naive patients with *EML4-ALK* who are treated with first-line platinum-based chemotherapy in a prospective cohort study. Our present study has shown that *EML4-ALK*-positive patients with advanced NSCLC manifest an aggressive clinical course similar to that of patients with wild-type tumors if the effective targeted therapy is not instituted. Our findings thus underline the importance of the development of ALK inhibitors for this molecularly defined population of NSCLC patients.

funding

This study was not supported by a sponsor or funding agency.

disclosure

The authors have declared no conflicts of interest.

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Cancer Science
The official journal of the Japanese Cancer Association

Clinical phase I study of elpamotide, a peptide vaccine for vascular endothelial growth factor receptor 2, in patients with advanced solid tumors

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(Received July 19, 2012/Revised August 22, 2012/Accepted August 28, 2012/Accepted manuscript online September 7, 2012/Article first published online October 14, 2012)

Targeting of tumor angiogenesis with vaccines is a potentially valuable approach to cancer treatment. Elpamotide is an immunogenic peptide derived from vascular endothelial growth factor receptor 2, which is expressed at a high level in vascular endothelial cells. We have now carried out a phase I study to evaluate safety, the maximum tolerated dose, and potential pharmacodynamic biomarkers for this vaccine. Ten HLA-A*24:02-positive patients with advanced refractory solid tumors received elpamotide s.c. at dose levels of 0.5, 1.0, or 2.0 mg once a week on a 28-day cycle. Five patients experienced an injection site reaction of grade 1 and 2, which was the most frequent adverse event. In the 1.0 mg cohort, one patient experienced proteinuria of grade 1 and another patient developed both hypertension and proteinuria of grade 1. No adverse events of grade 3 or higher were observed, and the maximum tolerated dose was therefore not achieved. The serum concentration of soluble vascular endothelial growth factor receptor 2 decreased significantly after elpamotide vaccination. Microarray analysis of gene expression in PBMCs indicated that several pathways related to T cell function and angiogenesis were affected by elpamotide vaccination, supporting the notion that this peptide induces an immune response that targets angiogenesis in the clinical setting. In conclusion, elpamotide is well tolerated and our biomarker analysis indicates that this anti-angiogenic vaccine is biologically active. Clinical trial registration no. UMIN000008336. (*Cancer Sci* 2012; 103: 2135–2138)

Angiogenesis, defined as the formation of new blood vessels from pre-existing vasculature, is essential for tumor growth and the spread of metastases.[1,2] Vascular endothelial growth factor (VEGF) is a pro-angiogenic molecule that plays a central role in angiogenesis, primarily through activation of VEGF receptor 2 (VEGFR2). Several approaches to the targeting of VEGF–VEGFR pathways, including those based on neutralizing antibodies to VEGF, small-molecule VEGFR tyrosine kinase inhibitors, and soluble VEGFR constructs (VEGF-Trap), are emerging as promising therapeutic options in clinical oncology.[3]

Vascular endothelial growth factor 2 has been a major target for anti-angiogenic therapy to date. Studies in mice have shown that tumor angiogenesis is inhibited as a result of cellular immune responses induced by vaccination with cDNA encoding mouse VEGFR2 or with a soluble fragment of the receptor.[4,5] On the basis of these findings, we have examined the possibility of developing a novel anti-angiogenic immunotherapy for cancer in the clinical setting. We previously identified peptide epitopes of human VEGFR2 and showed that CTLs induced by these peptides manifest potent and specific HLA class I-restricted cytotoxicity toward not only peptide-pulsed target cells but also endothelial cells expressing endogenous VEGFR2.[6] Furthermore, vaccination with peptides corresponding to these epitopes inhibited angiogenesis induced by tumor xenografts, resulting in marked suppression of tumor growth and prolongation of animal survival without the occurrence of fatal adverse events.[6]

We have now carried out a phase I clinical trial for treatment of HLA-A*24:02-positive patients with advanced refractory solid tumors by vaccination with the VEGFR2-169 peptide (elpamotide), which was previously shown to be the most effective among human VEGFR2 epitopic peptides tested for the ability to induce CTL precursors among PBMCs from cancer patients.[7] We examined the safety of this treatment as a primary endpoint, and the clinical and biological responses as secondary endpoints.

Patients and Methods

Patient eligibility. HLA-A*24:02-positive individuals aged ≥ 20 years with a histologically confirmed diagnosis of an advanced tumor refractory to standard therapy were included in the study if they had an Eastern Cooperative Oncology Group performance status of <2, a life expectancy of ≥ 3 months, and adequate or acceptable liver (serum bilirubin concentration of $\leq 2\times$ the upper limit of normal, and both aspartate aminotransferase and alanine aminotransferase levels in serum of $\leq 2.5\times$ the upper limit of normal) and bone marrow (absolute white blood cell count of $\geq 3000/mm^3$ and platelet count of $\geq 100\,000/mm^3$) function. Patients were excluded if they had symptomatic brain metastases, active bleeding, malignant ascites requiring drainage, or serious medical conditions such as uncontrolled hypertension, arrhythmia, or heart failure, or if they had been treated with an investigational drug within 4 weeks prior to study enrolment. Individuals were excluded if they had serious illness or concomitant non-oncological disease that was difficult to control by medication. All subjects received information about the nature and purpose of the study, and they provided written informed consent in accordance with institutional guidelines.

Study design. The study was designed as a single-center, open-label, dose-escalation phase I trial. The primary objective was to evaluate the tolerability-safety and dose-limiting toxicity (DLT) of elpamotide. Secondary objectives included determination of the maximum tolerated dose, preliminary assessment of antitumor activity and effects on peripheral blood biomarkers of angiogenesis in this patient population. The study was approved by the appropriate Institutional Review Board. Dose levels of elpamotide were 0.5, 1.0, and

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doi: 10.1111/cas.12014

2.0 mg per body injected s.c. once a week on a 28-day cycle. Intrapatient dose escalation was not permitted. If a patient experienced a drug-related DLT, the treatment with elpamotide was discontinued. The dose escalation–reduction scheme was based on the occurrence of drug-related DLTs within the first treatment cycle. If a DLT was not observed in any of the first three patients, the dose was escalated to the next level. If a DLT was observed in one of the first three patients, three additional patients were recruited to that dose level. If a DLT occurred in only one of the first six patients, dose escalation was permitted. If two or more of the six patients experienced a DLT, an independent data monitoring committee determined the dose escalation or reduction decision or stopped the recruitment of additional patients.

Safety and efficacy assessments. The safety and tolerability of elpamotide were assessed according to the Common Toxicity Criteria for Adverse Events version 3.0. A DLT was defined as a hematologic toxicity of grade 4 or a non-hematologic toxicity of grade 3 or 4. Objective tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors version 1.0.[8]

Circulating level of soluble VEGFR2. The concentration of soluble VEGFR2 (sVEGFR2) in serum was measured with ELISA (THERMOmax; Molecular Devices, Sunnyvale, CA, USA) before vaccination on day 1 and after OTS102 administration on days 8 and 29.

Microarray analysis. The PBMCs were isolated from 3 mL whole blood with the use of an Accuspin system (Sigma-Aldrich, St. Louis, MO, USA) and were then immediately suspended in an RNA stabilization solution (Isogen; Nippongene, Tokyo, Japan) and stored at −80°C. Total RNA was subsequently extracted from the cells and its quality checked as described previously.[9] The RNA was subjected to microarray analysis (Affymetrix, Santa Clara, CA, USA) as described.[10] Analysis of the microarray data was carried out with BRB ArrayTools software version 3.6.1 (http://linus.nci.nih.gov/BRB-ArrayTools.html) developed by R. Simon and A. Peng. In brief, a $\log_2$ transformation was applied to the raw data, and global normalization was used to calculate the median expression level over the entire array. Genes were excluded if the proportion of data missing or filtered out was >20%. Genes that passed the filtering criteria were then considered for further analysis. Pathway (gene set) analysis was carried out with the BRB ArrayTools software. The level of statistical significance was set at $P = 0.01$. A P-value was first computed for each gene, and the set of P-values was then summarized by LS and KS statistics. The gene set comparison tool analyzes 285 predefined BioCarta gene sets for differential expression among predefined classes (pre- *vs.* post-treatment).

Other statistical analysis. Serum sVEGFR2 levels at baseline (pretreatment) were compared with those on days 8 or 29 with Student's paired t-test. A P-value of <0.05 was considered statistically significant.

Results

Patient demographics. The characteristics of the 10 HLA-A*24:02-positive patients enrolled in the study are shown in Table 1. The patients included four with non-small-cell lung cancer, three with gastric cancer, two with colorectal cancer, and one with thyroid cancer, all of whom were refractory to standard therapy. Doses of elpamotide for the escalation protocol included 0.5, 1.0, and 2.0 mg. Nine patients completed the first cycle of four injections with elpamotide, with one patient at the dose level of 2.0 mg being withdrawn from the study after two doses of the vaccine because of disease progression. Five patients were subjected to further cycles of vaccination. The median duration of treatment was 58 days (range, 14–279 days), with a median of 8 (range, 2–33) elpamotide vaccinations.

Table 1. Characteristics of HLA-A*24:02-positive patients with advanced refractory solid tumors (n = 10) vaccinated with elpamotide

Characteristics	Peptide dose		
	0.5 mg (n = 3)	1.0 mg (n = 3)	2.0 mg (n = 4)
Median age (range), years	58 (58–65)	64 (58–70)	57 (30–84)
Male/female	1/2	1/2	3/1
Performance status (0/1)	1/2	0/3	0/4
Non-small-cell lung cancer	1	1	2
Gastric cancer	0	1	2
Colorectal cancer	1	1	0
Thyroid cancer	1	0	0

Safety. All 10 patients received at least one dose of the study treatment and were evaluated for safety (Table 2). No patient showed a toxicity of grade 3 or higher. Five patients (50%) (two in the 0.5 mg cohort, one in the 1.0 mg cohort, and two in the 2.0 mg cohort) developed immunologic reactions, erythema, or induration of grade 1 or 2 at injection sites. In the 1.0 mg cohort, one patient developed proteinuria of grade 1 and another developed both hypertension and proteinuria of grade 1. No DLT was thus observed in the trial.

Tumor response. Nine patients were evaluated for tumor response. Although no complete or partial response was observed, two patients had stable disease for at least two treatment cycles (56 days). A 58-year-old female patient with advanced thyroid cancer who had multiple metastases in her lungs and muscle achieved stable disease that persisted for >5 months after the 15th vaccination with elpamotide at 0.5 mg. A 70-year-old male with advanced gastric cancer had been treated with three prior chemotherapy regimens. Given that the tumor continued to grow despite chemotherapy, he was enrolled in the elpamotide 1.0 mg cohort. Tumor size as evaluated by computed tomography remained stable for 2 months after initiation of elpamotide treatment, with stable disease being declared after the eighth vaccination. The patient subsequently received another cycle of four vaccinations. During this third cycle of treatment, ascites was detected by computed tomography and progressive disease was declared.

Pathway analysis. To determine whether elpamotide vaccination induced systemic immunologic effects, we examined the gene expression profiles of PBMCs from all 10 patients before

Table 2. Summary of toxicities of grades (G) 1/2 or 3

Adverse events	Peptide dose						Total
	0.5 mg (n = 3)		1.0 mg (n = 3)		2.0 mg (n = 4)		
	G1/2	G3	G1/2	G3	G1/2	G3	
Reaction at injection site	2	0	1	0	2	0	5
Nasopharyngitis	1	0	0	0	1	0	2
Anorexia	1	0	0	0	1	0	2
Nausea	1	0	0	0	0	0	1
Vomiting	1	0	0	0	0	0	1
Diarrhea	0	0	0	0	1	0	1
Fatigue	0	0	1	0	0	0	1
Fever	0	0	0	0	1	0	1
Hypertension	0	0	1	0	0	0	1
Proteinuria	0	0	2	0	0	0	2

doi: 10.1111/cas.12014

vaccination, and on days 8 and 29 after the onset of vaccination. Pathway analysis of microarray data revealed that 17 pathways were selected from among 285 BioCarta pathways at the nominal significance level of $P = 0.01$ for the LS or KS permutation tests (Table 3). The pathways with the most differentially expressed genes included those related to angiogenesis and T cell function (Table 3), supporting the notion that elpamotide vaccination indeed affects angiogenesis and T cell activation in the clinical setting.

Serum level of sVEGFR2. The circulating level of sVEGFR2 was previously found to be reduced by other angiogenesis inhibitors that directly target VEGFR2. We determined the serum concentration of sVEGFR2 as a potential biomarker for elpamotide vaccination. The serum concentration of sVEGFR2 decreased significantly ($P = 0.026$) over the first 4 weeks of treatment (Fig. 1). The decrease in sVEGFR2 level tended to be larger at the higher dose levels of elpamotide, although this trend was not significant.

Table 3. Pathways with most differentially expressed genes between pre- and post-treatment among 285 BioCarta pathways

Pathway descriptions	No. of genes	LS *P*-value	KS *P*-value	Related pathways
VEGF, hypoxia, and angiogenesis	23	0.0018	0.0270	Angiogenesis
Hypoxia-inducible factor in the cardiovascular system	26	0.0055	0.0023	Angiogenesis
Role of nicotinic acetylcholine	13	0.0075	0.1485	N/A
Melanocyte development and pigmentation pathway	14	0.0093	0.1461	N/A
Transcription regulation by methyltransferase of *CARM1*	20	0.0097	0.0378	N/A
Classical complement pathway	6	0.0141	0.0018	N/A
Role of Tob in T cell activation	26	0.0146	0.0097	T cell
Lectin-induced complement pathway	6	0.0180	0.0018	N/A
Deregulation of CDK5 in Alzheimer's disease	20	0.0193	0.0054	N/A
IL-12- and Stat4-dependent signaling pathway in Th1 development	37	0.0254	0.0078	T cell
T cell receptor and CD3 complex	15	0.0283	0.0023	T cell
NOS2-dependent IL-12 pathway in NK cells	14	0.0350	0.0031	T cell
T cytotoxic cell surface molecules	28	0.0354	0.0072	T cell
T helper cell surface molecules	28	0.0354	0.0072	T cell
Role of MEF2D in T cell apoptosis	31	0.0358	0.0097	T cell
HIV-induced T cell apoptosis	24	0.0442	0.0012	T cell
ADP-ribosylation factor	38	0.0485	0.0087	N/A

CARM1, coactivator-associated arginine methyltransferase 1; IL, interleukin; NK, natural killer; Stat4, signal transducer and activator of transcription-4; N/A, not applicable; VEGF, vascular endothelial growth factor.

Discussion

The targeting of tumor angiogenesis with vaccines has potential advantages over such targeting of tumor cells directly in cancer therapy.[4–6,11] First, tumor endothelial cells are more accessible to the immune system than are tumor cells located at a distance from the vessels.[12] Tumor endothelial cells are thus readily accessed by lymphocytes in the bloodstream, and CTLs can directly damage endothelial cells without penetration into the tumor tissue. In addition, the lysis of even a small number of endothelial cells within the tumor vasculature may result in the disruption of vessel integrity, leading to inhibition of the growth of numerous tumor cells. Endothelial cells are thus a promising target for cancer immunotherapy. Second, the loss or downregulation of HLA molecules on tumor cells is thought to be a major reason for the limited clinical efficacy of vaccines that target tumor cells.[13–15] Given that such HLA loss has not been described for endothelial cells of newly formed tumor vessels, the development of vaccines that target vascular endothelial cells in tumor tissue may overcome the problem of immune-escape of tumor cells.

Vascular endothelial growth factor receptor 2 is a functional molecule associated with neovascularization and is highly expressed in newly-induced tumor vessels but not in normal vessels. The VEGFR2-169 peptide (elpamotide) derived from VEGFR2 has been previously characterized by induction of peptide-specific CTLs capable of killing VEGFR2-expressing human endothelial cells.[6,7] The present phase I study was carried out to examine the safety of elpamotide for HLA-A*24:02-positive patients with advanced tumors. Injection site reactions of grade 1 or 2 were the most frequent vaccine-related adverse events. Specific toxicities that have often been associated with anti-angiogenic treatment with antibodies to VEGF or VEGFR tyrosine kinase inhibitors include hyperten-

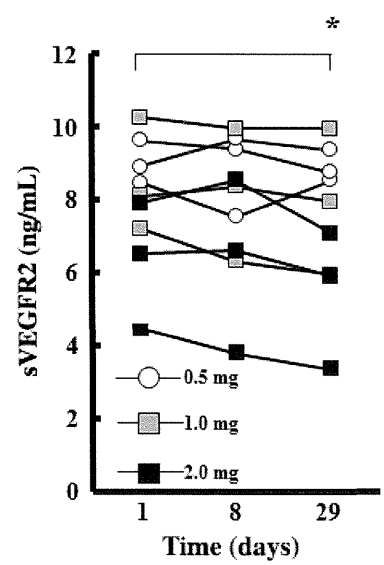

Fig. 1. Serum concentrations of soluble vascular endothelial growth factor receptor 2 (sVEGFR2) before and after elpamotide vaccination. Serum samples were collected at baseline (day 1) as well on days 8 and 29 for determination of sVEGFR2 concentration. *$P = 0.026$ (paired *t*-test) for comparison of the mean values for nine patients between days 1 and 29.

sion and proteinuria.[16,17] These toxicities occurred only at a low grade in two patients in the present study. No adverse events of grade 3 or higher were observed, indicating that elpamotide vaccination is safe and well tolerated.

Although *ex vivo* and *in vitro* studies have provided insight into the specific effects of peptide immunotherapy, they cannot substitute for studies carried out *in vivo*. To date, however, there has been no valid and widely accepted *in vivo* analysis to achieve proof of concept during clinical development of cancer vaccines. Microarray technology has allowed the identification of genes related to a given process in a hypothesis-free approach. The recent introduction of this technology to the field of cancer research has provided insights related to the more accurate classification of cancer, better definition of prognosis, and novel approaches to therapy. Microarray analysis has also proved to be a powerful tool for the identification and characterization of genes related to the ontogeny, differentiation, and activation of immune cells.[18] We have now applied such analysis to PBMCs obtained from patients in order to monitor the biological activity of elpamotide. To facilitate the interpretation of the enormous amount of microarray data, we examined gene sets related to biologically relevant pathways rather than individual genes. The results of our analysis indicate that several pathways related to T cell function and angiogenesis were significantly affected by a single treatment with elpamotide, supporting the notion that this peptide induces an immune response that targets angiogenesis. Our present study thus suggests that microarray analysis is a promising approach to achieving proof of concept during early clinical trials of cancer vaccines. We further explored if the changes in gene expression correlated with treatment response; however, definitive differences between responders (stable disease) ($n = 2$) and non-responders ($n = 7$) were not detected, perhaps due to small sample size. Further investigation to validate whether it will be useful for monitoring the treatment response is warranted.

Given that most angiogenesis inhibitors are cytostatic, it has been difficult to assess the biological effects of these agents in the early phase of clinical trials. Therefore, there is a need for validated biomarkers to monitor biological activity. The circulating level of sVEGFR2 was previously found to be reduced by other angiogenesis inhibitors that directly target VEGFR2,[19–21] although the mechanism underlying this consistent effect is not fully understood.[16,17] In the present study, serum sVEGFR2 concentrations showed a time-dependent decrease at all elpamotide dose levels studied, and this effect tended to be greater at the higher dose levels, suggesting that sVEGFR2 is a potential pharmacodynamic marker of drug exposure.

Inhibition of angiogenesis has provided new treatment avenues for cancer patients; however, there are no reliable biomarkers available to predict therapy response. Although tumor evaluation was not the primary objective of the present study, and the small sample size precludes any conclusions regarding treatment efficacy, the identification of predictive biomarkers to stratify cancer patients is vital to move this anti-angiogenic vaccine therapy forward. A randomized, controlled clinical trial of elpamotide for advanced cancer patients is being carried out in an effort to find such biomarkers.

In conclusion, elpamotide shows an acceptable safety profile for patients with advanced solid tumors. The preliminary evaluation of the biological activity of elpamotide with the use of microarray analysis as well as our serum marker (sVEGFR2) and disease stabilization data indicate that this agent is indeed biologically active.

Acknowledgments

We thank R. Simon and A. Peng for providing the BRB ArrayTools software.

Disclosure Statement

Takuya Tsunoda was employed in a leadership position by OncoTherapy Science (Tokyo, Japan). Kazuhiko Nakagawa received research funding for this study from OncoTherapy Science.

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doi: 10.1111/cas.12014

Expression changes in arrestin β 1 and genetic variation in catechol-O-methyltransferase are biomarkers for the response to morphine treatment in cancer patients

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Received November 1, 2011; Accepted December 5, 2011

DOI: 10.3892/or.2012.1660

Abstract. Genetic differences in individuals with regard to opioid-receptor signaling create clinical difficulties for opioid treatment; consequently, useful pharmacodynamic and predictive biomarkers are needed. In this prospective study, we studied gene expression changes in peripheral blood leukocytes using a microarray and real-time RT-PCR analysis to identify pharmacodynamic biomarkers for monitoring the effect of morphine in a cohort of opioid-treatment-naïve cancer patients. We also examined genetic variations in opioid receptor mu 1 (*OPRM1*, 118A ›G) and catechol-O-methyltransferase (*COMT*, 472G ›A) to evaluate predictive biomarkers of the treatment outcome of morphine. The plasma concentration of morphine was measured using a liquid chromatography-tandem mass spectrometry method. Microarray analysis revealed that the mRNA expression levels of arrestin β 1 (*ARRB1*) were significantly down-regulated by morphine treatment. Real-time RT-PCR analysis against independent samples confirmed the results (P=0.003) and changes during treatment were negatively correlated with the plasma morphine concentration (R=-0.42). No correlation was observed between the genotype of *OPRM1* and morphine treatment; however, the plasma concentration of morphine and the required dose of morphine were significantly lower for the A/A genotype of *COMT* (vs. A/G+G/G, P=0.008 and 0.03). We found that changes in the expression of *ARRB1* may be a novel pharmacodynamic biomarker and the *COMT* 472G ›A genotype may be a predictive biomarker of the response to morphine treatment.

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Key words: arrestin, catechol-O-methyltransferase, morphine, microarray, opioid receptor μ 1

Introduction

Pharmacogenetic, pharmacokinetic and pharmacodynamic variations among individuals result in a wide variety of responses to pain sensation and to analgesics; therefore, intensive investigations of biomarkers for opioid treatment have been performed to improve the effectiveness of morphine treatment (1).

The opioid receptors are G-protein coupled receptors (GPCRs), and three types of receptors μ, δ and κ-opioid receptors (*OPRM1*, *OPRD1* and *OPRK1*) are known to serve as receptors for morphine (2). Among them, *OPRM1* generated the main analgesic effect induced by morphine in a knock-out study performed in mice (3). Agonists for opioid receptors induce the activation of GPCRs, triggering the activation of various downstream molecules (2). A regulator of the G-protein signaling (RGS)-protein family negatively regulates opioid-receptor signaling by accelerating the deactivation of G proteins, and the regulators RGS2 and RGS9 are thought to be involved in resistance to morphine (4-6). In addition, G-protein coupled receptor kinase (GRK) phosphorylates the opioid receptors, leading to the binding of arrestin β 1 and 2 (ARRB1 and 2) to the opioid receptors (7). Thus, GRKs and ARRBs negatively regulate opioid-receptor signaling and are thought to be involved in resistance to morphine (8,9). To identify pharmacodynamic biomarkers that are capable

of monitoring the drug effect, we examined the gene expression changes in opioid signaling-related molecules using a microarray and real-time RT-PCR analysis in peripheral blood leucocytes (PBLs).

Meanwhile, genetic variants associated with varying pain sensitivity and responses to morphine are thought to be potential biomarkers for predicting the outcome of morphine treatment (1,10). In this study, we also evaluated two functional genetic variants, *OPRM1* 118A›G and catecholamine-O-methyltransferase gene (*COMT*) 472G›A (also known as Val158Met). The 118A›G variant of *OPRM1* leads to a change in amino acids at position 40, affecting a putative glycosylation site of the receptor and biologically altering receptor activity (1). The enzyme activity of *COMT* is genetically defined as high in G/G, intermediate in G/A and low in A/A, and its genotype is thought to be associated with the effect of opioid-signaling (11).

In this prospective study, we examined gene expression to explore possible pharmacodynamic biomarkers and to evaluate the use of functional genetic variants as predictive biomarkers of the response to morphine treatment in a cohort of opioid-treatment-naïve cancer patients.

Materials and methods

Patients and samples. This prospective study was conducted between 2009 and 2011 at the Kinki University Faculty of Medicine and Sakai Hospital, Kinki University Faculty of Medicine. Clinicopathological features including age, gender, ECOG performance status (PS) and type of primary malignant neoplasm were recorded. Morphine treatment was performed according to the standard method including titration (NCCN Guidelines™, Adult Cancer Pain). The required doses of morphine on Day 1 and on Day 8 are thought to be associated with the results of titration and the dose in the stationary phase, respectively.

PBL samples were obtained at baseline (pretreatment) and on Day 1 for the gene expression analyses. PBL samples for DNA were obtained at baseline. To measure the plasma concentrations of morphine, blood samples were collected on Days 1 and 8. The separated plasma were stocked at -80˚C until use. The present study was approved by the institutional review boards of both centers and written informed consent was obtained from all the patients.

RNA extraction. Each 2.5-ml whole blood sample was stored in a PAX gene Blood RNA tube (Qiagen, Hilden, Germany). RNA was extracted according to the manufacturer's protocol (Qiagen). Then, massively containing globin mRNA was removed using a biotinylated Globin-capture oligonucleotides-based method and the GLOBINclear™ kit (Ambion, Austin, TX). The quality and quantity of RNA obtained from these samples were verified using a NanoDrop2000 spectrophotometer (Cole-Parmer, Vernon Hills, IL).

Real-time reverse transcription PCR. The methods used in this section have been previously described (12). *GAPD* was used to normalize the expression levels in the subsequent quantitative analyses. The primers used for real-time RT-PCR were purchased from Takara (Otsu, Japan) as follows: *OPRM1* forward, 5'-TCA ATG TCT GCA ACT GGA TCC TC-3' and reverse, 5'-CAC TGG CAT AAT GAA GGC GAA G-3'; *OPRD1* forward, 5'-CTG GGC AAC GTG CTT GTC A-3' and reverse, 5'-CAT CAG GTA CTT GGC ACT CTG GAA-3'; *OPRK1* forward, 5'-CAC TTC ACG TGC TCT TAC AGC GTT A-3' and reverse, 5'-CCC TTG TGG GCA CAT ACA GCT AC-3'; *ARRB1* forward, 5'-GAG AAC GAG ACG CCA GTA GAT ACC A-3' and reverse, 5'-GGC GAG CAA AGT CCT CAA ATA CA-3'; *ARRB2* forward, 5'-ACC AAC CTG GCT TCC AGC A-3' and reverse, 5'-AAA GGC AGC TCC ACA GAG ACA TC-3'; *GRK5* forward, 5'-GGA GCT GAA CGT GTT TGG ACC TA-3' and reverse, 5'-AGC TGG GCG AAC TCT TGG AA-3'; *RGS9* forward, 5'-GCA CAA ACC CAC ATT TAC ATG CTC-3' and reverse, 5'-GCT TTG GCC AGC ATG TCC TTA-3'; *GAPD* forward, 5'-GCA CCG TCA AGG CTG AGA AC-3' and reverse, 5'-ATG GTG GTG AAG ACG CCA GT-3'.

Microarray analysis. The microarray procedure was performed according to the Affymetrix protocols (Santa Clara, CA), as described previously (13). Briefly, cRNA was synthesized using the GeneChip® 3'-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix). The labeled cRNAs were then purified and used for the construction of the probes. Hybridization was performed using the Affymetrix Gene Chip HG-U133 Plus 2.0 array for 16 h at 45˚C. The signal intensities were measured using a GeneChip® Scanner 3000 (Affymetrix) and converted to numerical data using GeneChip Operating Software, Ver. 1 (Affymetrix).

Genotyping. The genotype was evaluated for *OPRM1* 118A›G (rs1799971, p.Asn40Asp) and *COMT* 472G›A (rs4680, p.Val158Met). Genomic DNA isolated from blood samples using a QIAamp® DNA Blood Mini Kit (Qiagen) were amplified with the following primers: for *OPRM1* forward, 5'-AAG TCT CGG TGC TCC TGG CTA CC-3' and reverse, 5'-GTT TCC GAA GAG CCC CAC CAC GC-3'; and for *COMT* forward, 5'-GAT TCA GGA GCA CCA GCC CTC C-3' and reverse (intronic), 5'-CAC TGA GGG GCC TGG TGA TAG TG-3'. Each PCR reaction was performed in a 20-μl volume containing 20 ng of template, 0.5 μM of each primer, Ampdirect Plus (Shimadzu Corp., Kyoto, Japan) and 0.5 units of NovaTaq™ DNA Polymerase (Merck, Darmstadt, Germany). The amplification was performed for 35 cycles (95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 45 sec). The resulting PCR fragments consisting of 320 bp (*OPRM1*) and 210 bp (*COMT*) were directly sequenced with the corresponding forward and reverse primers, respectively.

Measurement of plasma concentration of morphine. The plasma concentration of morphine was measured using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. Morphine was purchased from Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Imipramine, an internal standard (IS), was obtained from Sigma-Aldrich (St. Louis, MO, USA). Pretreatment of the plasma samples was performed using protein precipitation. Briefly, 100 μl of plasma was mixed with 250 μl of IS solution (1 ng/ml imipramine in methanol). After vortexing (30 sec) and centrifugation (13,000 rpm, 5 min), the supernatant was directly analyzed using an autosampler. An

Table I. Clinical characteristics of study patients.

Characteristics	No. of patients
Age (years)	
<65	14
≥65	34
Gender	
Male	25
Female	23
PS	
0-2	32
3-4	16
Tumor types	
Lung cancer	20
CRC	8
Gastric cancer	5
Others	15
Required dose of morphine	
Day 1	
20 mg	3
30 mg	36
60 mg	6
90 mg	2
NE	1
Day 8	
20 mg	5
30 mg	20
40 mg	1
60 mg	9
90 mg	1
120 mg	1
NE	11

PS, performance status; CRC, colorectal cancer; Others, other primary tumor types; NE, not evaluated.

LC-MS/MS device was equipped with an Acquity UPLC (Ultra Performance LC) system and a Xevo TQ MS (Waters, Milford, MA, USA). Chromatographic separations were obtained under gradient conditions using an ACQUITY UPLC BEH C18 Column (100 mm x 2.1 mm ID, 1.7-μm particle size; Waters). The mobile phase consisted of eluent A (10 mmol/l ammonium formate) and eluent B (methanol). The flow rate was 0.5 ml/min and the gradient was from 2 to 60% B in 3.5 min, then an increase to 98% B in 0.5 min, holding at 98% B for 1 min and resetting to the initial conditions. The total run time was 8.5 min per sample. The column temperature was 45˚C, the sample temperature was 10˚C and the injection volume was 5 μl. The retention times of morphine and imipramine (IS) were 1.69 and 4.31 min, respectively. The mass spectrometer was operated in a positive electrospray mode. The capillary voltage was 0.5 kV and the desolvation temperature was 500˚C. The multiple reaction monitoring mode detected morphine and imipramine (IS) as follows: transitions, 286.4→152.3 and 281.2→86.0; cone voltages, 42 and 28 V; collision energies, 48 and 16 V, respectively. The chromatographic data were acquired and analyzed using MassLynx software, equipped with QuanLynx (Waters). Standard curves were prepared for a concentration range of 0.5-50 ng/ml for morphine. The inter- and intra-day variabilities in precision (expressed as the coefficient of variation) for morphine ranged from 4.2 to 7.7% and from 4.4 to 4.7%, respectively. The average accuracies for morphine were between 100.4 and 106.1%.

Statistical analysis. Differences between groups were analyzed using the Student's t-test or the Fisher's exact test. A P-value of <0.05 was considered statistically significant. All analyses were performed using JMP (SAS Institute, Cary, NC). A microarray analysis was performed using BRB-Array Tools software, Ver. 3.6.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html), as described previously (13,14).

Results

Patient results. A total of 48 patients with opioid-treatment naïve and histologically confirmed malignant neoplasms who were scheduled to undergo opioid treatment were evaluated in this study (Fig. 1A). All 48 patients and a total of 96 samples (baseline and Day 1) were evaluated in the gene expression analysis. Forty-one patients and samples were evaluated in the genotype analysis because the DNA samples were insufficient in 7 cases. The plasma concentration of morphine was determined for 47 samples on Day 1 and for 43 samples on Day 8.

The patient characteristics are summarized in Table I. The median age was 69 years (40-85 years); 25 patients were men and 23 patients were women. Sixty-seven percent of the patients had a PS of 0-2 and 42% had advanced lung cancer. The other primary tumors were 8 colorectal cancers, 5 gastric cancers, 4 unknown primary cancers, 2 pancreas cancers, 2 breast cancers, 2 gallbladder cancers, 1 renal cell carcinoma, 1 bladder cancer, 1 malignant lymphoma, 1 malignant pheochromocytoma and 1 skin cancer. The median required dose of morphine on Day 1 was 30 mg (20-90 mg), while that on Day 8 was 30 mg (20-120 mg).

Down-regulation of ARRB1 mRNA expression and morphine treatment. To identify pharmacodynamic biomarkers for monitoring the effect of morphine, we examined changes in gene expression during morphine treatment (baseline vs. Day 1) using a microarray analysis for 20 samples from 10 cases and validated the results using real-time RT-PCR for 76 samples from 38 cases, focusing on opioid receptor signaling. A schema for opioid receptor signaling is shown (Fig. 1B). The microarray analysis revealed that the mRNA expression levels of *ARRB1* and *GRK5* were significantly down-regulated by morphine treatment (P=0.01 and 0.001, Table II). Interestingly, down-regulated genes including *ARRB1*, *GRK5* and *RGS9* (P=0.054) are known as negative regulators of opioid receptor signaling. The gene expressions of the opioid receptors were not changed. To confirm these results, we examined the gene expressions of these genes including *OPRM1*, *OPRD1*,

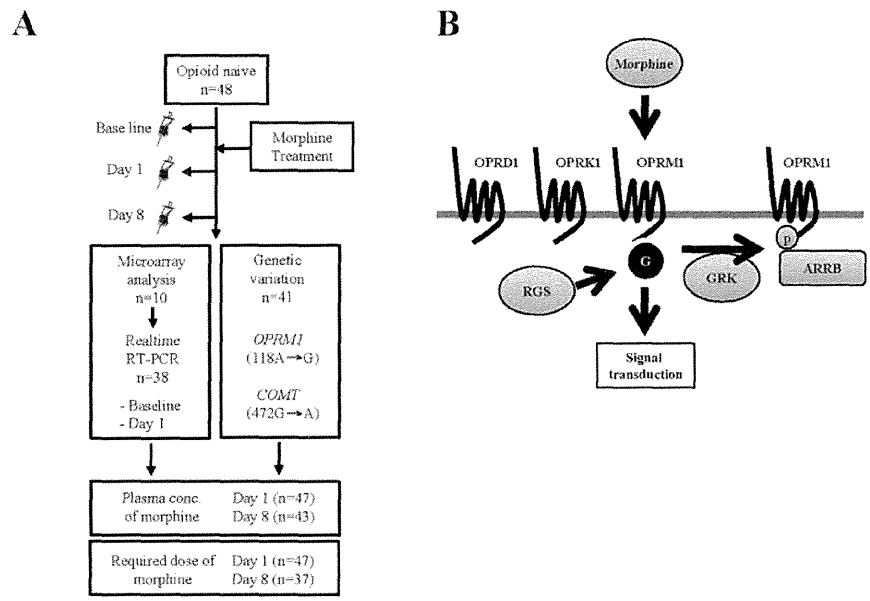

Figure 1. Schemas of study design and opioid receptor signaling. (A) Flow diagram of study. (B) Schema of opioid receptor signaling. OPRM1, D1 and K1 represent opioid receptor μ 1, δ 1 and κ 1, respectively; ARRB1 and 2 represent arrestin β 1 and 2; GRK, G protein-coupled receptor kinase; RGS, regulator of G-protein signaling; G, G protein; p, phosphorylation.

Table II. Gene expression changes in opioid signal-related molecules during morphine treatment.

		Microarray (n=10)			Real-time RT-PCR (n=38)		
Symbol	Name	Base line	Day 1	P-value[a]	Base line	Day 1	P-value[a]
OPRM1	Opioid receptor, μ 1	10.8±1.6	10.9±1.4	0.83	0.3±0.9	1.4±7.9	0.40
OPRD1	Opioid receptor, δ 1	11.3±4.2	10.0±0.0	0.37	0.2±0.4	0.3±1.4	0.46
OPRK1	Opioid receptor, κ 1	13.7±4.4	12.6±3.5	0.31	0.2±0.5	0.2±0.3	0.68
ARRB1	Arrestin β 1	123.7±40.2	101.0±30.9	0.01[b]	48.6±18.1	41.8±17.0	0.003[b]
ARRB2	Arrestin β 2	1193.5±476.5	1158.3±317.6	0.77	416.6±177.4	430.9±164.3	0.56
GRK1	G protein-coupled receptor kinase 1	10.3±1.3	11.3±2.7	0.36	ND	ND	ND
GRK4	G protein-coupled receptor kinase 4	11.2±3.6	10.9±2.5	0.82	ND	ND	ND
GRK5	G protein-coupled receptor kinase 5	419.6±121.1	346.7±137.4	0.001[b]	14.9±6.9	13.8±6.1	0.12
GRK6	G protein-coupled receptor kinase 6	464.4±87.5	457.3±120.8	0.87	ND	ND	ND
RGS2	Regulator of G-protein signaling 2	5776.5±1845.2	5872.8±1847.0	0.83	ND	ND	ND
RGS9	Regulator of G-protein signaling 9	26.7±16.5	17.6±7.9	0.05	2.8±2.4	2.5±1.7	0.17

Gene expression changes were examined using microarray and real-time RT-PCR. Peripheral blood leukocytes sampled during morphine treatment at baseline (pretreatment) and Day 1 (after treatment) were used for the analysis. [a]Comparisons between baseline vs. Day 1. The P-values were calculated using a t-test. [b]$P<0.05$. Data are shown as the average ± standard deviation.

OPRK1, *ARRB1*, *ARRB2*, *GRK5* and *RGS9* using real-time RT-PCR in 38 independent cases. The mRNA expression level of *ARRB1* was significantly and reproducibly down-regulated by morphine treatment (P=0.003, Table II and Fig. 2A). This result strongly suggests that *ARRB1* may be a promising and pharmacodynamic biomarker of morphine.

Next, we evaluated whether the down-regulation of *ARRB1* was correlated with the plasma concentration of morphine or the required dose. A moderate and weak inversed correlation was observed between the down-regulation of *ARRB1* and the plasma concentration of morphine (R=-0.42, Fig. 2B) or the required dose of morphine (R=-0.19, Fig. 2C). The results suggest that a higher plasma concentration or a higher dose of morphine induces the significant down-regulation of *ARRB1* and the change in *ARRB1* expression may be useful as a monitoring marker for morphine, although further studies are necessary.

COMT genotype is involved in outcome of morphine treatment. To find predictive biomarkers of the treatment outcome of morphine, we performed a functional genotype analysis

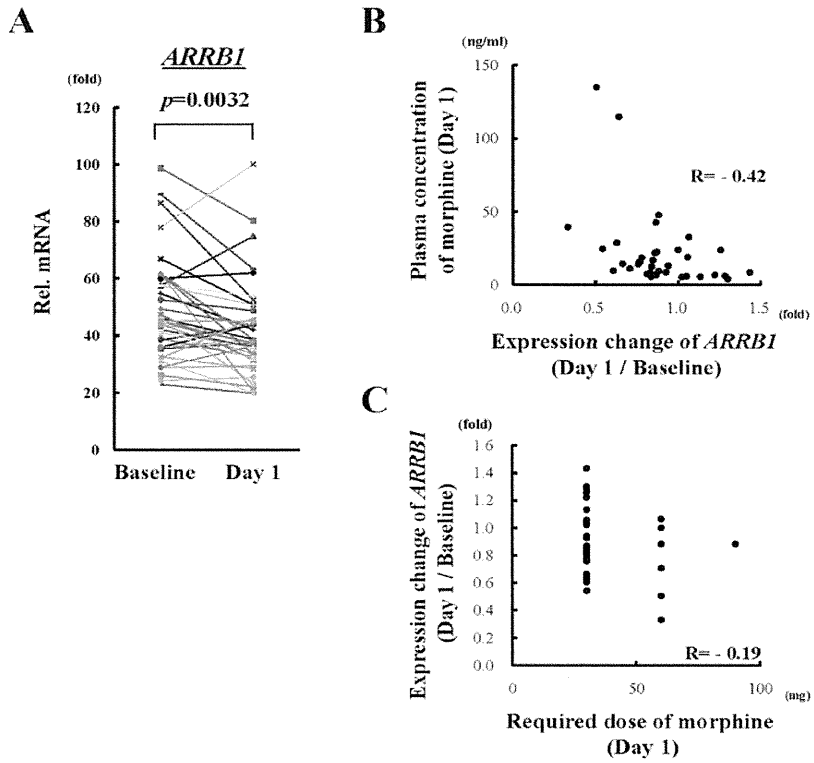

Figure 2. Down-regulation of arrestin β 1 (*ARRB1*) and outcome of morphine treatment. (A) A real-time RT-PCR analysis of peripheral blood leukocytes obtained at baseline (pretreatment) and on Day 1 showed that the mRNA level of *ARRB1* was significantly down-regulated during morphine treatment. Rel mRNA, normalized mRNA expression levels (*ARRB1/GAPD*x10^6). (B) Expression changes in *ARRB1* and plasma concentration of morphine treatment on Day 1. R, correlation coefficient. (C) Expression changes of *ARRB1* and required dose of morphine on Day 1.

Table III. Genotypes and treatment outcome of morphine.

		OPRM1 118A ›G				*COMT* 472G ›A			
Symbol	Days	A/A	A/G	G/G	P-value[a]	G/G	G/A	A/A	P-value[b]
Plasma concentration	D1	26.1±36.1	21.0±23.2	15.1±18.3	0.45	34.1±35.7	11.9±6.2	8.7±4.0	0.008[c]
of morphine (ng/ml)	D8	28.0±20.0	29.6±25.2	28.1±22.2	0.94	33.0±21.2	23.1±23.2	36.7±26.3	0.56
Required dose of	D1	43.1±23.9	32.2±9.0	34.0±15.2	0.78	43.7±21.4	28.9±3.2	30.0±0.0	0.03[c]
morphine (mg)	D8	38.9±16.2	37.1±15.4	36.0±15.2	0.82	40.0±15.4	34.6±15.1	40.0±17.3	0.81

Genotypes were evaluated for *OPRM1* 118A ›G (rs1799971, p.Asn40Asp) and *COMT* 472G ›A (rs4680, p.Val158Met). The treatment outcome of morphine was examined using the plasma concentration of morphine (Days 1 and 8) and the required dose of morphine (Days 1 and 8). [a]Comparisons between G/G vs. A/A+A/G of the *OPRM1*. [b]Comparisons between A/A vs. G/G+G/A of *COMT*. The P-values were calculated using a t-test. [c]P<0.05. Data are shown as the average ± standard deviation.

of *OPRM1* 118A ›G (rs1799971, p.Asn40Asp) and *COMT* 472G ›A (rs4680, p.Val158Met). The treatment outcome of morphine was examined based on the plasma concentration of morphine (Days 1 and 8) and the required dose (Days 1 and 8) according to genotype. No correlation was observed between the *OPRM1* 118A ›G genotype and the plasma concentration or the required dose of morphine (Table III). However, the plasma morphine concentration on Day 1 was significantly lower in patients with the A/A genotype of COMT, compared with those with the A/G+G/G genotypes (A/A: n=4, 8.7±4.0 ng/ml; G/A: n=18, 11.9±6.2 ng/ml; G/G: n=19, 34.1±35.7 ng/ml; P=0.008, Fig. 3A). In addition, the required dose of morphine on Day 1 was also significantly lower for the A/A genotype of *COMT*, compared with the A/G+G/G genotypes (A/A, 30.0±0.0 mg; G/A, 28.9±3.2 mg; G/G, 43.7±21.4 mg; P=0.03, Fig. 3B). On the other hand, the genotype was not correlated with the treatment outcome of morphine on Day 8. Collectively, our results indicate that the *COMT* genotype is involved in the outcome of morphine treatment, suggesting that it may be useful as a predictive biomarker for morphine treatment.

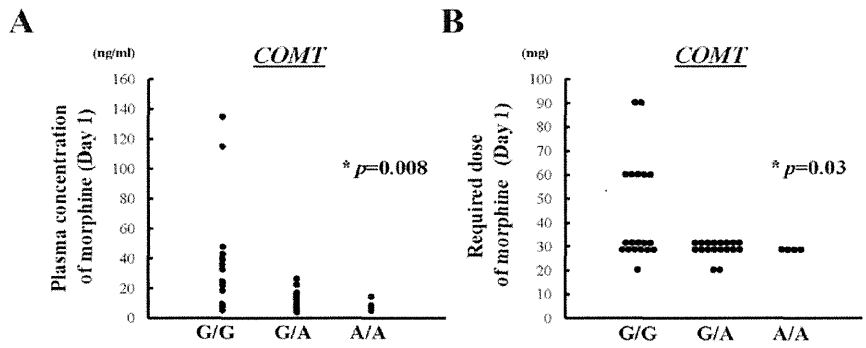

Figure 3. Catechol-O-methyltransferase genotypes (*COMT* 472G ›A, rs4680, p.Val158Met) and outcome of morphine treatment. (A) Genotypes and plasma concentration of morphine treatment on Day 1. *Comparisons between A/A vs. G/G+G/A of *COMT*. (B) Genotypes and required dose of morphine on Day 1.

Discussion

Morphine activates opioid receptor signaling in the cells of the central nervous system (CNS). Unlike easily available tissues, such as blood cells, these CNS cells cannot be sampled and used for analysis; therefore, clinically useful pharmacodynamic biomarkers of morphine have remained largely unclear to date. We recently described an approach examining PBLs as surrogate tissues to evaluate drug response and found that it is a feasible, non-invasive and repeatable pharmacodynamic approach in clinical settings (15). In this study, we found that *ARRB1* mRNA expression is a reproducible and useful biomarker for monitoring the effects of morphine treatment using PBLs as surrogate tissues.

ARRB1 regulates the desensitization of numerous GPCRs including OPRM1, D1 and D2 dopamine receptors and emerging evidence has demonstrated that ARRB1 functions as a scaffold protein that links GPCRs to intracellular signaling, such as MAPK and as a transcription factor that translocates to the nucleus (16,17). A recent study showed that chronic morphine treatment blocked the agonist-induced redistribution of ARRB1 in stably *OPRM1*-transfected HEK293 cells through the persistent stimulation of MAPK activity and the authors concluded that chronic morphine treatment produces adaptational changes at the *ARRB1* level (18). These observations and our findings suggest that the drug response of PBLs to morphine mediates the down-regulation of *ARRB1* expression during morphine treatment and reflects the overall cellular response to opioid signaling in an individual.

COMT is one of the enzymes that inactivate catecholamines; therefore, it is regarded as key regulator of adrenergic, noradrenergic and dopaminergic signaling (19). Various diseases are thought to be involved in COMT function including mental disorders, suicidal behavior and personality traits, and tardive dyskinesia (11,21-23). Regarding the COMT genotype as it relates to cancer pain, individuals with a A/A (Met/Met) genotype had a lower regional opioid signal response to pain and a higher sensitivity to pain, compared with heterozygous individuals (24). On the other hand, several clinical studies have demonstrated that the required dose of morphine was lower in subjects with an A/A genotype of *COMT*, compared with others (25-27). These results are consistent with our result. The question why the required dose of morphine is lower in patients with an A/A genotype, even though they are more sensitive to pain, can be explained by a possibly elevated density of OPRM1 in patients with the A/A genotype (28). Our results indicate that *COMT* 472G ›A may be a predictive biomarker, although further studies are necessary.

Taken together, our results may provide novel insights into the relations between morphine treatment and *ARRB1* expression and the *COMT* 472G ›A genotype.

Acknowledgements

This study was supported by the Third-Term Comprehensive 10-Year Strategy for Cancer Control and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare (H22-037). We thank Mrs. Tomoko Kitayama, Mrs. Erina Hatashita, Mrs. Kiyoko Kuwata, Mrs. Haruka Yamaguchi, Mr. Hiromasa Wadaguri and Mrs. Akiko Mizumoto for their technical assistance. We also thank Dr Richard Simon and Dr Amy Peng for providing us with the BRB ArrayTools software.

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HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

FGF3/FGF4 Amplification and Multiple Lung Metastases in Responders to Sorafenib in Hepatocellular Carcinoma

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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 clinico-pathological features of these responders remain largely unclear. We analyzed the clinical and molecular backgrounds of 13 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 polymerase chain reaction–based copy number assay revealed that *FGF3/FGF4* amplification was observed in three of the 10 HCC samples from responders in which DNA was evaluable, whereas amplification was not observed in 38 patients with stable or progressive disease ($P = 0.006$). Fluorescence *in situ* hybridization analysis confirmed *FGF3* amplification. In addition, the clinico-pathological 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 hypersensitivity 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. (HEPATOLOGY 2013;57:1407-1415)

Abbreviations: 5FU, 5-fluorouracil; CGH, comparative genomic hybridization; DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FFPE, formalin-fixed, paraffin-embedded; FISH, fluorescence in situ *hybridization; HCC, hepatocellular carcinoma; IC_{50}, 50% inhibitory concentration; mRNA, messenger RNA; PCR, polymerase chain reaction; PIVKA-II, protein induced by vitamin K absence or antagonist-II; RPMI-1640, Roswell Park Memorial Institute 1640; RT-PCR, reverse-transcription PCR.*

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Received October 7, 2011; accepted June 25, 2012.

Supported by the Third-Term Comprehensive 10-Year Strategy for Cancer Control (K. N.), a National Cancer Center Research and Development Fund (H22-015, to M. K.), and a Grant-in-Aid for Scientific Research (23650627, to K. N.).

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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, treatment response, and duration of sorafenib

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DOI 10.1002/hep.25956

Potential conflict of interest: Nothing to report.

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

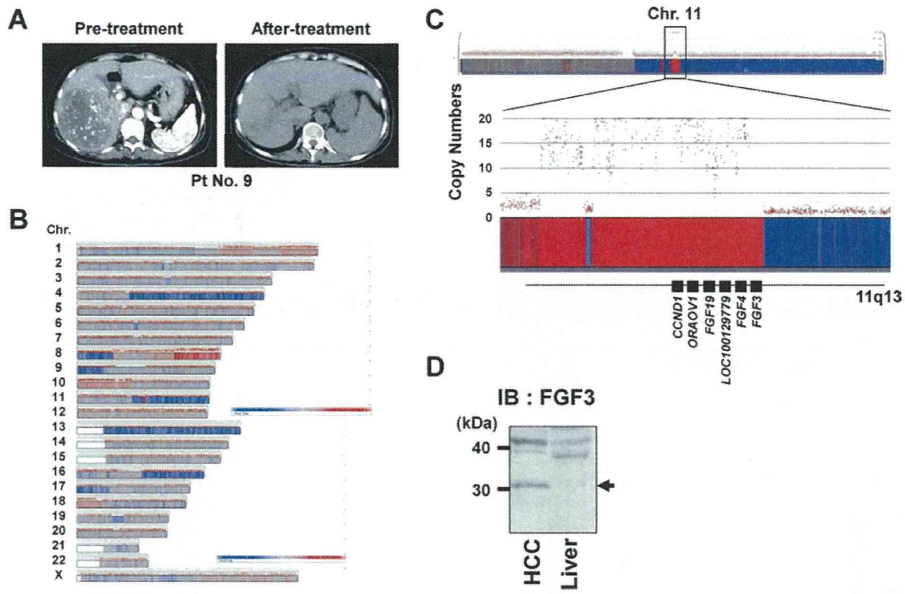

Fig. 1. HCC exhibiting a marked response to sorafenib treatment harbors *FGF3/FGF4* gene amplification. (A) Abdominal CT images obtained pretreatment (left panel) and 2 months after treatment (right panel). (B) CGH analysis of the tumor. Paired background liver tissue was used as a reference sample.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

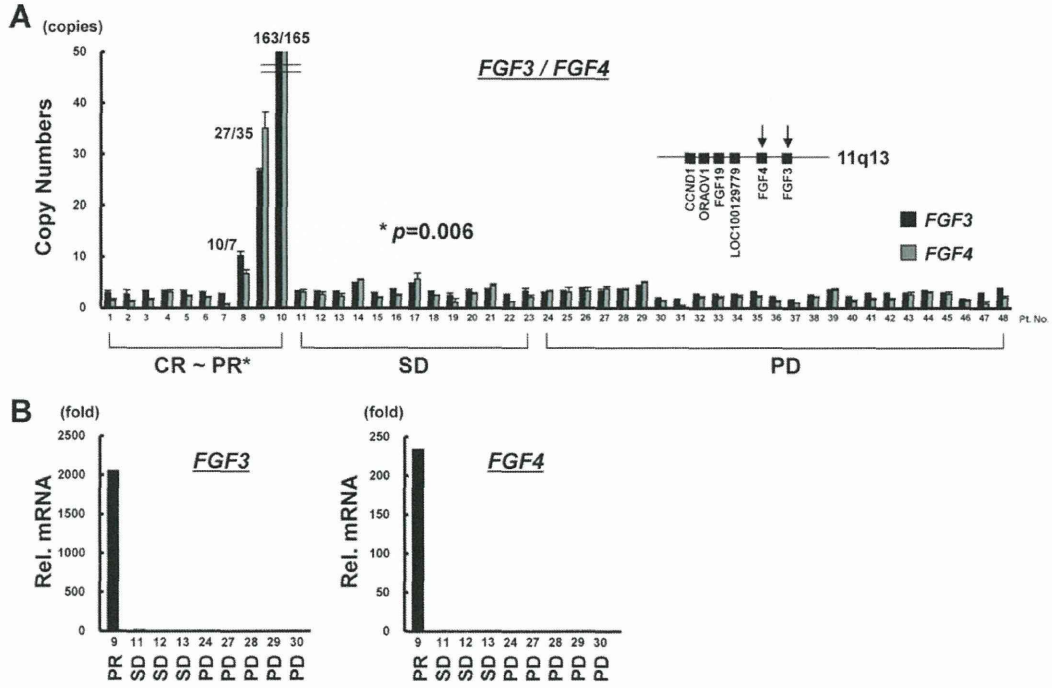

Fig. 2. *FGF3/FGF4* gene amplification is frequently observed in responders to sorafenib in HCC. (A) *FGF3/FGF4* gene amplification was determined using the TaqMan copy number assay in DNA samples obtained from 48 HCC samples that had been treated with sorafenib. *FGF3* amplification of >5 copies was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

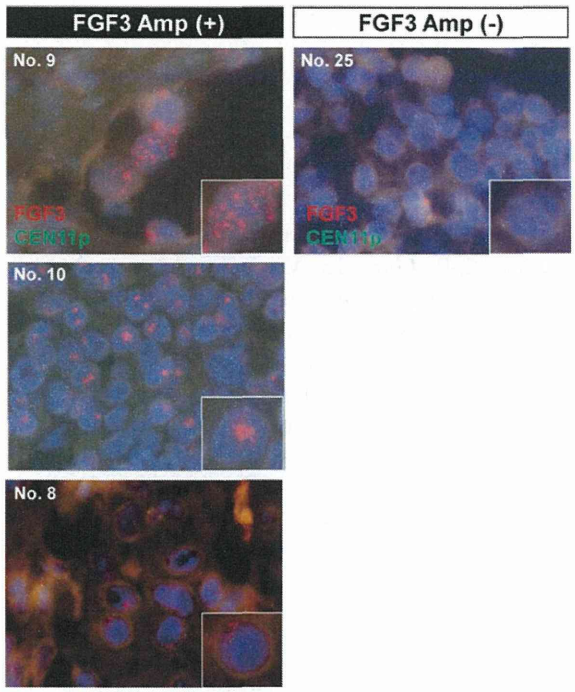

Fig. 3. FISH analysis of *FGF3*-amplified HCC. Patient numbers were indicated. Green staining indicates *CEN11P* loci; red staining indicates *FGF3* loci. High-power images are presented in each inset for a single cancer cell. Amp, gene amplification.

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.

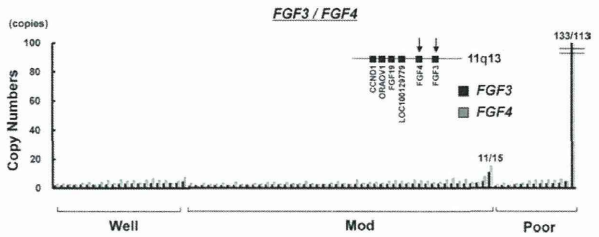

Fig. 4. *FGF3/FGF4* gene amplification in a series of HCC samples without sorafenib treatment. TaqMan copy number assay for *FGF3* and *FGF4* was used to examine DNA samples obtained from 82 surgical specimens. Human normal genomic DNA was used as a normal control. Well, well-differentiated HCC; Mod,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

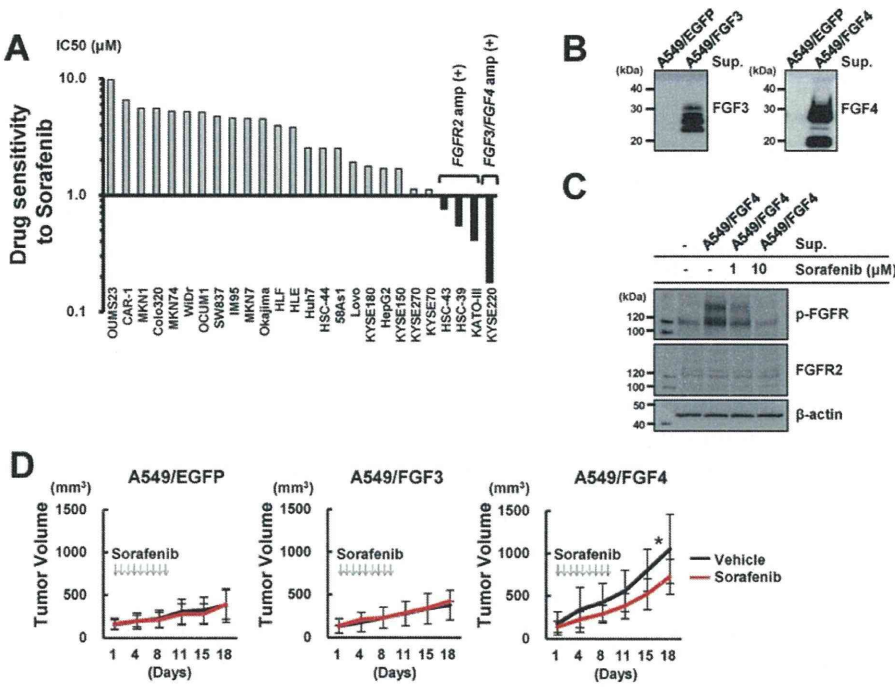

Fig. 5. FGF3 and FGF4 overexpression and drug sensitivity to sorafenib *in vitro* and *in vivo*. (A) Growth inhibitory assay examining sorafenib in various cancer cell lines *in vitro*. The growth inhibitory effect of sorafenib was examined using an MTT assay. The IC_{50} values of each cell line are 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