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_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 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 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 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.[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 non-cancerous FFPE tissue were used as a normal control.

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

The method was performed as described previously.[11] In blief, cDNA 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: 95ºC for 5 minutes, and 50 cycles of 95ºC for 10 seconds and 60ºC 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.[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

A western blot analysis was performed as described previously.[11] 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.[12]

Plasmid construction, viral production and stable transfectants

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

and Prime STAR^{TM} HS DNA polymerase (TaKaRa, Otsu, Japan) with following primers: *FGF3*, forward 5'-GG GAA TTC GCC GCC ATG GGC CTA ATC TGG CTG CTA-3' and reverse 5'-CC CTC GAG GCC CAG CTA GTG CGC ACT GGC CTC'; *FGF4*, forward 5'-GG GAA TTC GCC GCC ATG TCG GGG CCC GGG ACG GCC GCG GTA GCG C-3' and reverse 5'-CC CTC GAG GGA GGG TCA CAG CCT GGG GAG GAA GTG GGT GAC CTT C-3'. 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 the *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 $5x10^6$ 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 pre-treatment tumor size among the three

groups (n=20 in each group). The mice were then treated with a low dose of sorafenib (n=10, 15 mg/kg/day, p.o.) or the vehicle control (n=10, Cremophor EL/ethanol/water, p.o.) for 9 days. The tumor volume was calculated as the length x width^2 x 0.5 and was assessed every 2-3 days.

Statistical analysis

The statistical analyses were performed to test for differences between groups using the Student *t*-test or Fisher exact test. A *p* value of < 0.05 was considered statistically significant. All the 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 female was diagnosed as having histologically confirmed advanced HCC (Pt. No. 9, Fig. 1A, left panel) with multiple lung metastases. The patient received combination treatment with sorafenib, 5FU and interferon, and a subsequent treatment assessment showed a partial response. Since the disease was well-controlled by sorafenib treatment for 14 months (Fig. 1A, right panel), surgery was performed. To characterize this tumor molecularly, we performed an 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 which contains several genes including *fibroblast growth factor 3* (*FGF3*), *FGF4*, *cyclin D1* (*CCND1*) and *FGF19*, was highly amplified over 20 copies (Fig 1C). A western blot analysis showed that FGF3 was overexpressed in the HCC specimen, compared with the paired background liver specimen (Fig. 1D).

The 11q13 locus is known to be a frequently amplified region in several human cancers except for 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 ten (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 ± 0.8/6.7 ± 0.8, 26.7 ± 0.4/35.1 ± 3.1 and 162.5 ± 9.0/165.0 ± 12.5 copies in the amplified samples, while the copy numbers of *FGF3* for all the others 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_ mRNA

We examined the 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 (Pt. No. 9) expressed extremely high mRNA levels of *FGF3/FGF4*, compared with non-amplified samples (Fig. 2B). The results demonstrated that *FGF3/FGF4* gene amplification mediates the overexpression of *FGF3/FGF4* mRNAs and proteins (Fig. 2B and 1D).

FISH analysis confirmed _FGF3/FGF4_ gene amplification

We used a FISH analysis to examine *FGF3/FGF4* amplification and to verify the results of the above PCR-based DNA copy number assay. All *FGF3/FGF4*-amplified clinical samples were confirmed as exhibiting high level *FGF3*-amplification using a FISH analysis (Fig. 3). One patient showed multiple scattered signals (Pt. No. 9), while two patients showed large clustered signals

(Pt. Nos. 10 and 8). Non-amplified HCC (Pt. No. 25) showed a negative result for gene amplification. These results clearly demonstrated the presence of *FGF3/FGF4*-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, while the other was a moderately differentiated tumor.

Clinicopathological features of responders to sorafenib

Detailed clinicopathological features of sorafenib-responders were shown in Table 1. Comparison of clinical factors including age, sex, viral status, AFP level, PIVKA-II, clinical stage, primary tumor size, metastatic status, histological type and tumor response between responders and non-responders was described in

Table 2. Notably, multiple lung metastases over five nodules was significantly higher among responders to sorafenib (responders, 5/13, 38%; non-responders, 2/42, 5%; p=0.006). Although the difference was not significant, poorly differentiated HCC tended to be more common among responders to sorafenib (responders, 5/13, 38%; non-responders, 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), while 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 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 (Figure 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 sorafenib (15 mg/kg/day, p.o.) or without sorafenib (vehicle control, p.o.). *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 (Figure 5D). Notably, the low-dose sorafenib treatment significantly inhibited the growth of the A549/FGF4 tumors, while it was not effective against A549/EGFP and A549/FGF3 tumors (Figure 5D). These results suggested that the 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 *cyclin D1* (*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 largely unclear. Relatively small cohort studies have shown that 1 out of 20 HCC exhibited *FGF3* amplification as determined using a CGH analysis,[17] and 3 out of 45 HCC examined using a Southern blot analysis had a copy number of over 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 HCCs.

Tumor shrinkage might be due to the mixed effect (sorafenib + 5FU + interferon) of combination therapy in the initially described patient (Pt. No. 9). However, during this patient's long clinical course, tumor regrowth was observed following withdrawal of sorafenib because of oral hemorrhage, and tumor re-shrinkage 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 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 non-responders (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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