Hepatocellular carcinoma (HCC) is the sixth most common cancer-related cause of death in the world annually, and the development of new primary tumors, recurrences, and metastasis are the most common causes of mortality among patients with HCC.[1,2] Sorafenib (Nexavar; Bayer Healthcare Pharmaceuticals Inc.) is a small molecule kinase inhibitor that is classified as an anti-angiogenic inhibitor.[3] Sorafenib inhibits the kinase activities of Raf-1 and B-Raf in addition to vascular endothelial growth factor receptors, platelet-derived growth factor receptor β, Flt-3, and c-KIT. Two large randomized controlled trials reported a significant clinical benefit of single-agent sorafenib in extending overall survival in both Western and Asian patients with advanced unresectable HCC.[4,5] Consequently, sorafenib is now used as a standard therapy for HCC. The mechanisms of action that lead to these remarkably prolonged overall survival periods are thought to result from the anti-angiogenic effects of sorafenib and its characteristic inhibitory effect on Raf-1 and B-Raf signaling. In these trials, a partial response was observed in 0.7% (2/299) and 3.3% (5/150) of the patients treated with sorafenib.[4-5]

Recently, emerging evidence has demonstrated that some responders exhibit rapid tumor regression as a result of sorafenib treatment for HCC. Complete responses were observed in two patients with advanced HCC and multiple lung metastases, with rapid tumor regression observed even after short-term treatment with sorafenib.[6,7] The drastic tumor response to sorafenib seems to be similar to the tumor response obtained using other tyrosine kinase inhibitors to target a deregulated signal in cancer cells. For example, constitutively active mutations of epidermal growth factor receptor (EGFR) tyrosine kinase in non–small cell lung cancer are associated with a striking treatment response to gefitinib, a selective EGFR tyrosine kinase inhibitor.[8,9] We hypothesized that these HCC cells may harbor a genetic background conducive to a drastic response to sorafenib, rather than the typical anti-angiogenic effect. In this study, we retrospectively searched for genetic changes using mainly formalin-fixed, paraffin-embedded (FFPE) samples from patients with HCC who had undergone sorafenib treatment.

Patients and Methods

Reagent and Cell Culture. Sorafenib was provided by Bayer Healthcare Pharmaceuticals Inc. (Montville, NJ). All cell lines used in this study were maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Sigma, St. Louis, MO) except for IM95, OUMS23, Colo320, WiDr, HLF, HLE, Huh7, and HepG2 (Dulbecco's modified Eagle's medium [DMEM]; Nissui Pharmaceutical, Tokyo, Japan); LoVo (F12; Nissui Pharmaceutical, Tokyo, Japan); KYSE180, KYSE220, and KYSE270 (RPMI-1640:F12, 1:1); KYSE150 (F12); and KYSE70 (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) or 2% FBS for the KYSE series plus penicillin and streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. These cell lines were obtained from the American Type Culture Collection (Manassas, VA) and the Japanese Collection of Research Bioresources Collection (Sennan-shi, Osaka, Japan).

Patients and Samples. The inclusion criteria for the study were as follows: patients with histologically confirmed HCC who had been treated with sorafenib, from whom pretreatment tumor samples were available. Finally, the clinical characteristics of a total of 55 cases of HCC from 12 medical centers were evaluated retrospectively. In the gene copy number analysis, four samples were excluded because of an insufficient quantity of DNA, two samples were excluded because of the poor quality of the DNA and two samples were response not evaluable. One not evaluable sample was poor DNA quality. Thus, the copy number assay was performed using the remaining 48 samples. Meanwhile, a series of 82 HCC samples were obtained from frozen specimens of surgical specimens at the Kinki University Faculty of Medicine. The tumor response was evaluated using computerized tomography according to the Response Evaluation Criteria in Solid Tumors; the response was then classified as a complete response, a partial response, stable disease, progressive disease, or not evaluable. The clinico-pathological features evaluated included age, sex, viral infection, alpha-fetoprotein level, protein induced by vitamin K absence or antagonist-II (PIVKA-II), clinical stage, primary tumor size, metastatic lesion, histological type,

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treatment response, and duration of sorafenib treatment. The present study was approved by the institutional review boards of all the centers involved in the study, and informed consent was obtained from the patients.

Isolation of Genomic DNA. Genomic DNA samples were extracted from deparaffinized tissue sections preserved as FFPE tissue using a QIAamp DNA Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA samples were extracted from surgical frozen sections using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. The DNA concentration was determined using the NanoDrop2000 (Thermo Scientific, Waltham, MA).

Comparative Genomic Hybridization Analysis. The Genome-wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA) was used to perform array comparative genomic hybridization (CGH) on genomic DNA from HCC and paired liver samples according to the manufacturer's instructions. A total of 250 ng of genomic DNA was digested with both Nsp I and Sty I in independent parallel reactions, subjected to restriction enzymes, ligated to the adaptor, and amplified using polymerase chain reaction (PCR) with a universal primer and TITANIUM Taq DNA Polymerase (Clontech, Palo Alt, CA). The PCR products were quantified, fragmented, end-labeled, and hybridized onto a Genome-wide Human SNP6.0 Array. After washing and staining in Fluidics Station 450 (Affymetrix), the arrays were scanned to generate CEL files using the GeneChip Scanner 3000 and GeneChip Operating Software version 1.4. In the array CGH analysis, sample-specific copy number changes were analyzed using Partek Genomic Suite 6.4 software (Partek Inc., St. Louis, MO).

Copy Number Assay. The copy numbers for *FGF3* and *FGF4* were determined using commercially available and predesigned TaqMan Copy Number Assays according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) as described.[10] The primer IDs used for the *FGFs* were as follows: *FGF3*, Hs06336027_cn; *FGF4*, HS01235235_cn. The *TERT* locus was used for the internal reference copy number. Human Genomic DNA (Clontech) and DNA from noncancerous FFPE tissue were used as a normal control.

Real-Time Reverse-Transcription PCR. Real-time reverse-transcription PCR (RT-PCR) was performed as described.[11] In brief, complementary DNA was prepared from the total RNA obtained from each surgical frozen section using a GeneAmp RNA-PCR kit (Applied Biosystems). Real-time RT-PCR amplification was performed using a Thermal Cycler Dice (TaKaRa, Otsu, Japan) in accordance with the manufacturer's instructions under the following conditions: 95°C for 5 minutes, followed by 50 cycles of 95°C for 10 seconds and 60°C for 30 seconds. The primers used for the real-time RT-PCR were as follows: *FGF3*, 5′-TTT GGA GAT AAC GGC AGT GGA-3′ (forward) and 5′-CGT ATT ATA GCC CAG CTC GTG GA-3′ (reverse); *FGF4*, 5′-GAG CAG CAA GGG CAA GCT CTA-3′ (forward) and 5′-ACC TTC ATG GTG GGC GAC A-3′ (reverse); *GAPD*, 5′-GCA CCG TCA AGG CTG AGA AC-3′ (forward) and 5′-ATG GTG GTG AAG ACG CCA GT-3′ (reverse). *GAPD* was used to normalize expression levels in the subsequent quantitative analyses.

Fluorescence* In Situ *Hybridization Analysis. Fluorescence *in situ* hybridization (FISH) was performed as described.[10] Probes designed to detect the *FGF3* gene and *CEN11p* on chromosome 11 were labeled with fluorescein isothiocyanate or Texas red and were designed to hybridize to the adjacent genomic sequence spanning approximately 0.32 Mb and 0.63 Mb, respectively. The probes were generated from appropriate clones from a library of human genomic clones (GSP Laboratory, Kawasaki, Japan).

Immunoblotting. Western blot analysis was performed as described.[11] The following antibodies were used: monoclonal FGF3 (R&D Systems, Minneapolis, MN), FGF4 and FGFR2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and phosphorylated FGFR and horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA). NIH-3T3 cells were exposed to the indicated concentrations of sorafenib for 2 hours and were then stimulated with FGF4-conditioned medium for 20 minutes.

Cell Growth Inhibitory Assay. To evaluate growth inhibition in the presence of various concentrations of sorafenib, we used an MTT assay as described.[12]

Plasmid Construction, Viral Production, and Stable Transfectants. The methods used in this section have been described.[12] The complementary DNA fragment encoding human full-length *FGF3* or *FGF4* was isolated using PCR and Prime STAR HS DNA polymerase (TaKaRa, Otsu, Japan) with following primers: *FGF3*, 5′-GG GAA TTC GCC GCC ATG GGC CTA ATC TGG CTG CTA-3′ (forward) and 5′-CC CTC GAG GCC CAG CTA GTG CGC ACT GGC CTC-3′ (reverse); *FGF4*, 5′-GG GAA TTC GCC GCC ATG TCG GGG CCC GGG ACG GCC GCG GTA GCG C-3′ (forward) and 5′-CC CTC GAG

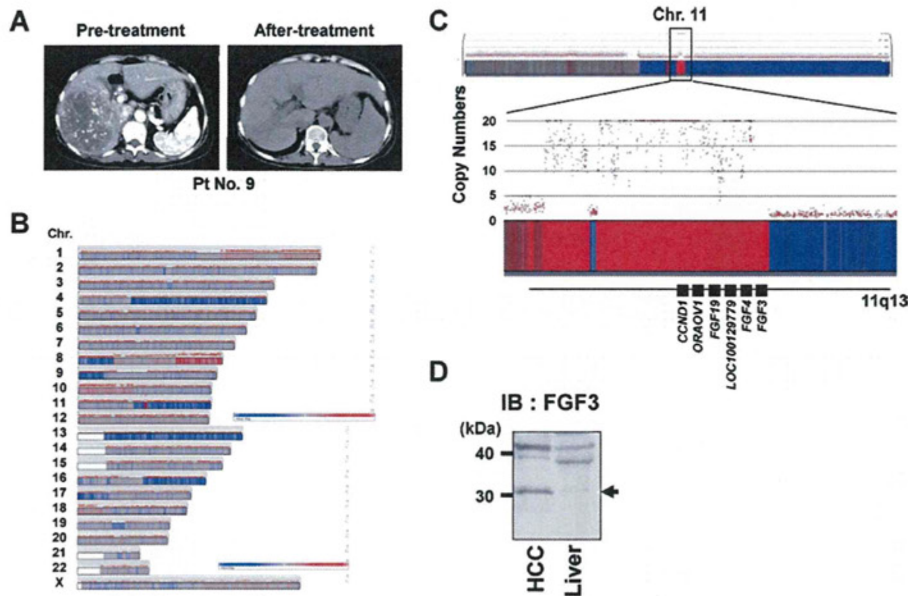

Fig. 1. HCC exhibiting a marked response to sorafenib treatment harbors *FGF3/FGF4* gene amplification. (A) Abdominal CT images obtained pretreatment (left panel) and 2 months after treatment (right panel). (B) CGH analysis of the tumor. Paired background liver tissue was used as a reference sample. A gain (>4 copies, red) and a loss (<0.5 copies, blue) of genomic copy number are shown. (C) Whole copy numbers of chromosome 11 are shown. A highly amplified region is described in the lower panel. (D) Western blot analysis of FGF3 (arrow) in HCC and paired background liver samples. IB, immunoblotting.

GGA GGG TCA CAG CCT GGG GAG GAA GTG GGT GAC CTT C-3′ (reverse). The stable transfectants expressing *EGFP* or *FGF3* or *FGF4* for each cell line were designated as A549/EGFP, A549/FGF3, and A549/FGF4.

Xenograft Studies. Nude mice (BALB/c nu/nu, 6-week-old females; CLEA Japan Inc., Tokyo) were used for *in vivo* studies and were cared for in accordance with the recommendations for the handling of laboratory animals for biomedical research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kinki University. Mice were subcutaneously inoculated with a total of 5×10^6 A549/EGFP, A549/FGF3, or A549/FGF4 cells. Two weeks after inoculation, the mice were randomized according to tumor size into two groups to equalize the mean pretreatment tumor size among the three groups (n = 20 mice per group). The mice were then treated with a low dose of oral sorafenib (n = 10, 15 mg/kg/day) or vehicle control (n = 10, Cremophor EL/ethanol/water) for 9 days. Tumor volume was calculated as length $\times$ width^2 $\times$ 0.5 and was assessed every 2 to 3 days.

Statistical Analysis. The statistical analyses were performed to test for differences between groups using the Student *t* test or Fisher's exact test. $P < 0.05$ was considered statistically significant. All analyses were performed using PAWS Statistics 18 (SPSS Japan Inc., Tokyo, Japan).

Results

Responder to Sorafenib Who Harbored* FGF3/FGF4 *Gene Amplification. A 58-year-old woman was diagnosed as having histologically confirmed advanced HCC (Fig. 1A, left panel) with multiple lung metastases. She received combination treatment with sorafenib, 5-fluorouracil (5FU), and interferon, and a subsequent treatment assessment revealed a partial response. Because the disease was well controlled with sorafenib treatment for 14 months (Fig. 1A, right panel), surgery was performed. To characterize this tumor molecularly, we performed array CGH analysis using frozen surgical specimens of the HCC region and paired background liver tissue as a reference control. The array CGH analysis revealed a low-level gain in the genomic DNA copy number for 1q, 8q, 10p, and 18p and a high level gain at 11q13 (Fig. 1B). Interestingly, the 11q13 region, a rare amplicons in HCC that contains several genes, including *FGF3*, *FGF4*, *CCND1*, and *FGF19*, was highly amplified over 20 copies (Fig. 1C). Western blot analysis revealed that FGF3 was overexpressed in the HCC specimen compared with the paired background liver specimen (Fig. 1D).

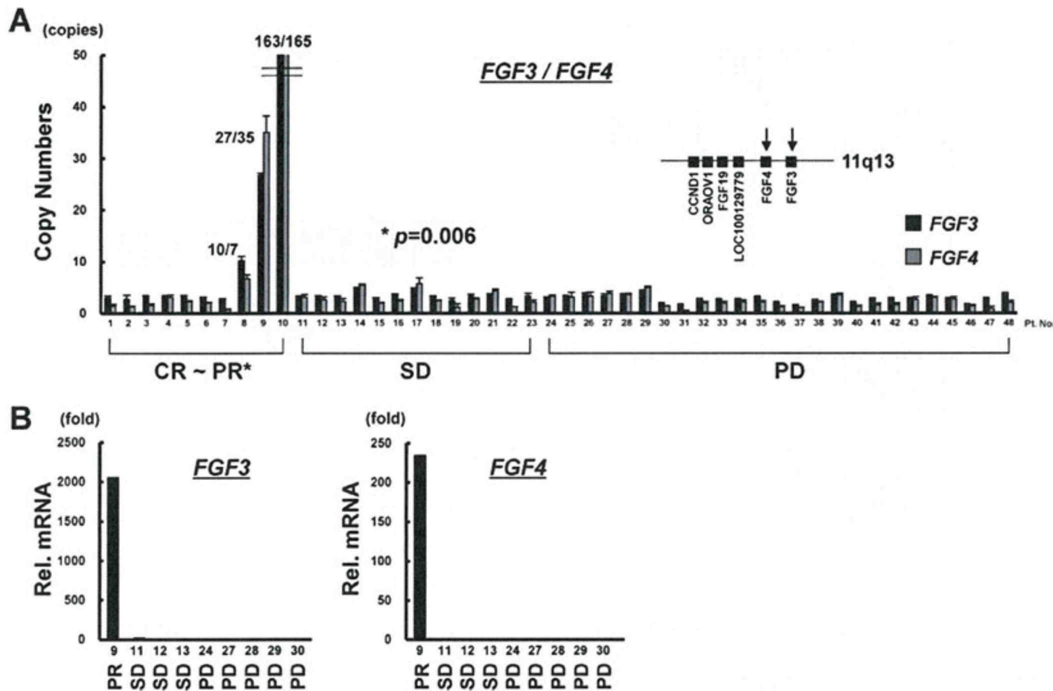

Fig. 2. *FGF3/FGF4* gene amplification is frequently observed in responders to sorafenib in HCC. (A) *FGF3/FGF4* gene amplification was determined using the TaqMan copy number assay in DNA samples obtained from 48 HCC samples that had been treated with sorafenib. *FGF3* amplification of >5 copies was observed in three of the sorafenib responders. *Complete response + partial response versus stable disease + progressive disease. (B) *FGF3/FGF4* gene amplification mediates the overexpression of *FGF3/FGF4* mRNA. The mRNA expression levels of *FGF3* and *FGF4* were examined in nine HCC samples that were available as frozen samples among 48 HCC samples that were treated with sorafenib. Rel. mRNA, *target gene/GAPD* $\times 10^6$.

The 11q13 locus is known to be a frequently amplified region in several human cancers except HCC.[13] Thus, we hypothesized that the amplification of 11q13 may be involved in a marked response to sorafenib.

FGF3/FGF4 ***Gene Amplification Is Frequently Observed in Responders to Sorafenib.*** To address the question of whether *FGF3/FGF4* gene amplification is also found in the HCC of other responders to sorafenib, we examined HCC specimens collected from 11 other medical centers in Japan. Because most of the HCC samples were collected as FFPE samples, we used a TaqMan Copy number assay.[10] A copy number assay revealed that *FGF3/FGF4* amplification was observed in three of the 10 (30%) HCC samples that responded to sorafenib, whereas no amplification was observed in the 38 specimens from patients with stable or progressive disease ($P = 0.006$, Fig. 2A). The copy numbers for *FGF3/FGF4* were $10.2 \pm 0.8/6.7 \pm 0.8$, $26.7 \pm 0.4/35.1 \pm 3.1$, and $162.5 \pm 9.0/165.0 \pm 12.5$ copies in the amplified samples, whereas the copy numbers of *FGF3* for all the other samples were below 5 copies. The correlation between the *FGF3* locus and the *FGF4* locus copy numbers was very high ($R = 0.998$), indicating that the DNA copy number assay for *FGF3/FGF4* was a sensitive and reproducible method.

FGF3/FGF4 ***Gene Amplification Mediates the Overexpression of*** **FGF3/FGF4** ***Messenger RNA.*** We examined the messenger RNA (mRNA) expression levels of *FGF3/FGF4* in nine HCC samples that were available as frozen samples among the 48 sorafenib-treated samples, as shown in Fig. 2A. One amplified sample expressed extremely high mRNA levels of *FGF3/FGF4* compared with nonamplified samples (Fig. 2B). The results demonstrated that *FGF3/FGF4* gene amplification mediates the overexpression of *FGF3/FGF4* mRNAs and proteins (Figs. 2B and 1D).

FISH Analysis Confirmed **FGF3/FGF4** ***Gene Amplification.*** We used FISH analysis to examine *FGF3/FGF4* amplification and to verify the results of the above-described PCR-based DNA copy number assay. All *FGF3/FGF4*-amplified clinical samples were confirmed as exhibiting high-level *FGF3* amplification using FISH analysis (Fig. 3). One patient showed multiple scattered signals, whereas two patients showed large clustered signals. Nonamplified HCC yielded a negative result for gene amplification. These results clearly demonstrate the presence of *FGF3/FGF4*-

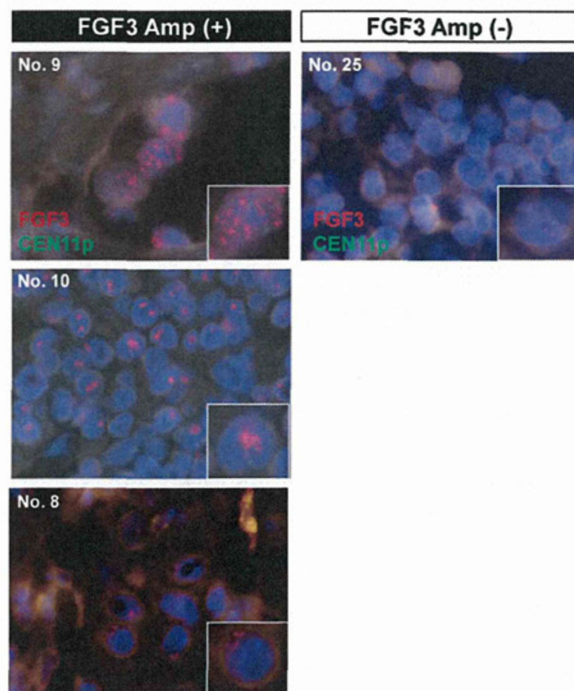

Fig. 3. FISH analysis of *FGF3*-amplified HCC. Patient numbers were indicated. Green staining indicates *CEN11P* loci; red staining indicates *FGF3* loci. High-power images are presented in each inset for a single cancer cell. Amp, gene amplification.

amplified HCC among the clinical samples, and the FISH analysis results were consistent with those for the copy number assay.

Frequency of* FGF3/FGF4 *Gene Amplification in HCC. To determine the frequency of *FGF3/FGF4* gene amplification in HCC, we performed a copy number assay for HCC samples without sorafenib treatment in a series of surgical specimens. Two of the 82 (2.4%) HCC samples exhibited *FGF3/FGF4* gene amplification, with copy numbers of 10.7/15.3 and 133.3/112.7 copies, respectively (Fig. 4). One amplified HCC was a poorly differentiated tumor, whereas the other was a moderately differentiated tumor.

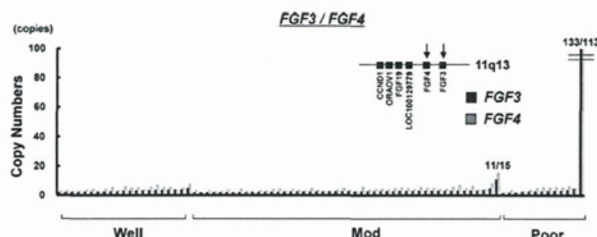

Fig. 4. *FGF3/FGF4* gene amplification in a series of HCC samples without sorafenib treatment. TaqMan copy number assay for *FGF3* and *FGF4* was used to examine DNA samples obtained from 82 surgical specimens. Human normal genomic DNA was used as a normal control. Well, well-differentiated HCC; Mod, moderately differentiated HCC; Poor, poorly differentiated HCC.

Clinicopathological Features of Responders to Sorafenib. The clinico-pathological features of the sorafenib responders are shown in Table 1. A comparison of clinical factors (age, sex, viral status, alpha-fetoprotein level, PIVKA-II, clinical stage, primary tumor size, metastatic status, histological type, and tumor response between responders and nonresponders) is given in Table 2. Notably, multiple lung metastases over five nodules was significantly higher among responders to sorafenib (responders, 5/13 [38%]; nonresponders, 2/42 [5%]; $P = 0.006$). Although the difference was not significant, poorly differentiated HCC tended to be

Table 1. Clinico-pathological Characteristics in Sorafenib Responders

Patient No.	Age, Years	Sex	Viral Status	AFP, ng/mL	PIVKA-II, mAU/mL	Clinical Stage	HCC in the Liver	Lung Metastasis	Other Metastases	Histological Type	Combination Treatment	Treatment Response	*FGF3/FGF4* Amplification
1	52	M	B	198	140	IV	2 cm, ×3	multi	Adrenal gland	Mod	(−)	PR	(−)
2	63	M	B	24	1,983	III	6 cm	(−)	(−)	Mod	(−)	CR	(−)
3	58	M	C	16	14	III	9 cm, multiple	(−)	(−)	Well	(−)	PR	(−)
4	62	M	B	8	130	IV	(−)	×3	(−)	Mod-Poor	(−)	PR	(−)
5	47	F	C	1,872	728	IV	2 cm, multiple	Multiple	(−)	Poor	+TAI	CR	(−)
6	66	M	C	290	18,507*	IV	5 cm	(−)	(−)	Mod	(−)	CR	(−)
7	71	M	C	404,100	1,328	IV	5 cm, multiple	Multiple	(−)	Poor	(−)	CR	(−)
8	66	M	Non	49	7,173	IV	(−)	×2	Pleural, LN	Mod	(−)	PR	Amplification
9	58	F	B	715	101	IV	11 cm	Multiple	(−)	Combination†	+5FU/IFN	PR	Amplification
10	80	F	C	378	21	III	3 cm, ×3	(−)	(−)	Poor, Mod‡	(−)	CR	Amplification
11	57	M	C	46,835	2,730	IV	14 cm, multiple	Multiple	(−)	Mod	(−)	CR	ND
12	77	M	B	435	71,000	IV	4 cm, multiple	(−)	(−)	Mod	(−)	PR	ND
13	84	M	Non	5,410	847,000*	IV	13 cm, multiple	(−)	(−)	Poor	(−)	PR	ND

Abbreviations: AFP, alpha-fetoprotein; CR, complete response; F, female; IFN, interferon; LN, lymph node; M, male; Mod, moderately differentiated; ND, not done; Non, non-B, non-C; Poor, poorly differentiated; PR, partial response; TAI, transcatheter arterial infusion; Well, well differentiated.

*Warfarin treatment (+).

†HCC with cholangiocarcinoma component.

‡From two different HCC nodules.

Table 2. Clinicopathological Characteristics and *FGF3/FGF4* Gene Amplification in Responders and Nonresponders to Sorafenib

Characteristic	Responders (n = 13)	Nonresponders (n = 42)	*P* Value*
Age, years (range)	63 (47-84)	66 (22-89)	0.98
Sex, M/F	10/3	30/12	0.97
Viral status, no.			0.69
HBV	5	10	
HCV	6	16	
B+C	0	1	
Non-B, non-C	2	15	
AFP, ng/mL (range)	378 (8-404,100)	56 (2-114,248)	0.33
PIVKA-II, mAU/mL (range)	728 (14-847,000)	81 (11-147,000)	0.78
Clinical stage, no.			0.73
II	0	1	
III	3	13	
IV	10	28	
Primary tumor, cm (range)	5 (0-14)	3 (0-15)	0.20
Lung metastasis, no.			0.13
(−)	6	31	
(+)	7	11	
Multiple lung metastases, no.			0.006
<5	8	40	
≥5	5	2	
Other metastases, no.			0.24
(−)	11	26	
(+)	2	16	
Histological type, no.			0.13
Well	1	7	
Moderate	6	26	
Poor	5	6	
Combination†	1	3	
Response, no.			ND
Complete response	6	–	
Partial response	7	–	
Stable disease	–	16	
Progressive disease	–	24	
Not evaluable	–	2	

Abbreviations: AFP, alpha-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus; ND, not done.

**P* values of viral status and histological type were calculated between HBV versus HCV and poorly differentiated versus nonpoorly differentiated.

†HCC with cholangiocarcinoma component.

more common among responders to sorafenib (responders, 5/13 [38%]; nonresponders, 6/42 [14%]; $P = 0.13$). These results suggest that multiple lung metastases and a poorly differentiated histology may be clinical biomarkers for sorafenib treatment in patients with HCC.

Sorafenib Potently Inhibits Cellular Growth in* FGF3/FGF4*-Amplified and* FGFR2*-Amplified Cell Lines. We examined the growth inhibitory effect of sorafenib in various cancer cell lines to evaluate whether activated FGFR signaling is involved in the response to sorafenib. Among 26 cell lines, KYSE220 was the only *FGF3/FGF4*-amplified cell line (data not shown), and HSC-43, HSC-39, and KATOIII were the only *FGFR2*-amplified cell lines.[14] Sorafenib potently inhibited cellular growth in these four cell lines at a sub-μM 50% inhibitory concentration (IC_{50}) (Fig. 5A). The IC_{50} values were as follows: HSC43, 0.8 μM; HSC39, 0.6 μM; KATOIII, 0.4 μM; and KYSE220, 0.18 μM. These results suggest that activated FGFR signaling may be involved in the response to sorafenib.

***Sorafenib Inhibits Tumor Growth in* FGF4*-Introducing Cell Lines* In Vivo.** Finally, we established cancer cell lines stably overexpressing *EGFP*, *FGF3*, or *FGF4* to examine the relationship between the gene function of *FGF3* or *FGF4* and drug sensitivity to sorafenib *in vivo*. Western blotting confirmed that exogenously expressed FGF3 and FGF4 were secreted into the culture medium (Fig. 5B). Sorafenib inhibited the FGF4-conditioned, medium-mediated expression levels of phosphorylated FGFR (Figure 5C). A similar result was obtained using recombinant FGF4 (data not shown). Mice inoculated with these cell lines were treated with a low dose of oral sorafenib (15 mg/kg/day) or without sorafenib (vehicle control). *FGF3* overexpression did not increase the tumor volume compared with EGFP tumors; however, *FGF4* overexpression aggressively increased tumor volume and clearly enhanced the malignant phenotype (Fig. 5D). Notably, the low-dose sorafenib treatment significantly inhibited the growth of the A549/FGF4 tumors, whereas it was not effective against A549/EGFP and A549/FGF3 tumors (Fig. 5D). These results suggest that overexpression of *FGF4* is partially involved in the response to sorafenib.

Discussion

The *FGF3* gene was first identified and characterized based on its similarity to the mouse *fgf3/int-2* gene, which is a proto-oncogene activated in virally induced mammary tumors in mice.[15] Meanwhile, the *FGF4* gene was first identified in gastric cancer as an oncogene *HST*, which has the ability to induce the neoplastic transformation of NIH-3T3 cells upon transfection.[16] These genes were initially regarded as proto-oncogenes. *FGF3* and *FGF4* genes are located side-by-side and are also closely located to the *FGF19* and *CCND1* genes (within 0.2 Mb of the 11q13 region).[13] The 11q13 region is known as a gene-dense region, and gene amplification of this region is frequently observed in various solid cancers (including breast cancer, squamous cell carcinoma of the head and neck, esophageal cancer, and melanoma) at frequencies of 13%-60%.[13] On the other hand, the frequency of *FGF3/FGF4* amplification in HCC remains

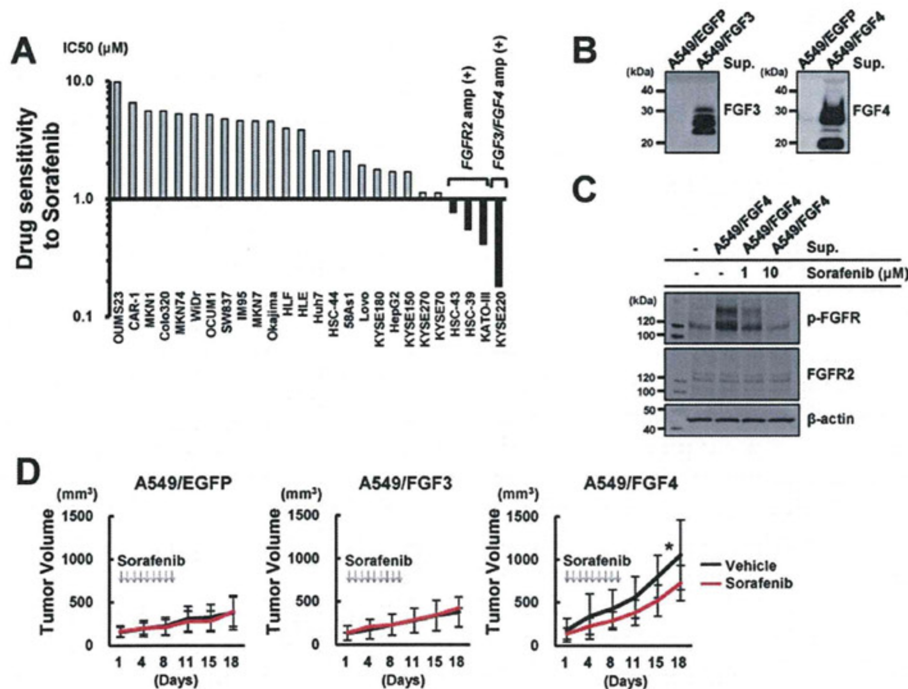

Fig. 5. FGF3 and FGF4 overexpression and drug sensitivity to sorafenib *in vitro* and *in vivo*. (A) Growth inhibitory assay examining sorafenib in various cancer cell lines *in vitro*. The growth inhibitory effect of sorafenib was examined using an MTT assay. The IC_{50} values of each cell line are shown in the graph. The black bars show that the IC_{50} values were below 1 μM. Amp, gene amplification. (B) Cancer cell lines stably overexpressing *EGFP*, *FGF3*, or *FGF4* were established and designated as A549/EGFP, A549/FGF3, and A549/FGF4. Western blot analysis confirmed that exogenously expressed FGF3 and FGF4 were secreted into the culture medium. Sup., supernatant. (C) NIH-3T3 cells were exposed to indicated concentrations of sorafenib for 2 hours and were then stimulated with FGF4-conditioned medium for 20 minutes. (D) Mice inoculated with A549/EGFP, A549/FGF3, or A549/FGF4 (n = 20 each) were treated with a low dose of oral sorafenib (n = 10, 15 mg/kg/day) or without (n = 10, vehicle control). $^{*}P < 0.05$.

largely unclear. Relatively small cohort studies have reported that one out of 20 HCCs exhibited *FGF3* amplification as determined via CGH analysis,[17] and 3 out of 45 HCCs examined using Southern blot analysis had a copy number >5;[18] meanwhile, amplification was not detected in 0 out of 42 surgically resected HCCs.[19] In the present study, two of the 82 (2.4%) HCC samples exhibited *FGF3/FGF4* gene amplification in the HCC series. If only 2%-3% of HCC patients harbor the *FGF3/FGF4* amplification, its value as a biomarker seems to be limited in clinics because a frequency of 2%-3% is too low to stratify the patients for specific targeted therapy. However, a combination of biomarkers—including *FGF3/FGF4* amplification, lung metastasis, tumor differentiation, and other unrevealed dysregulation of FGFR signaling—may increase the response prediction. In addition, 2%-3% of *FGF3/FGF4* amplification may be a promising therapeutic target for future FGFR-targeted therapies in the treatment of HCC.

Tumor shrinkage might be due to the mixed effect (sorafenib + 5FU + interferon) of combination therapy in the initially described patient. However, during this patient's long clinical course, tumor regrowth was observed following withdrawal of sorafenib because of oral hemorrhage, and tumor reshrinkage was observed when sorafenib treatment recommenced. Thus, we considered that tumor shrinkage might be achieved by the effect of sorafenib on its own, rather than 5FU + interferon.

Regarding determinants of drug sensitivity to sorafenib, the mechanism of hypersensitivity in the gastric cancer cell lines HSC-39, HSC-43, and KATO-III is *FGFR2* gene amplification and is thought to be the addiction of these cell lines to this gene,[14] since sorafenib has a relatively weak but significant inhibitory effect on FGFR1 at a concentration of 580 ± 100 nM.[3] This result suggests that the blockade of FGFR signaling by sorafenib may lead to a significant treatment response, at least in *FGFR2*-amplified cells. In this study, we found that *FGF4*, but not *FGF3* overexpression, was partially involved in the sensitivity to sorafenib *in vivo*. The limitations of the study are the small number of responder patients and the potential bias in their selection because of the retrospective study design. Further clinical study of responders to

sorafenib is necessary. We are presently undertaking a prospective molecular translational study (2010-2012) in a cohort of Japanese patients with sorafenib-treated HCC.

Multiple lung metastases were frequently observed among responders to sorafenib (38%) but were less common among nonresponders (5%). Based on a Japanese follow-up survey of patients with primary HCC, lung metastasis was observed in 7% (169/2355) of the patients at the time of autopsy.[20] Another study demonstrated that 15% of patients were found to have extrahepatic metastases, and lung metastasis was detected in 6% of 995 consecutive HCC patients.[21] When compared with these data from large-scale studies, the frequency of lung metastasis among responders to sorafenib seems quite high. In addition, a poorly differentiated histological type tended to be more common among responders, although the correlation was not significant.

In conclusion, we found that *FGF3/FGF4* gene amplification, multiple lung metastases, and a poorly differentiated histological type may be involved in the response to sorafenib.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Incidence of hepatocellular carcinoma in HCV-infected patients with normal alanine aminotransferase levels categorized by Japanese treatment guidelines

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Abstract

Background This study was conducted to evaluate Japanese treatment guidelines for patients with chronic hepatitis C virus (HCV) infection and normal alanine aminotransferase (N-ALT) levels from the viewpoint of the incidence of hepatocellular carcinoma (HCC).

Methods Four groups of patients with chronic HCV infection treated with pegylated interferon (Peg-IFN) plus ribavirin, and classified according to the N-ALT guidelines, were examined for HCC incidence: group A ($n = 353$), ALT $\leq$30 IU/L and platelet (PLT) $\geq$15 $\times$ 10^4/mm^3; group B ($n = 123$), ALT $\leq$30 IU/L and PLT <15 $\times$ 10^4/mm^3; group C ($n = 233$), 30 < ALT $\leq$ 40 IU/L and PLT $\geq$15 $\times$ 10^4/mm^3; and group D ($n = 100$), 30 < ALT $\leq$ 40 IU/L and PLT <15 $\times$ 10^4/mm^3. The mean observation period was 36.2 ± 16.5 months

Results In groups A and C, the HCC incidence was low even in patients with non-response (NR) (cumulative rates at 3 years, 0.0 and 2.9 %, respectively). In groups B and D, 14.5 and 5.3 % of NR patients had developed HCC at 3 years, but none of the patients with sustained virologic response (SVR) or relapse had developed HCC. In group B, no patients with mild fibrosis developed HCC irrespective of the antiviral effect of the treatment. Among patients with PLT <15 $\times$ 10^4/mm^3 (group B plus group D), the HCC incidence was significantly lower in patients with SVR and relapse than in NR patients ($p < 0.001$, $p = 0.021$, respectively).

Conclusion These results suggest that N-ALT patients with PLT <15 $\times$ 10^4/mm^3 could be candidates for early antiviral therapy.

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Introduction

Continuous hepatitis C virus (HCV) infection causes liver inflammation and can lead to liver fibrosis, which may progress to cirrhosis and hepatocellular carcinoma (HCC) [1–4]. Because HCV carriers with persistent normal alanine aminotransferase (PNALT) levels have minimal liver inflammation and the progression of liver fibrosis in such patients is slow, they are generally considered to be at low risk for carcinogenesis [5–7]. Moreover, patients with PNALT had not been considered as candidates for antiviral therapy in the era of interferon (IFN) monotherapy because of reports of ALT flare-up owing to antiviral therapy in some cases (47–67 %) [8–10].

However, in recent years, the antiviral efficacy of pegylated IFN (Peg-IFN) plus ribavirin combination therapy for patients with chronic HCV infection has been reported to be equivalent for patients with normal alanine aminotransferase (N-ALT) levels and those with elevated ALT levels [11–15]. In addition, for patients with PNALT, there have been fewer cases of ALT flare-up caused by Peg-IFN plus ribavirin combination therapy than with IFN monotherapy [12, 15]. Thus, patients with chronic HCV infection and N-ALT have come to be treated with Peg-IFN plus ribavirin combination therapy.

Treatment guidelines for patients with chronic HCV infection and N-ALT levels have been prepared by a Japanese group conducting "Research on Hepatitis" supported by Health and Labour Sciences Research Grants from the Japanese Government. In these guidelines, HCV carriers with N-ALT ($\leq$40 IU/L) are categorized into four groups according to their ALT levels ($\leq$30 or $\geq$31 IU/L) and platelet (PLT) counts ($\geq$15 or $<$15 $\times$ 10^4/mm^3). Briefly, the therapeutic strategies are as follows: patients with ALT levels of more than 31 IU/L are candidates for antiviral treatment, but observation is recommended for patients with ALT levels of $<$30 IU/L. However, the goal of antiviral treatment is to improve the long-term prognosis, including inhibition of HCC. Therefore, the indication of antiviral therapy for patients with chronic HCV infection and N-ALT should be decided based on whether or not Peg-IFN plus ribavirin combination therapy can suppress the cumulative rate of HCC incidence and improve prognosis. It is thus very important to examine the effect of inhibition of HCC induced by antiviral therapy in patients with chronic HCV infection and N-ALT.

In the present study, we evaluated the treatment guidelines for patients with chronic HCV infection and N-ALT from the viewpoint of HCC inhibition by analyzing the differences in the cumulative rates of HCC incidence among the above four groups. The treatment guidelines also recommend that if patients with ALT $\leq$30 IU/L and PLT $<$15 $\times$ 10^4/mm^3 have moderate to severe liver fibrosis (F2–4), they should receive antiviral therapy. We also evaluated the effect of Peg-IFN plus ribavirin on HCC incidence according to the degree of fibrosis in this group.

Patients and methods

This retrospective study was conducted by Osaka University and institutions participating in the Osaka Liver Forum. Among patients with chronic HCV infection who had received Peg-IFN plus ribavirin combination therapy from December 2004 to December 2009, four groups of patients, classified according to the N-ALT guidelines, who had not suffered from HCC, were examined for their HCC incidence: group A ($n = 353$), ALT $\leq$30 IU/L and PLT $\geq$15 $\times$ 10^4/mm^3; group B ($n = 123$), ALT $\leq$30 IU/L and PLT $<$15 $\times$ 10^4/mm^3; group C ($n = 233$), 30 $<$ ALT $\leq$ 40 IU/L and PLT $\geq$15 $\times$ 10^4/mm^3; and group D ($n = 100$), 30 $<$ ALT $\leq$ 40 IU/L and PLT $<$15 $\times$ 10^4/mm^3. The Kaplan–Meier method was used to examine the cumulative rates of HCC incidence in the four groups. Excluded from this study were patients who developed HCC within 12 months from the start of Peg-IFN plus ribavirin combination therapy, patients with co-infection with hepatitis B or human immunodeficiency virus, patients with drug-induced or alcoholic liver disorders, and patients with autoimmune hepatitis. The protocol was performed after obtaining informed consent from each patient before treatment in accordance with the ethical guidelines of the Declaration of Helsinki amended in 2008. This study was approved by the Institutional Review Board and registered in the Universal Hospital Medical Information Network (UMIN) Clinical Trials Registry (UMIN unique trial number, C000000197).

Treatment protocol

All patients received Peg-IFN alpha-2b (PEGINTRON; Merck & Co., Whitehouse Station, NJ, USA) plus ribavirin (REBETOL; MSD) for the duration of the study. Peg-IFN alpha-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight (body weight $\leq$60 kg, 600 mg;

60–80 kg, 800 mg; >80 kg, 1000 mg), according to the standard treatment protocol for Japanese patients. Dose modification according to the intensity of the hematological adverse effects followed, as a rule, the manufacturer's drug information. The dose of Peg-IFN alpha-2b was reduced to 50 % of the assigned dose if the white blood cell (WBC) count declined to <1500/mm^3, the neutrophil count declined to <750/mm^3, or the PLT count declined to <8 × 10^4/mm^3, and was discontinued if the WBC count declined to <1000/mm^3, the neutrophil count declined to <500/mm^3, or the PLT count declined to <5 × 10^4/mm^3. Ribavirin was also reduced, from 1000 to 600 mg, or 800 to 600 mg, or 600 to 400 mg, if the hemoglobin (Hb) level decreased to <10 g/dL, and was discontinued if the Hb level decreased to <8.5 g/dL. Both Peg-IFN alpha-2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. During this therapy, no medicine containing iron or hematopoetic growth factors, such as erythropoietin alpha, or granulocyte–macrophage colony-stimulating factor, was administered. The serum HCV RNA levels were qualitatively analyzed using the COBAS AMPLICOR HCV Test, version 2.0 (lower limit of detection 50 IU/mL; Roche Diagnostics, Branchburg, NJ, USA), and the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 KIU/ml). In the patients with HCV genotype 1, as a rule, treatment duration was 48 weeks, but the patients with detectable HCV RNA (≥50 IU/mL) at week 12 and undetectable HCV RNA (<50 IU/mL) at week 24 were treated for 72 weeks. Patients with HCV genotype 2 were treated for 24 weeks.

Definition of virologic response

A sustained virologic response (SVR) was defined as undetectable HCV RNA at the end of treatment and at 24 weeks after completion of treatment. A relapse was defined as undetectable HCV RNA at the end of treatment but detectable HCV RNA at 24 weeks after completion of treatment. A non-response (NR) was defined as detectable HCV RNA at the end of treatment.

Histological evaluation

Liver biopsy was performed immediately before initiation of the Peg-IFN plus ribavirin combination therapy. Liver biopsy specimens were scored using the METAVIR system, and the grade of activity and stage of fibrosis were evaluated [16].

HCC surveillance

Ultrasonography or computed tomography (CT) was carried out before the initiation of the Peg-IFN plus ribavirin combination therapy and every 3–6 months during the follow-up period. New space-occupying lesions detected or suspected at the time of ultrasonography were further examined by CT or hepatic angiography. HCC was diagnosed by the presence of typical hypervascular characteristics on angiography, in addition to the findings from CT. If no typical image of HCC was observed, fine-needle aspiration biopsy was carried out, with the patient's consent, or the patient was carefully followed until a diagnosis was possible with a definite observation by CT or angiography.

End point

The observation period was defined as the period from the start of Peg-IFN plus ribavirin combination therapy. Patients who developed HCC and patients whose treatments were switched to other types of IFN therapy were defined as censored cases at that point in time.

Statistical analysis

Baseline data for various demographic, biochemical, and virologic characteristics of the patients were expressed as means ± SD. To analyze differences between baseline data among the four groups, analysis of variance or the χ^2 test was performed. The Kaplan–Meier method was used to calculate the cumulative incidence of HCC. The prognostic relevance of clinical variables and HCC incidence was evaluated by univariate analysis with the log-rank test. A value of $p < 0.05$ (two-tailed) was considered to indicate significance. The statistical software used for this analysis was IBM SPSS for Windows v. 19.0.0 (SPSS, Armonk, NY, USA).

Results

Baseline characteristics of patients categorized by the treatment guidelines

The baseline clinical features of the patients are shown in Table 1. There were significant differences in age; sex; body mass index (BMI); HCV genotype; past history of IFN therapy; grade and stage of liver histology; WBC, neutrophil, and PLT counts; Hb levels; and virologic response among the four groups. The mean ages of the patients in groups B and D were significantly higher than those of the patients in groups A and C. The proportion of males was lowest in group A (26 %) and highest in group C (41 %). The proportion of patients with progression of liver fibrosis (F3–4) diagnosed by the METAVIR score was 7.8 % among all patients tested and highest in group D (22.5 %). In groups B and D, peripheral blood cell counts (WBC, neutrophils, Hb, PLT) were significantly lower and the

Table 1 Baseline characteristics of the patients with chronic HCV infection and normal ALT levels

	Group A ALT ≤30 IU/L PLT count ≥15 × 10^4/mm^3	Group B ALT ≤30 IU/L PLT count <15 × 10^4/mm^3	Group C 30 < ALT ≤ 40 IU/L PLT count ≥15 × 10^4/mm^3	Group D 30 < ALT ≤ 40 IU/L PLT count <15 × 10^4/mm^3	*p* value
Number of patients	353	123	233	100	
Age (years)	55.6 ± 11.3	60.3 ± 8.4	54.6 ± 11.8	60.7 ± 8.6	<0.001
Sex: male/female	95/258	44/79	95/138	35/65	0.005
BMI (kg/m^2)	22.6 ± 3.3	22.1 ± 3.0	23.2 ± 3.4	22.3 ± 2.6	0.029
HCV genotype: 1/2	203/144	86/35	180/52	81/16	<0.001
HCV RNA (KIU/mL), mean ± SD	2333 ± 1664	2276 ± 1478	2261 ± 1599	2354 ± 1644	0.998
Past IFN therapy: naïve/experienced[a]	266/81	79/41	173/52	63/33	0.018
Histology[b]: activity: A0/A1/A2/A3	32/179/48/1	6/64/23/0	20/105/36/1	0/46/24/1	0.026
Fibrosis: F0/F1/F2/F3/F4	41/169/40/9/1	4/49/29/7/5	16/107/31/7/1	0/34/21/13/3	<0.001
White blood cell count (/mm^3)	5543 ± 1606	4405 ± 1211	5601 ± 1638	4677 ± 1337	<0.001
Neutrophil count (/mm^3)	3008 ± 1213	2332 ± 948	2999 ± 1243	2578 ± 1026	<0.001
Hemoglobin (g/dL)	13.3 ± 1.3	13.3 ± 1.4	13.9 ± 1.4	13.3 ± 1.3	<0.001
Platelet count (×10^4/mm^3)	21.1 ± 4.7	12.2 ± 2.1	21.3 ± 4.8	12.1 ± 2.2	<0.001
ALT (IU/L)	22.8 ± 5.2	23.5 ± 5.4	35.4 ± 2.9	35.8 ± 2.9	<0.001
Virologic response: SVR/relapse/NR	218/82/53	59/32/32	133/51/49	44/26/30	0.005

BMI body mass index, *ALT* alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *SVR* sustained virologic response, *NR* non-response, PLT platelet

[a] Virologic response to previous treatment was unknown for 22 patients

[b] Fibrosis stages are evaluated on a scale of 0–4 and activity grades are evaluated on a scale of 0–3 according to the METAVIR histological score. Fibrosis data were not available for 222 patients. Activity data were not available for 223 patients

numbers of patients with progression of liver fibrosis were significantly higher than in groups A and C. The mean duration of the observation period was 36.2 ± 16.5 months.

Antiviral efficacy of Peg-IFN plus ribavirin combination therapy

In genotype 1 patients, the rates of SVR, relapse, and NR were 50.7, 25.1, and 24.1 %, respectively, in group A; 39.5, 24.4, and 36.0 % in group B; 52.2, 23.9, and 23.9 % in group C; and 39.5, 25.1, and 35.2 % in group D. Although there was no significant difference in the treatment effect among the four groups, the SVR rate was significantly higher in groups A and C than that in groups B and D (groups A and C: SVR 51.4 %, relapse 24.5 %, NR 24.0 %; groups B and D: SVR 39.5 %, relapse 25.1 %, NR 35.2 %, $p = 0.012$). In genotype 2 patients, the rates of SVR, relapse, and NR were 77.8, 20.1, and 2.1 %, respectively, in group A; 65.7, 31.4, and 2.9 % in group B; 75.0, 15.4, and 9.6 % in group C; and 62.5, 31.3, and 6.3 % in group D. Although there was no significant difference in the treatment effect among the four groups, the SVR rate tended to be higher in groups A and C than that in groups B and D (groups A and C: SVR 77.0 %, relapse 18.9 %, NR 4.1 %; groups B and D: SVR 64.7 %, relapse 31.4 %, NR 8.9 %, $p = 0.152$).

Cumulative rate of HCC incidence according to the treatment effect of Peg-IFN plus ribavirin combination therapy

Eleven patients developed HCC during the observation period, and all were infected with HCV genotype 1. Figure 1 shows the cumulative rates of HCC incidence according to the treatment effect in the four groups.

In group A, no patients developed HCC during the 3 years of observation, regardless of the effect of Peg-IFN plus ribavirin combination therapy. Moreover, among those with SVR and relapse, no patients developed HCC during the 3-year observation period, while in NR patients the cumulative rate of HCC incidence at 5 years was 4.0 %. No significant difference in HCC incidence was found among the patients with SVR, relapse, and NR ($p = 0.071$) (Fig. 1a). In group C, no significant difference in HCC incidence was found among the patients with SVR, relapse, and NR (cumulative rates of HCC at 3 years, 2.2, 0.0, and 2.9 %, respectively; at 5 years, 3.7, 0.0, and 2.9 %, respectively, $p = 0.631$) (Fig. 1c). In group B, a marginally significant difference was found in HCC incidence among patients with SVR, relapse, and NR ($p = 0.054$), and patients with SVR had a significantly lower rate of HCC incidence than that of patients with NR (SVR vs. relapse, $p = 0.346$, SVR vs. NR, $p = 0.013$, relapse vs.

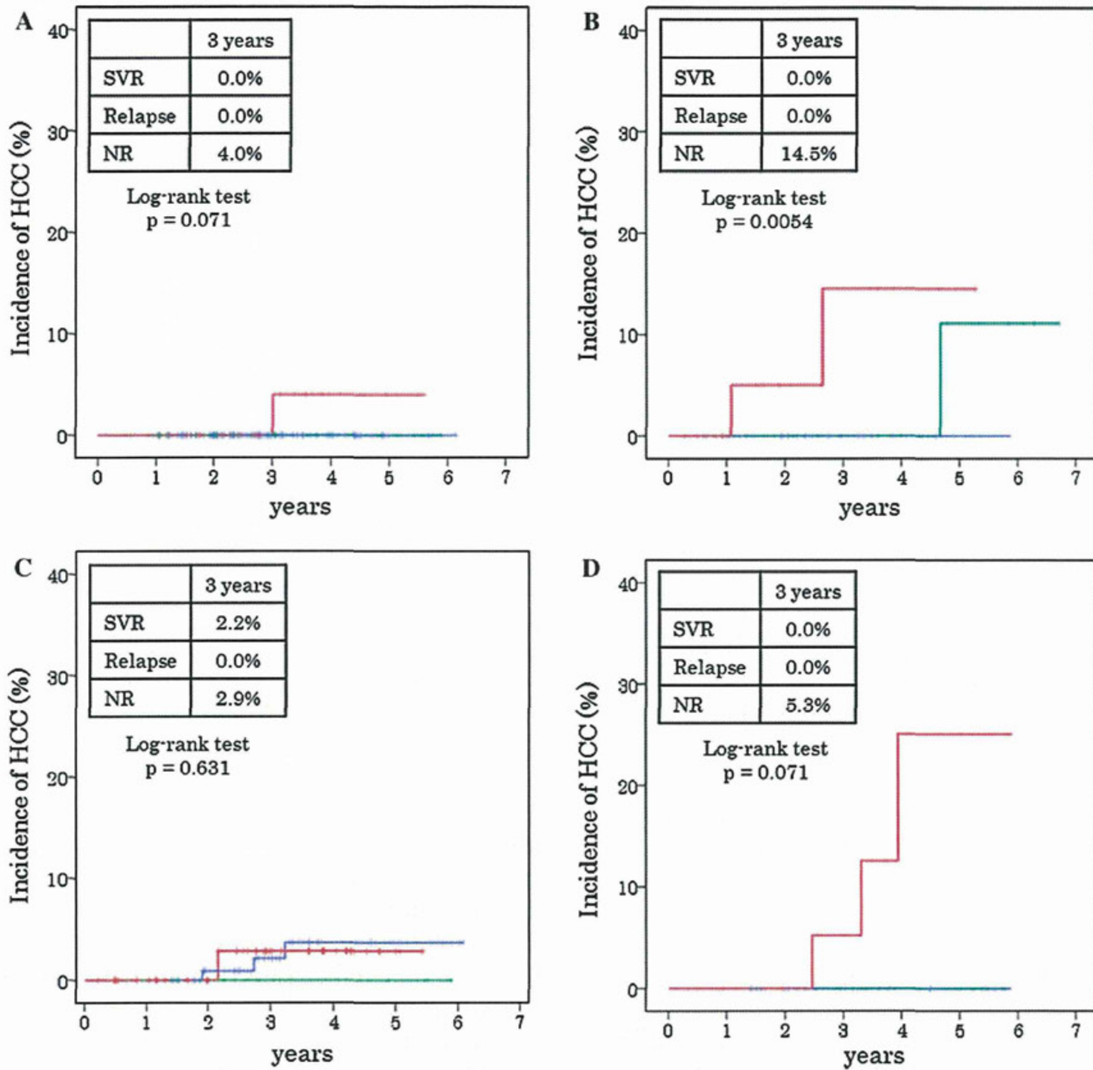

Fig. 1 Cumulative rates of hepatocellular carcinoma (*HCC*) incidence in groups A, B, C, and D, categorized according to the treatment effect of pegylated interferon (Peg-IFN) plus ribavirin combination therapy. **a** Group A (patients with alanine aminotransferase [ALT] level ≤30 IU/L and platelet [PLT] count ≥15 × 10^4/mm^3), **b** group B (patients with ALT ≤30 IU/L and PLT <15 × 10^4/mm^3), **c** group C (patients with 30 < ALT ≤ 40 IU/L and PLT ≥15 × 10^4/mm^3), **d** group D (patients with 30 < ALT ≤ 40 IU/L and PLT < 15 × 10^4/mm^3). *Blue line* patients with sustained virologic response (*SVR*), *green line* patients with relapse, *red line* patients with non-response (*NR*)

NR, $p = 0.250$). Of the NR patients, 14.5 % had developed HCC at 3 years, while none of the SVR or relapse patients had developed HCC at 3 years (Fig. 1b). In group D, there was a significant difference in HCC incidence among patients with SVR, relapse, and NR ($p = 0.006$), and patients with SVR or relapse had a significantly lower rate of HCC incidence than patients with NR (SVR vs. NR, $p = 0.012$, relapse vs. NR, $p = 0.047$). In the NR patients, 5.3 % had developed HCC at 3 years and 25.0 % had developed HCC at 5 years, but none of the SVR or relapse patients had developed HCC at 3 years (Fig. 1d).

In the analysis of the differences in the cumulative rates of HCC incidence in the patients with 30 < ALT ≤ 40 IU/L (group C plus group D), the p value for a significant difference was 0.059 among the patients with SVR, relapse, and NR (Fig. 2). In the analysis of the differences in the cumulative rates of HCC incidence among the patients with PLT counts of less than 15 × 10^4/mm^3 (group B plus group D), there was a significant difference in HCC incidence among patients with SVR, relapse, and NR ($p < 0.001$), and patients with SVR or relapse had a significantly lower rate of HCC incidence than patients with NR (cumulative rates of HCC incidence at

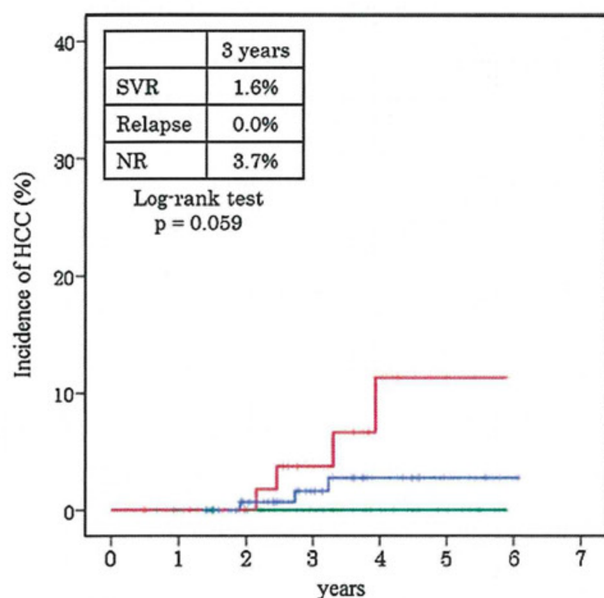

Fig. 2 Cumulative rates of HCC incidence according to ALT levels. Cumulative rates of HCC incidence in patients with ALT levels of 30 < ALT ≤ 40 IU/L (group C plus group D). *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

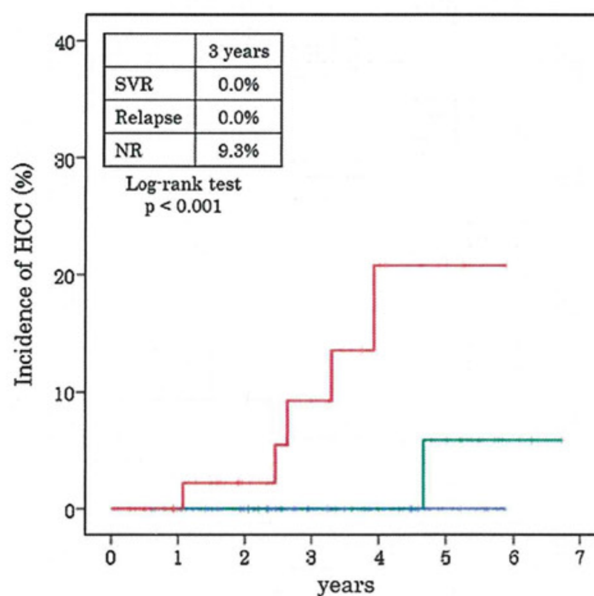

Fig. 3 Cumulative rates of HCC incidence according to PLT counts. Cumulative rates of HCC incidence in patients with PLT counts of <15 × 10^4/mm^3 (group B plus group D). *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

3 years, 0.0, 0.0, and 9.3 %, respectively; at 5 years, 0.0, 11.1, and 20.8 %, respectively; SVR vs. NR, $p < 0.001$, relapse vs. NR, $p = 0.021$) (Fig. 3).

Cumulative rate of HCC incidence in group B according to the stage of liver fibrosis

Based on the pattern of the Japanese treatment guidelines, we categorized the patients in group B into two groups according to the stage of liver fibrosis (F0–1 or F2–4) and compared the cumulative rates of HCC incidence. Patients with no fibrosis or mild fibrosis (F0–1) showed no HCC development regardless of the virologic response (SVR, relapse, or NR). Of note, in those with moderate to severe fibrosis (F2–4) in group B, there was no significant difference in HCC incidence among patients with SVR, relapse, and NR ($p = 0.174$), although SVR patients tended have a lower rate of HCC incidence than NR patients (SVR vs. relapse, $p = 0.414$, SVR vs. NR, $p = 0.071$, relapse vs. NR, $p = 0.383$). No patient in the SVR or relapse groups developed HCC, while the cumulative rate of HCC incidence at 3 years for the NR group was 25.0 % (Fig. 4).

Discussion

Patients with chronic HCV infection and N-ALT have been reported to show the possibility of ALT flare-up during the natural course of the disease (22–27 %) [17, 18] and to develop moderate to severe progression of liver fibrosis (5–30 %) [18–21]. However, very low cumulative incidences of HCC have been reported among patients with average ALT integration values less than or equal to 20 IU/L (5-year, 0.0 %, 10-year, 3.6 %) [22]. Therefore, it remains controversial whether HCV eradication by antiviral therapy can reduce the incidence of HCC in patients with chronic HCV infection and N-ALT [23–26].

The definition of N-ALT remains unclear because its cutoff value is still under consideration [22, 27, 28]. In Japan, treatment guidelines for patients with chronic HCV infection and N-ALT define N-ALT as serum ALT levels of ≤40 U/L, and the therapeutic strategy is decided after categorizing patients into four groups according to ALT levels and PLT counts. However, the indication of antiviral therapy should be based on whether or not HCC incidence can be suppressed by the antiviral therapy. Therefore, we examined the treatment guidelines from the viewpoint of inhibiting HCC in patients with chronic HCV infection and N-ALT.

In the present study, the antiviral efficacy of Peg-IFN plus ribavirin combination therapy for patients with chronic HCV infection and N-ALT was almost equivalent to the efficacy in those with elevated ALT levels, as previously reported [11–15]. The SVR rate was significantly higher in groups A and C than in groups B and D for patients with genotype 1, and the same tendency was found

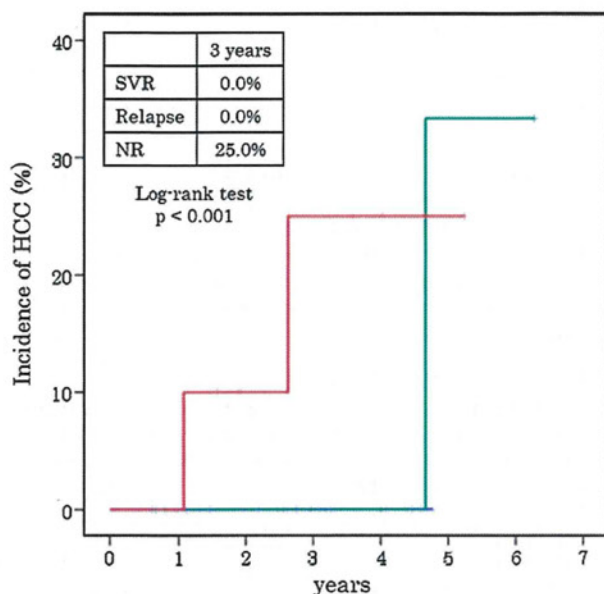

Fig. 4 Cumulative rates of HCC incidence in group B patients ($ALT \leq 30$ IU/L and $PLT < 15 \times 10^4/mm^3$) with moderate to severe liver fibrosis (F2–4), according to the treatment effect of Peg-IFN plus ribavirin combination therapy. *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

for those with genotype 2. The reason for this was considered to be that groups B and D included many patients with moderate to severe liver fibrosis (F3–4, 17.0 %), which can lead to a lower SVR rate [23, 29, 30].

The present study revealed the cumulative rates of HCC incidence according to the treatment effect in the four groups. In group D, the cumulative rate of HCC incidence in the SVR and relapse patients was significantly lower than that for the NR patients. This result supports the recommendation by the treatment guidelines that patients in group D be managed in the same way as patients with chronic hepatitis C (CH-C) and elevated ALT levels.

In group B patients, the treatment guidelines recommend antiviral therapy for those who have moderate to severe liver fibrosis (F2–4). In our present study, patients with no fibrosis to mild fibrosis (F0–1) did not develop HCC, and in the patients with moderate to severe fibrosis (F2–4), the cumulative rate of HCC incidence tended to be lower in the SVR group than that in the NR group ($p = 0.071$). These results also indicate the appropriateness of the Japanese treatment guidelines. However, further study is needed because of the small number of cases studied here.

It appears that group A patients have time to wait for therapy with the next generation of direct antiviral agents (DAAs), such as Peg-IFN plus ribavirin plus a second-generation protease inhibitor, because none of the patients had developed HCC at 3 years. Even in group C, for which the treatment guidelines recommend antiviral therapy, there was no significant difference in the cumulative rate of HCC incidence among the SVR, relapse, and NR patients, with the incidence being below 5 % at 3 years. Accordingly, patients with PLT counts of more than $15 \times 10^4/mm^3$ (groups A or C) have time to wait until the next generation of DAAs becomes available, because patients with PLT counts of more than $15 \times 10^4/mm^3$ have a low 3-year carcinogenesis rate.

The Japanese treatment guidelines recommend antiviral therapy for patients with $30 < ALT \leq 40$ IU/L levels. However, in the present study, in the patients with $30 < ALT \leq 40$ IU/L levels, the p value for a significant difference in the cumulative rate of HCC incidence among the patients with SVR, relapse, and NR was 0.059. This result indicates that the patients with $30 < ALT \leq 40$ IU/L levels have the potential to be candidates for antiviral therapy, and further study is needed to clarify this. However, these patients may not be candidates for immediate antiviral therapy because the cumulative rates of HCC incidence at 3 years in the patients with SVR, relapse, and NR were low (cumulative rates of HCC at 3 years: 1.6, 0.0, and 3.7 %). On the other hand, as mentioned above, in the patients with PLT counts of $<15 \times 10^4/mm^3$, the cumulative rate of HCC incidence was significantly lower in the SVR and relapse patients than that in the NR patients (cumulative rates of HCC at 3 years: 0.0, 0.0, and 9.3 %; at 5 years: 0.0, 11.1, and 20.8 %; $p < 0.001$). This result suggests that patients with PLT counts of $<15 \times 10^4/mm^3$ may be candidates for antiviral therapy.

A limitation of this study was that the incidence of HCC was not compared between a treatment group and a non-treatment group. This study showed the suppressive effect of antiviral therapy on HCC incidence by comparing patients according to the treatment's antiviral effect. Peg-IFN plus ribavirin combination therapy has become acceptable for patients with chronic HCV infection and N-ALT levels. However, if there were no difference in HCC incidence between patients with SVR and non-SVR in the group receiving Peg-IFN plus ribavirin combination therapy, it would not be necessary for patients with chronic HCV infection and N-ALT to receive this therapy. In this study, we compared the incidence of HCC according to the treatment effect in HCV-infected patients with N-ALT levels categorized by the Japanese treatment guidelines. Indeed, although our results did not demonstrate that N-ALT patients should be treated, they indicated that it could be appropriate to treat N-ALT patients, because the incidence of HCC in these patients with SVR was suppressed compared with that in the NR patients.

In conclusion, in patients with N-ALT and PLT counts of $<15 \times 10^4/mm^3$ who received Peg-IFN plus ribavirin

combination therapy, the cumulative rate of HCC incidence was significantly lower in those with SVR or relapse than in those with NR. Therefore, HCV-infected patients with N-ALT and PLT counts of $<15 \times 10^4/mm^3$ could be candidates for early antiviral therapy for the purpose of reducing the risk of developing HCC.

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Lower incidence of hepatocellular carcinoma in patients with transient virologic response to peginterferon and ribavirin combination therapy: Is it really the effect of the therapy?

To the Editor:

Ogawa *et al.* reported interesting and important findings, based on a large prospective cohort, regarding the effect of combination therapy with peginterferon and ribavirin on the incidence of hepatocellular carcinoma (HCC) [1]. They reported lower incidence of HCC after treatment in patients with transient virological response (TVR, defined as relapse or breakthrough), as well as in patients with sustained virological response (SVR), relative to patients with non-virological response (NVR). The suppressive effect of this antiviral therapy on the development of HCC in patients with SVR has been established by several reports and can be explained by the eradication of hepatitis C virus (HCV), resulting in the release of inflammation and improvement of liver fibrosis [2]. However, it is unclear why the incidence of HCC after treatment was also lower in patients with TVR than in those with NVR, despite the persistence of viremia after treatment. Ogawa *et al.* attributed this observation to the preventive effect of complete HCV suppression during therapy on the development of HCC.

Previously reported viral and host factors that are strongly associated with response to antiviral therapy with peginterferon and ribavirin [3,4] may also be associated with the pathogenesis of HCC. Amino acid substitutions in the HCV core region, a viral factor reportedly associated with the response to peginterferon and ribavirin therapy in patients with HCV genotype 1b [3] (i.e., the vast majority of subjects in the study by Ogawa *et al.*), are also associated with the development of HCC [5]. Regarding host factors associated with the response to combination therapy [4], genetic polymorphisms near the *IL28B* gene are reportedly associated with hepatic steatosis [6] and interact with amino acid substitutions in the HCV core region [5,7]. Both hepatic steatosis and amino acid substitutions in the HCV core region are associated with the development of HCC [5,8]. In addition, amino acid substitutions in the HCV core region are reportedly associated with the development of HCC, even in patients who achieved SVR [9]. Ogawa *et al.* reported, without providing detailed data, a higher incidence of HCC in patients bearing the non-TT genotype of rs8099917 near the *IL28B* gene, which is unfavorable to response to the combination therapy; this observation is also consistent with our previous report [10].

These results suggest that differences in HCC incidence based on the outcome of antiviral combination therapy are mainly attributable to these viral and host factors. It is possible that Ogawa *et al.* simply classified patients based on the likelihood of developing HCC upon observing the response to the combination therapy (i.e., TVR and NVR). It would be interesting if the authors were to analyze the incidence of HCC in relation to the outcome of combination therapy based on these host and viral factors. In addition, it would be interesting to investigate genetic polymorphisms near the *IL28B* gene and amino acid substitutions at residue 70 of the HCV core region, in the 13 patients who developed HCC despite the achievement of SVR.

Given the existence of factors associated with both therapeutic response and incidence of HCC, one should be cautious in drawing the conclusion that lower incidence of HCC in patients with TVR, relative to those with NVR, actually reflects the "suppressive effect" of peginterferon and ribavirin combination therapy on hepatocarcinogenesis.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Letter to the Editor

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Glycoproteomic Discovery of Serological Biomarker Candidates for HCV/HBV Infection-Associated Liver Fibrosis and Hepatocellular Carcinoma

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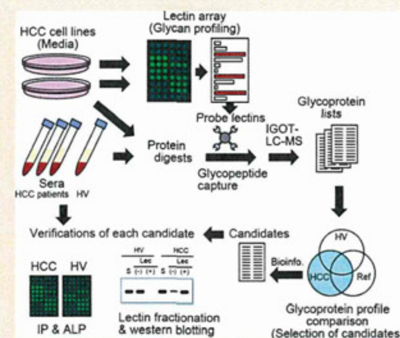
Supporting Information

ABSTRACT: We previously proposed a high-throughput strategy to discover serological biomarker candidates of cancer. This strategy focuses on a series of candidate glycoproteins that are specifically expressed in the original tissues (cells) of the target cancer and that carry glycan structures associated with carcinogenesis [Narimatsu, H., et al. *FEBS J.* **2010**, *277*(1), 95–105]. Here, we examined the effectiveness of our strategy in identifying biomarkers to assess progression of liver fibrosis and for the early detection of hepatocellular carcinoma (HCC). On the basis of the results of lectin array analyses in culture media of hepatoma cell lines, we captured glycopeptides carrying AAL-ligands (fucosylated glycans) or DSA-ligands (branched glycans) from digests of culture media proteins and sera from HCC patients with a background of liver cirrhosis (LC). Glycoproteins were identified by the IGOT-LC–MS method. In all, 21 candidates were selected from 744 AAL-bound glycoproteins for further verification according to (i) their abundance in serum, (ii) their specific expression in liver, and (iii) the availability of antibodies to the glycoproteins. All selected candidates showed enhancement of AAL-reactivity in sera of HCC patients compared with that of healthy volunteers (HV). These results indicate that our glycoproteomic strategy is effective for identifying multiple glyco-biomarker candidates in a high-throughput manner.



KEYWORDS: *glycoprotein, glycan alteration, hepatocellular carcinoma, liver fibrosis, biomarker, lectin array, glycoproteomics*

■ INTRODUCTION

In Japan, hepatocellular carcinoma (HCC) often develops in association with chronic liver diseases such as liver cirrhosis (LC) caused by persistent infection with hepatitis B or C virus followed by liver fibrogenesis.[1] The annual incidence of HCC increases with the degree of liver fibrosis and reaches 7–8% for the LC patients (fibrosis stage = F4).[2] Therefore, it is important to understand the degree of progression of liver fibrosis for patients with chronic hepatitis. This will enable identification of high-risk patients with progressed liver fibrosis, who should then undergo further detailed examinations such as computed tomography (CT) to detect evidence of early stage HCC. Presently, the progression of liver fibrosis is diagnosed by percutaneous biopsy; however, the method is both invasive and painful.[3,4] In addition, this method may result in sampling errors because the portion of the organ subjected to examination is very small.[5,6] These concerns prompted us to develop a serum biomarker to quantitatively evaluate the progression of liver fibrosis. Hence, we aimed to identify a marker that is applicable to a rapid, low cost, accurate, sensitive, and minimally invasive test. Furthermore, development of a serum HCC biomarker with increased sensitivity is required in order to diagnose early stage HCC at a size that is applicable for treatment such as radiofrequency ablation, thereby enhancing survival rates.[7,8]

Thus far, serological biomarkers have been screened by proteomic analyses using sera of HCC patients.[9,10] However, early stage cancer cells often express almost the same proteome as their originating cells because they have a common lineage. Hence, it is difficult to discover, verify, and validate practical serological marker candidates by comparing the levels of individual protein concentrations in serum between patients and healthy or disease controls. The problem can be seen by reference to a simple example. Suppose that the cancerous liver cells comprise 1% of the entire liver and secrete a 10-fold excess of a certain protein into the blood compared with the noncancerous cells. The blood concentration of such a protein in the cancer patient will be 1.1-fold that of the healthy individual.

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