death agonist	BID	NM_197967
0.0019	3.82	Programmed cell death 8	AIFM1	NM_145813

expression resulting in hepatic fibrosis, steatosis, and eventually HCC development, peretinoin was previously shown to significantly repress the development of hepatic fibrosis and tumors [27].

Although gene expression profiling analysis was conducted using the remnant liver after definitive treatment in the present study, past similar research has demonstrated the possibility of predicting recurrent metachronous and multicentric HCC [37,38]. The exact mechanisms of how the expression profile of non-tumor tissues might determine the recurrence risk are not known. However, the degree of differentiation of hepatocytes and microenvironments such as angiogenesis and fibrogenesis in non-tumor lesions of the liver is likely to be closely associated with hepatocarcinogenesis. Interestingly, patients with pre-activated peretinoin-response genes were resistant to HCC recurrence for the entire observation period (4.5 years).

This study demonstrated that the patient response to peretinoin during the early period of administration could predict HCC recurrence and, potentially, patient survival. However, it should be noted that the current study protocol consisted of 600 mg peretinoin as the subsequent maintenance treatment for all patients after the 8-week start phase (Figure 1A). In addition, we did not conduct a placebo control to observe serial changes