

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.<sup>10</sup> 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.<sup>11</sup> 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.<sup>10</sup> 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.<sup>11</sup> 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.<sup>12</sup>

**Plasmid Construction, Viral Production, and Stable Transfectants.** The methods used in this section have been described.<sup>12</sup> 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

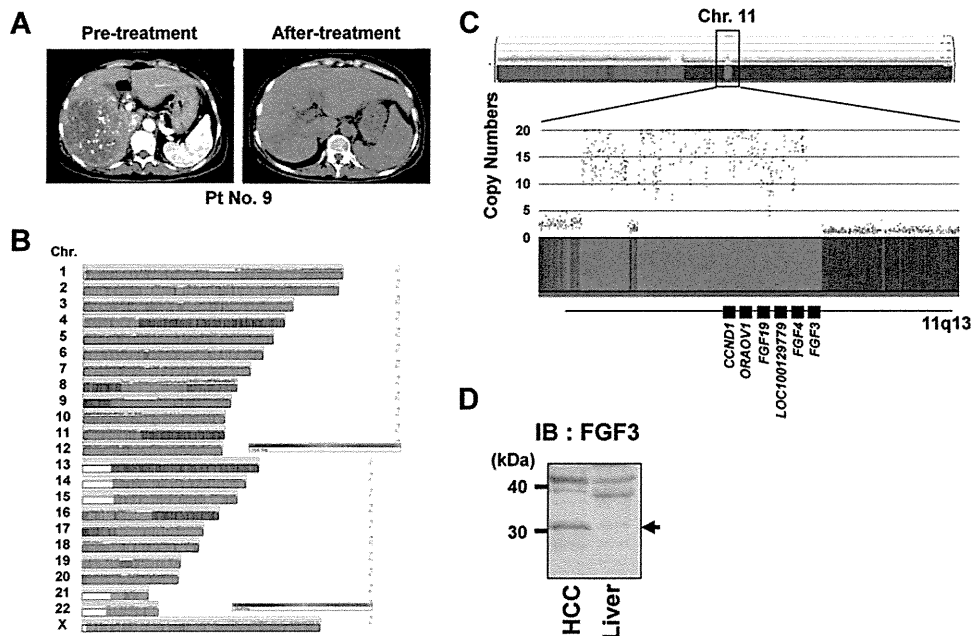


Fig. 1. HCC exhibiting a marked response to sorafenib treatment harbors *FGF3/FGF4* gene amplification. (A) Abdominal CT images obtained pretreatment (left panel) and 2 months after treatment (right panel). (B) CGH analysis of the tumor. Paired background liver tissue was used as a reference sample. 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  $5 \times 10^6$  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<sup>2</sup>  $\times$  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 amplicon 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).

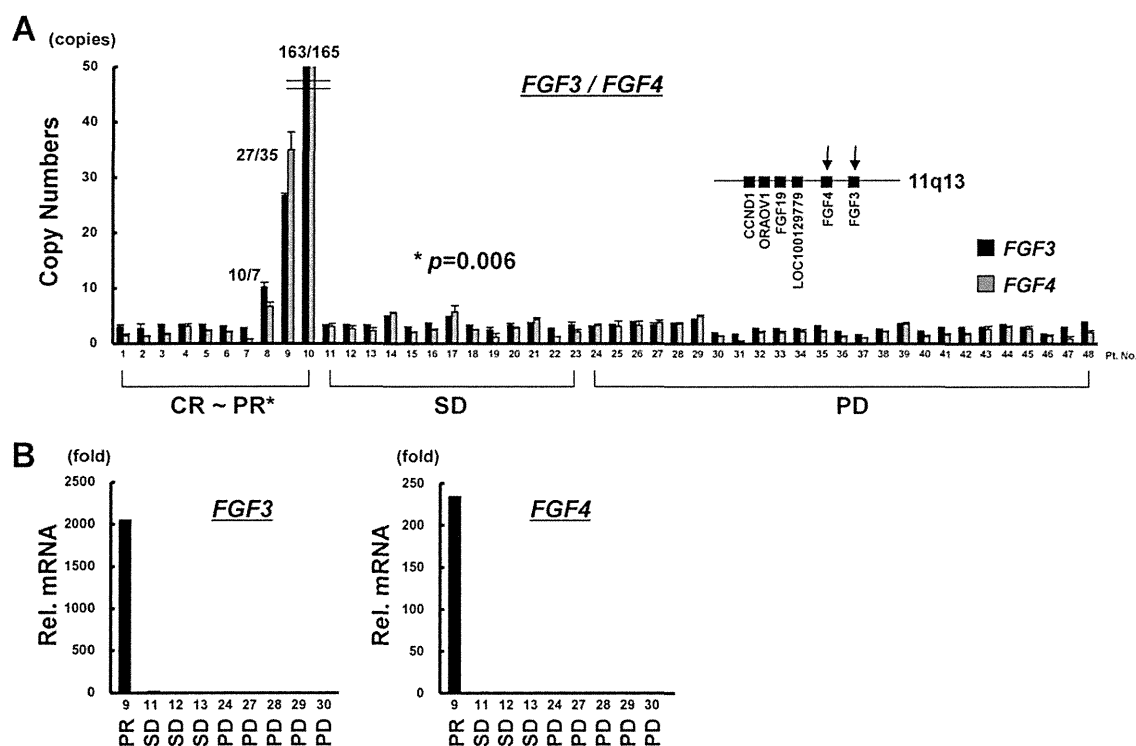


Fig. 2. *FGF3/FGF4* gene amplification is frequently observed in responders to sorafenib in HCC. (A) *FGF3/FGF4* gene amplification was determined using the TaqMan copy number assay in DNA samples obtained from 48 HCC samples that had been treated with sorafenib. *FGF3* amplification of  $>5$  copies 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.<sup>13</sup> 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.<sup>10</sup> 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*-

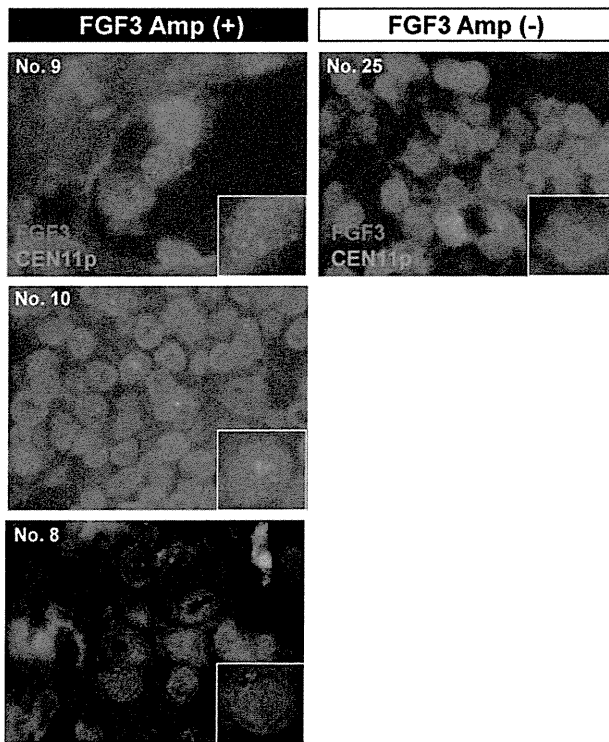


Fig. 3. FISH analysis of *FGF3*-amplified HCC. Patient numbers were indicated. Green staining indicates *CEN11P* loci; red staining indicates *FGF3* loci. High-power images are presented in each inset for a single cancer cell. Amp, gene amplification.

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

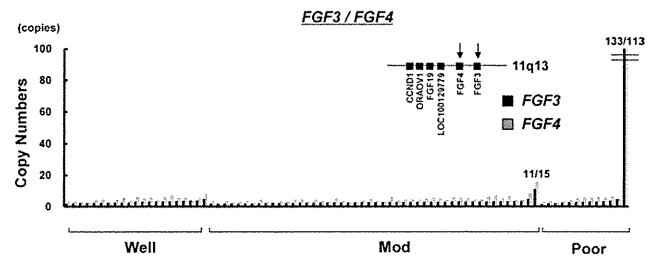


Fig. 4. *FGF3/FGF4* gene amplification in a series of HCC samples without sorafenib treatment. TaqMan copy number assay for *FGF3* and *FGF4* was used to examine DNA samples obtained from 82 surgical specimens. Human normal genomic DNA was used as a normal control. Well, well-differentiated HCC; Mod,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

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.

**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	<i>FGF3/FGF4</i> 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.<sup>14</sup> Sorafenib

potently inhibited cellular growth in these four cell lines at a sub- $\mu\text{M}$  50% inhibitory concentration ( $\text{IC}_{50}$ ) (Fig. 5A). The  $\text{IC}_{50}$  values were as follows: HSC43, 0.8  $\mu\text{M}$ ; HSC39, 0.6  $\mu\text{M}$ ; KATOIII, 0.4  $\mu\text{M}$ ; and KYSE220, 0.18  $\mu\text{M}$ . 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.<sup>15</sup> 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.<sup>16</sup> 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).<sup>13</sup> 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%.<sup>13</sup> On the other hand, the frequency of *FGF3/FGF4* amplification in HCC remains

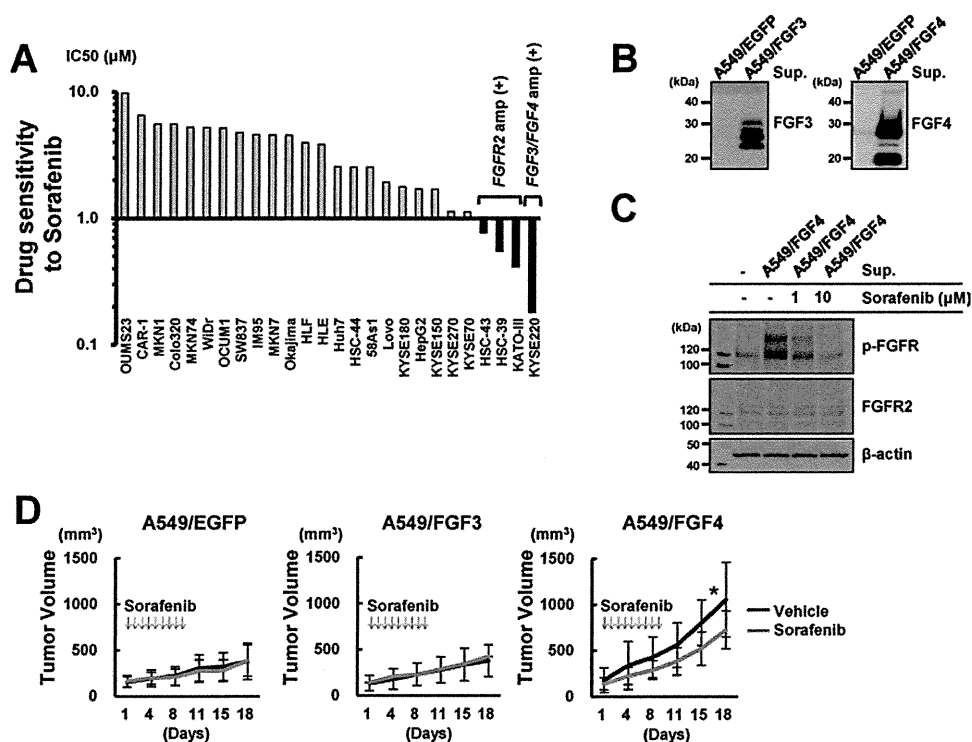


Fig. 5. FGF3 and FGF4 overexpression and drug sensitivity to sorafenib *in vitro* and *in vivo*. (A) Growth inhibitory assay examining sorafenib in various cancer cell lines *in vitro*. The growth inhibitory effect of sorafenib was examined using an MIT assay. The IC<sub>50</sub> values of each cell line are shown in the graph. The black bars show that the IC<sub>50</sub> 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,<sup>17</sup> and 3 out of 45 HCCs examined using Southern blot analysis had a copy number  $>5$ ;<sup>18</sup> meanwhile, amplification was not detected in 0 out of 42 surgically resected HCCs.<sup>19</sup> 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,<sup>14</sup> since sorafenib has a relatively weak but significant inhibitory effect on FGFR1 at a concentration of  $580 \pm 100$  nM.<sup>3</sup> 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.<sup>20</sup> 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.<sup>21</sup> 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.

## References

- Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, et al. Cancer statistics, 2005. *Ca Cancer J Clin* 2005;55:10-30.
- Yamamoto J, Kosuge T, Takayama T, Shimada K, Yamasaki S, Ozaki H, et al. Recurrence of hepatocellular carcinoma after surgery. *Br J Surg* 1996;83:1219-1222.
- Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* 2004;64:7099-7109.
- Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;359:378-390.
- Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, 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.
- So BJ, Bekaii-Saab T, Bloomston MA, Patel T. Complete clinical response of metastatic hepatocellular carcinoma to sorafenib in a patient with hemochromatosis: a case report. *J Hematol Oncol* 2008;1:18.
- Nakazawa T, Hidaka H, Shibuya A, Koizumi W. Rapid regression of advanced hepatocellular carcinoma associated with elevation of des-gamma-carboxyprothrombin after short-term treatment with sorafenib—a report of two cases. *Case Rep Oncol* 2010;3:298-303.
- Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497-1500.
- Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129-2139.
- Matsumoto K, Arao T, Hamaguchi T, Shimada Y, Kato K, Oda I, et al. FGFR2 gene amplification and clinicopathological features in gastric cancer. *Br J Cancer* 2012;14:727-732.
- Matsumoto K, Arao T, Tanaka K, Kaneda H, Kudo K, Fujita Y, et al. mTOR signal and hypoxia-inducible factor-1 alpha regulate CD133 expression in cancer cells. *Cancer Res* 2009;69:7160-7164.
- Kaneda H, Arao T, Tanaka K, Tamura D, Aomatsu K, Kudo K, et al. FOXQ1 is overexpressed in colorectal cancer and enhances tumorigenicity and tumor growth. *Cancer Res* 2010;70:2053-2063.
- Ormandy CJ, Musgrove EA, Hui R, Daly RJ, Sutherland RL. Cyclin D1, EMS1 and 11q13 amplification in breast cancer. *Breast Cancer Res Treat* 2003;78:323-335.
- Takeda M, Arao T, Yokote H, Komatsu T, Yanagihara K, Sasaki H, et al. AZD2171 shows potent antitumor activity against gastric cancer over-expressing fibroblast growth factor receptor 2/keratinocyte growth factor receptor. *Clin Cancer Res* 2007;13:3051-3057.
- Peters G, Brookes S, Smith R, Dickson C. Tumorigenesis by mouse mammary tumor virus: evidence for a common region for provirus integration in mammary tumors. *Cell* 1983;33:369-377.
- Sakamoto H, Mori M, Taira M, Yoshida T, Matsukawa S, Shimizu K, et al. Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa. *Proc Natl Acad Sci U S A* 1986;83:3997-4001.
- Takeo S, Arai H, Kusano N, Harada T, Furuya T, Kawauchi S, et al. Examination of oncogene amplification by genomic DNA microarray in hepatocellular carcinomas: comparison with comparative genomic hybridization analysis. *Cancer Genet Cytogenet* 2001;130:127-132.
- Nishida N, Fukuda Y, Komeda T, Kita R, Sando T, Furukawa M, et al. Amplification and overexpression of the cyclin D1 gene in aggressive human hepatocellular carcinoma. *Cancer Res* 1994;54:3107-3110.
- Chochi Y, Kawauchi S, Nakao M, Furuya T, Hashimoto K, Oga A, et al. A copy number gain of the 6p arm is linked with advanced hepatocellular carcinoma: an array-based comparative genomic hybridization study. *J Pathol* 2009;217:677-684.
- Ikai I, Arai S, Ichida T, Okita K, Omata M, Kojiro M, et al. Report of the 16th follow-up survey of primary liver cancer. *Hepatol Res* 2005;32:163-172.
- Uka K, Aikata H, Takaki S, Shirakawa H, Jeong SC, Yamashina K, et al. Clinical features and prognosis of patients with extrahepatic metastases from hepatocellular carcinoma. *World J Gastroenterol* 2007;13:414-420.

## FGF3/FGF4 amplification and multiple lung metastases in responders to sorafenib in hepatocellular carcinoma

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**Running Title:** Analysis of responders to sorafenib in HCC

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## Abstract

The response rate to sorafenib in hepatocellular carcinoma (HCC) is relatively low (0.7 – 3%), however, rapid and drastic tumor regression is occasionally observed. The molecular backgrounds and clinicopathological features of these responders remain largely unclear. We analyzed the clinical and molecular backgrounds of thirteen responders to sorafenib with significant tumor shrinkage in a retrospective study. A comparative genomic hybridization analysis using one frozen HCC sample from a responder demonstrated that the 11q13 region, a rare amplicon in HCC including the loci for *FGF3* and *FGF4*, was highly amplified. A real-time PCR-based copy number assay revealed that *FGF3/FGF4* amplification was observed in three of the ten HCC samples from responders in which DNA was evaluable, whereas amplification was not observed in 38 patients with stable or progressive disease ( $p=0.006$ ). A fluorescence in situ hybridization analysis confirmed *FGF3* amplification. In addition, the clinicopathological features showed that multiple lung metastases (5/13,  $p=0.006$ ) and a poorly differentiated histological type (5/13,  $p=0.13$ ) were frequently observed in responders. A growth inhibitory assay showed that only one *FGF3/FGF4*-amplified and three *FGFR2*-amplified cancer cell lines

exhibited hyper-sensitivity to sorafenib *in vitro*. Finally, an *in vivo* study revealed that treatment with a low dose of sorafenib was partially effective for stably and exogenously expressed *FGF4* tumors, while being less effective in tumors expressing *EGFP* or *FGF3*. *Conclusion: FGF3/FGF4* amplification was observed in around 2% of HCCs. Although the sample size was relatively small, *FGF3/FGF4* amplification, a poorly differentiated histological type, and multiple lung metastases were frequently observed in responders to sorafenib. Our findings may provide a novel insight into the molecular background of HCC and sorafenib responders, warranting further prospective biomarker studies.

## Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer-related 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.<sup>1-2</sup> Sorafenib (Nexavar; Bayer Healthcare Pharmaceuticals Inc.) is a small molecule kinase inhibitor that is classified as an anti-angiogenic inhibitor.<sup>3</sup> Sorafenib inhibits the kinase activities of Raf-1 and B-Raf in addition to vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptor  $\beta$  (PDGFR- $\beta$ ), 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.<sup>4-5</sup> 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 (PR) was observed in 0.7% (2/299) and 3.3% (5/150) of the patients treated with sorafenib.<sup>4-5</sup>

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.<sup>6-7</sup> 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 EGFR tyrosine kinase in non-small cell lung cancer are associated with a striking treatment response to gefitinib, a selective EGFR tyrosine kinase inhibitor.<sup>8-9</sup> 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.

## Materials and methods

### Reagent and cell culture

Sorafenib was provided by Bayer Healthcare Pharmaceuticals Inc. (Montville, NJ). All the cell lines used in this study were maintained in RPMI-1640 medium (Sigma, St. Louis, MO) except for IM95, OUMS23, Colo320, WiDr, HLF, HLE, Huh7, and HepG2 (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<sub>2</sub> 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 twelve medical centers were retrospectively evaluated. 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 (NE). One NE 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 (CR), a partial response (PR), stable disease (SD), progressive disease (PD) or NE. The clinicopathological features evaluated included age, sex, viral infection, alpha-fetoprotein (AFP) level, protein induced by vitamin K absence or antagonist-II (PIVKA-II), clinical stage, primary tumor size, metastatic lesion, histological type, 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 (CGH) analysis**

The Genome-wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA) was used to perform an array-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 PCR with a universal primer and TITANIUM Taq DNA Polymerase (Clontech, Palo Alto, 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 ver.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 pre-designed TaqMan Copy Number Assays according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) as previously described.<sup>10</sup> 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 non-cancerous FFPE tissue were used as a normal control.

### **Real-time reverse-transcription PCR (RT-PCR)**

The method was performed as described previously.<sup>11</sup> In brief, cDNA was prepared from the total RNA obtained from each surgical frozen section using a GeneAmp<sup>®</sup> 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, and 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*, forward 5'-TTT GGA GAT AAC GGC AGT GGA-3' and reverse 5'-CGT ATT ATA GCC CAG CTC GTG GA-3'; *FGF4*, forward 5'-GAG CAG CAA GGG CAA GCT CTA-3' and reverse 5'-ACC TTC ATG GTG GGC GAC A -3'; *GAPD*, forward 5'-GCA CCG TCA AGG CTG AGA AC-3' and reverse 5'-ATG GTG GTG AAG ACG CCA GT-3'. *GAPD* was used to normalize the expression levels in the subsequent quantitative analyses.

#### **Fluorescence *in situ* hybridization analysis**

The method of fluorescence *in situ* hybridization (FISH) was performed as described previously.<sup>10</sup> 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**

A western blot analysis was performed as described previously.<sup>11</sup> The following antibodies were used: monoclonal FGF3 (R&D Systems, Minneapolis, MN), FGF4 and FGFR2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and phospho-FGFR and HRP-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA). The 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 previously described.<sup>12</sup>

### **Plasmid construction, viral production and stable transfectants**

The methods used in this section have been previously described.<sup>12</sup> The cDNA fragment encoding human full-length *FGF3* or *FGF4* were isolated using PCR