Pazopanib, a Novel Multitargeted Kinase Inhibitor, Shows Potent In Vitro Antitumor Activity in Gastric Cancer Cell Lines with FGFR2 Amplification

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Abstract

Pazopanib is an orally bioavailable, ATP-competitive, multitargeted tyrosine kinase inhibitor mainly targeting VEGFR2 and PDGFR tyrosine kinases, but the biologic sequences of pazopanib activities beyond antiangiogenesis are poorly defined. We used a panel of 38 gastric cancer cell lines to test the efficacy of pazopanib. In a growth inhibition assay, genomic changes indicated that pazopanib had differential effects on cell growth. Treatment of the KATO-III, OCUM-2M, SNU-16, and HSC-39 gastric cancer cell lines harboring FGFR2 amplification with pazopanib resulted in marked decreases of cell survival with IC50 in ranges of 0.1 to 2.0 μmol/L, whereas the same treatment of those cell lines without FGFR2 amplification had no growth-inhibitory effects. In the ectopic FGFR2-expressing model, treatment with the indicated concentrations of pazopanib significantly inhibited cell growth and colony formation by FGFR2-expressing NIH 3T3 cells with wild-type (WT) FGFR2 and mutant FGFR2 (S252W). Pazopanib also selectively suppressed constitutive FGFR2 signaling and phosphorylation of downstream effectors. In cell-cycle analysis, FGFR2-amplified cells underwent cell-cycle arrest at the G1–S phase after pazopanib treatment, whereas there were no significant effects on cell-cycle progression in cells without FGFR2 amplification treated with pazopanib. In addition, pazopanib increased a substantial fraction of sub-G1 only in FGFR2-amplified cells. These findings show that the activation of FGFR2 signaling by amplification may be a critical mediator of cell proliferation in a small subset of gastric cancer patients and that pazopanib may provide genotype-correlated clinical benefits beyond the setting of highly vascular tumors. Mol Cancer Ther; 13(11); 1–10. ©2014 AACR.

Introduction

Gastric cancer is the leading cause of cancer-related death worldwide with an incidence of 18.9 deaths/100,000 people per year (1). There are approximately 934,000 new cases of gastric cancer each year worldwide, with 56% of the new cases occurring in East Asia (2). According to the Central Tumor Registry data for 2002, gastric cancer accounts for 20.8% of all cancers in Korea (3). Although overall survival (OS) rates of gastric cancer have improved after the implementation of a national screening program in adults over 40 years in Korea, a large proportion of patients is still diagnosed at metastatic stages. Typically, fluoropyrimidines and platinum compounds form the backbone of chemotherapy for patients with advanced gastric cancer (AGC) and have substantially improved outcomes compared with single-agent chemotherapy or best supportive care (4). However, the median survival time following cytotoxic chemotherapy is still less than 12 months, and thus metastatic gastric cancer remains a therapeutic challenge for medical oncologists. The role of molecularly targeted therapy has not been adequately explored in gastric cancer when compared with other common solid tumors such as non–small cell lung cancer, breast, or colorectal cancer.

There is increasing evidence that suggests that sensitivity to molecularly targeted agents among diverse human cancers is well correlated with underlying genetic alterations of the tumor. Therefore, identifying critical genes that play pivotal roles in controlling tumor growth and survival will establish the basis for developing therapeutic targets. The fibroblast growth factor receptor...
(FGFR) tyrosine kinase family is composed of four kinases (FGFR 1, 2, 3, and 4) that differentially respond to 18 FGF ligands and have long been implicated as causes of cancer (5). Recent research has shown that members of the FGFR family are promising targets that are deregulated by amplification, point mutation, or translocation. Amplification or activation of FGFR1 has been reported in lung cancer and translocations involving FGFR3, and activating in FGFR3, have been associated with multiple myeloma and bladder cancer. Missense mutations of FGFR2 are also present in several types of human cancer, including lung squamous carcinoma, gastric cancer, and endometrial cancer (6–8). These cancers are shown to be sensitive to FGFR2 kinase inhibition in the cells bearing such FGFR2 mutations, implicating FGFR2 as a novel therapeutic target (7). In gastric cancer, FGFR2 was first identified as the K-sam gene amplification in the human gastric cancer cell line KATO-III, and is preferentially amplified or overexpressed in poorly differentiated or undifferentiated types of gastric cancers (9, 10). Recent preclinical studies have demonstrated a critical role for FGFR2 amplification in gastric cancer cell proliferation and survival, and detected antitumor activities of multitargeted tyrosine kinase inhibitors (MTKI) and pan-FGFR inhibitors in FGFR2-amplified gastric cancer cell lines, which suggests that FGFR2 amplification may be a promising therapeutic target in gastric cancer (11–15). Therefore, the present preclinical study primarily focused on the antitumor activities of pazopanib on FGFR2 signaling in gastric cancer.

Materials and Methods

Cell culture, cell growth assay, and colony formation assay
Thirty-eight genetically defined human gastric cancer cell lines representing broad genetic heterogeneity were assembled to establish an in vitro platform that could be used to potentially identify genotype-correlated sensitivities and to eventually develop novel therapeutic strategies in gastric cancer. Human gastric cancer cell lines AGS, KATO-III, MKN-1, MKN-45, N87, SNU-1, SNU-5, SNU-16, SNU216, SNU-484, SNU-520, SNU-601, SNU620, SNU-638, SNU-668, and SNU 719 cells were purchased from the Korean Cell Line Bank in 2008. MKN-7, NUGC-3, and NUGC-4 were purchased from the Health Science Research Resources (Osaka, Japan) in 2008. YCC-1, YCC-2, YCC-3, YCC-6, YCC-7, YCC-10, YCC-11, YCC-16, and HS-741T were kindly provided by Dr. Sun Young Rha (Yonsei Cancer Center, Seoul, Korea) in 2008. HSC-39, HSC-44, HSC-57 HSC-58, HSC-60, and HSC-69 were kindly provided by Dr. Kazuyoshi Yanagihara (Yasuda Women’s University, Hiroshima, Japan) in 2009, TMK-1 by Dr. Wataru Yasui (Hiroshima University, Hiroshima, Japan) in 2009, and OCUM-2M by Dr. Masa-kazu Yashiro (Osaka City University, Osaka, Japan) in 2008. FGFR2-expressing NIH 3T3 cells with retroviruses encoding isoform IIIb wild-type (WT) FGFR2 and FGFR2 S252W were kindly provided Dr. Matthew Meyerson (Dana-Farber Cancer Institute, Boston, MA) in 2010. YCC cell lines and NIH 3T3 lines were maintained in DMEM (Gibco-BRL) and supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 2 mmol/L glutamine. The other cell lines were cultured in RPMI-1640 medium (Gibco-BRL) and supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 2 mmol/L glutamine. All cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C. All cell lines have been authenticated using viability, morphology and growth curve analysis on a regular basis, and tested negative for Mycoplasma.

The inhibition of growth was assessed by the 5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoli-um (MTS) assay according to previously established methods (16). Pazopanib (GW786034) was kindly provided by GlaxoSmithKline (Brentford). Sorafenib and sunitinib were purchased from LC Laboratories. Stock solution of each compound was reconstituted in DMSO at a concentration of 10 mmol/L (final concentration of 0.1% DMSO for the MTS assay) and stored at −20°C. For the colony formation assay, FGFR2-expressing NIH 3T3 cells with WT FGFR2 and mutant FGFR2 (S252W) were used. Two hundred cells per well were seeded in 6-well plates and treated with 0.1 to 1.0 μmol/L of pazopanib for 3 days. Triplicate cultures of each cell type were maintained at 37°C for 14 days in an atmosphere of 5% CO₂, with fresh medium added after 7 days. Colonies, defined as groups of cells containing a minimum of 50 cells, were stained with 0.1% crystal violet and counted under an inverted phase contrast microscope CKX31SF (Olympus Biosystems). Colonies were photographed after 2 weeks and the percentage of relative number of colonies was expressed as (number of colonies from treated cells/number of colonies from controls) × 100. The assay was repeated three times with duplicate samples.

DNA sequencing for FGFR2
Analysis of DNA sequencing was performed for all known mutation sites of FGFR2 exons. DNA was extracted from five paraffin sections of 10-μm thickness containing a representative portion of each tumor block, using the QIAamp DNA Mini Kit (Qiagen). One hundred nanograms of DNA was amplified in a 20-μL reaction solution containing 2 μL of 10× buffer (Roche), 1.7 to 2.5 mmol/L of MgCl₂, 0.3μmol/L of each primer pairs, 250 μmol/L of deoxynucleotide triphosphate, and 2.5 U of DNA polymerase (Roche). Amplifications were performed using a 5-minute initial denaturation at 94°C, followed by 30 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C, and a 10-minute final extension at 72°C. Polymerase chain reaction (PCR) products were then 2% gel-purified with the QIAgent Gel Extraction Kit (Qiagen). DNA templates were processed for the DNA sequencing reaction using the ABI-PRISM BigDye Terminator version 3.1 (Applied Biosystems) with both forward
and reverse sequence-specific primers. Twenty nanograms of purified PCR products was used in a 20-μL sequencing reaction solution containing 8 μL of BigDye Terminator v3.1 and 0.1 μmol/L of the same PCR primer. Sequencing reactions were performed using 25 cycles of 10 seconds at 96°C, 5 seconds at 50°C, and 4 minutes at 60°C. Sequence data were generated with the ABI PRISM 3100 DNA Analyzer (Applied Biosystems) and analyzed by Sequencing Analyzing Software v5.3.1 (Applied Biosystems) to compare variations. Primers used for FGFR2 sequencing were as follows: exon 6, F: 5′-TCTGGCAT-GAGCTCCTAGC, R: 5′-CACCCTCTCTGGCTAGT; exon 7, F: 5′-ATGTTAGGAGGTAAGCC, R: 5′-ATCTCTAC-TGTGTTGAAT; exon 8, F: 5′-TGGTACAGTGCTAC-TACC, R: 5′-GCAACGCACTAGTACAGT; exon 11, F: 5′-TACAGTACGTCCCATG, R: 5′-CTTGTGGCTCA-TGCGTACGA; exon 13, F: 5′-AATCTAGCACCTCA-AGT, R: 5′-CGGTGCTGATTATCTGC.

**FGFR2 amplification with quantitative real-time PCR and FISH**

Quantitative real-time PCR (qRT-PCR) with TaqMan probes was performed to analyze FGFR2 copy number using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The FGFR2 primers were F: CCCCCCTCAATCATTCTC, R: ACCGCCGGCTT-GAAAC; the probe was labeled with the reporter dye 6-carboxyfluorescein (6-FAM-TCTGCTAGGCTTTTCTTTT-MGBFQ). The average FGFR2 copy number per cell was calculated from the differences in the threshold amplification cycles between EGFR and RNaseP.

Fluorescence in situ hybridization (FISH) was also performed according to the established protocol using an FGFR2 probe at chromosome arm 10q26 and chromosome 10 centromere probe (CEP10) purchased from Macrogen (Supplementary Data). Four-micrometer tumor sections were immersed in Tris-base and EDTA (TE), affinizing in xylene and dehydrating in ethanol. The sections were rehydrated in PBS, and then washed in phosphate-buffered saline (PBS), and then counterstained with DAPI. One hundred cells from each TMA core were analyzed and the number of FGFR2 and CEP10 signals was determined; tumors with FGFR2 to CEP10 copy number per cell was over 2 or the presence of ≥10% gene cluster were defined as amplified.

**Immunoprecipitation, Western blot analysis, and RTK array**

Cells grown under the previously specified conditions were lysed in the following lysis buffer composition: 20 mmol/L/L Tris (pH 7.4), 150 mmol/L/L NaCl, 1% NP40, 10% glycerol, 1 mmol/L/L EDTA, 1 mmol/L/L EGTA, 5 mmol/L/L sodium pyrophosphate, 50 mmol/L/L NaF, 10 mmol/L/L β-glycerophosphate, 1 mmol/L/L sodium vanadate, 0.5 mmol/L/L DTT, 4 μg/mL leupeptin, 4 μg/mL pepstatin, 4 μg/mL aprotinin, and 1 mmol/L/L phenylmethylsulfonyl fluoride. Lysates were centrifuged at 16,000 × g for 5 minutes at 4°C. The supernatant was used for subsequent procedures. For IPs, anti-FGFR2 antibody (Santa Cruz Biotechnology) was added to the lysate and then incubated with protein A/G agarose beads (Santa Cruz Biotechnology). IP's were washed three times with ice-cold lysis buffer before boiling in 2× Lamelli sample buffer. Western blot analyses were conducted after separation by SDS/PAGE electrophoresis and transfer to nitrocellulose membranes. Immunoblotting was performed according to the antibody manufacturer's recommendations. Antibody binding was detected using an enhanced chemiluminescence system (PerkinElmer, Waltham, MA, USA). The phospho-AKT (S473), AKT, cleaved PARP, and cleaved caspase-3 antibodies were from Cell Signaling Technology (Beverly, MA, USA). The phospho-FGFR and FGFR2 antibodies were from R&D Systems (Minneapolis, MN, USA). The FGFR2 antibody for IP was from Santa Cruz Biotechnology. The phosphotyrosine (4G10) antibody was from Upstate Biotechnology (Lake Placid, NY, USA).

At approximately 70% of cell confluence, protein extracts of KATO-III, OCUM-2M, MKN-45 and N87 cells were also prepared for the Human Phospho-RTK Array (R&D Systems, Minneapolis, MN, USA). This array can detect the phosphorylation level of 42 different receptor tyrosine kinases (RTK) on the same nitrocellulose membrane. Assays were performed in accordance with the manufacturer's instructions.

**Fluorescence-activated cell sorting for cell-cycle analysis**

The fluorescence-activated cell sorting (FACS) analysis was performed using methods similar to those described previously. Briefly, 1 to 1.5 × 10⁶ cells were seeded into 10-cm² plates, and the drugs were added 24 hours later. After 24 hours of treatments, the cells were trypsinized and fixed overnight in ethanol at 4°C. Fixed cells were then resuspended in 0.5% RNase A (Sigma), centrifuged, resuspended in 5 μmol/L propidium iodide (Sigma) and 38 mmol/L sodium citrate, incubated at room temperature for 30 minutes, and analyzed by the FACSCalibur flow cytometry (Becton Dickinson) with a cell-cycle test software, ModFit LT (Verity Software House Inc.). All experiments were repeated three times.

**Patients and tissue specimens**

The outcomes of 544 patients with stage II–IV (M0) gastric cancer who received adjuvant chemoradiation therapy after curative surgery were previously reported (17). Of these patients and an additional 23 stage IB patients who were included in a previous study, formalin-fixed paraffin-embedded primary tumor tissues were available from 482 patients. The postoperative adjuvant treatment that was adopted was the same as that used for...
the INT-0116 (SWOG-9008) trial (18). All patients provided written informed consent according to the institutional guidelines and the study was approved by the Institutional Review Board at Samsung Medical Center (Seoul, Korea).

Statistical analyses

Disease-free survival (DFS) was defined as the time from surgery to the first relapse of cancer, or death because of any cause. OS was calculated from the date of surgery to the date of death. OS and DFS were calculated using the Kaplan–Meier method. Correlation analyses were performed using the two-sided $\chi^2$ test or the Fisher exact test. Differences in DFS and OS were compared using the log-rank tests and the Cox proportional hazards analysis. $P$ values less than 0.05 were considered statistically significant.

Results

Correlation of sensitivity with MTKIs and FGFR2 amplification in human gastric cancer cell lines

The efficacy of MTKI, including pazopanib, sorafenib, and sunitinib, whose clinical activity is not well recognized in gastric cancer, was first tested using the in vitro platform for modeling genotype-correlated drug sensitivity in a panel of 37 gastric cancer lines. Among the 37 gastric cancer cell lines tested, most cells were not sensitive to MTKIs and some cells showed growth inhibition based on the MTKI. However, four gastric cancer cells (KATO-III, OCUM-2M, SNU-16, and HSC-39; 10.8%) exhibited dramatic sensitivity, which could indicate they share the same genetic mechanism(s) for underlying drug sensitivity to MTKIs (Fig. 1A).

To understand possible underlying genetic mechanisms of MTKI sensitivity, genetic alterations were correlated with their MTKI sensitivities. Of the four cell lines showing sensitivity to MTKIs, all were found to have high FGFR2 gene copy numbers by qRT-PCR (Fig. 1B). In addition, FISH analysis showed that the amplified gene copies were integrated within a chromosomal locus at 10p26, consistent with the so-called homogeneously staining regions (HSR) in these cell lines (Fig. 1C–H). The FISH results were consistent with the qRT-PCR results in identifying the subset of cell lines with FGFR2 amplification. However, any previously reported known oncogenic FGFR2 mutations were not found in the current study of the gastric cancer cell lines (data not shown; refs. 7, 19).

Pazopanib blocks proliferation and survival of gastric cancer cell lines with FGFR2 amplification

Gastric cancer cell lines were first treated with pazopanib to investigate whether inhibition of FGFR2 kinase activity is effective against gastric cancer cell lines harboring FGFR2 amplification and to test the potential therapeutic relevance of these findings. Treatment of the KATO-III, OCUM-2M, SNU-16, and HSC-39 gastric cancer cell lines harboring FGFR2 amplification with pazopanib resulted in marked decreases in cell survival with IC$_{50}$ in ranges of 0.1 to 2.0 $\mu$mol/L, whereas similar treatment of the cell lines without FGFR2 amplification had no effect (Fig. 2A). In addition, treatment with either sorafenib or sunitinib similarly showed significant growth inhibition in the gastric cancer lines harboring FGFR2 amplifications (Fig. 2B and C). To further test the efficacy of pazopanib against ectopic expression of FGFR2, FGFR2-expressing NIH 3T3 cells with WT FGFR2 and mutant FGFR2 (S252W) were used in a growth inhibition and colony formation assay. Treatment with the indicated concentrations of pazopanib significantly inhibited cell growth and colony formation by the FGFR2-expressing NIH 3T3 cells with WT FGFR2 and mutant FGFR2 (S252W), when compared with the NIH-3T3 cell with vector (Fig. 3A–C).

Cell-cycle analyses were performed to examine the mechanisms of growth inhibition of pazopanib in FGFR2-amplified cells. All cell lines were treated for 24 hours in the presence and absence of 1 $\mu$mol/L pazopanib. The FGFR2-amplified cells underwent cell-cycle arrest at the G1–S phase after pazopanib treatment, whereas there was no significant effect on cell-cycle progression in cells without FGFR2 amplification after treatment with pazopanib. In addition, pazopanib caused an increase in the substantial fraction of sub-G1 in FGFR2-amplified cells only (Fig. 4).

Pazopanib selectively inhibits FGFR2 phosphorylation and downstream signaling molecules in FGFR2-amplified gastric cancer cell lines

The effects of drug treatment on FGFR2-dependent signaling were tested to address the mechanism by which pazopanib triggers cell death in FGFR2-amplified cells. Pazopanib selectively suppressed the constitutive phospho-FGFR2 in FGFR2-amplified KATO-III cells, whereas pazopanib did not suppress the phospho-MET and phospho-HER2 in MKN-45 and N87 cells, respectively (Fig. 5A). Most importantly, treatment with this concentration of pazopanib also effectively abrogated the baseline phosphorylation of downstream effectors of growth factor receptors such as ERK-1/2 and AKT (Fig. 5B and C). Sorafenib and sunitinib similarly downregulated FGFR2 phosphorylation and downstream signaling were tested to address the mechanism by which pazopanib triggers cell death in FGFR2-amplified cells. Thus, constitutive activation of these proliferative and survival pathways in FGFR2-amplified cells appears to depend specifically on baseline FGFR2 signaling. In contrast, in cells without FGFR2 amplification where FGFR2 is not constitutively activated, pazopanib had no effect on signaling. This indicates that these effectors are likely to be activated through alternative growth factor receptors (Fig. 5A).

Induction of apoptosis has been linked to suppression of essential growth factor–mediated survival pathways and increased fraction of sub-G1 in cell-cycle analysis. PARP cleavage in Western blot analyses demonstrated apoptosis in FGFR2-amplified cells treated with pazopanib, but not in cells without FGFR2 amplification (Figs. 4A and B).
These findings suggest that FGFR2 signaling plays a critical role in cell survival in these FGFR2-addicted cell lines.

**Correlations between FGFR2 amplification and clinical outcomes**

To assess FGFR2 amplification in clinical gastric cancer cases, tumor specimens from 482 patients with AGC were analyzed by qRT-PCR using TaqMan probes and dual-color FISH (Fig. 6). Of the 482 patients, 24 (5%) of the patients had FGFR2 copy numbers greater than 4.0 copies. FISH and qRT-PCR analyses were also consistent in identifying FGFR2 amplification in tissue specimens, with higher-fold amplification apparent by FISH (Fig. 6). This presumably reflects the effect of low-level copy-number variability in the control locus used in qRT-PCR analysis, resulting in underestimation of the true extent of FGFR2 amplification. However, FGFR2 amplification was not associated with clinicopathologic parameters such as age, sex, histology, Bormann type, Lauren classification, tumor location, or tumor–node–metastasis (TNM) staging. In addition, FGFR2 amplification was not related to clinical outcomes (data not shown).

**Discussion**

This study shows that FGFR2 is amplified in a subset of gastric cancer and is closely associated with sensitivity to Pazopanib for FGFR2 Amplification in Gastric Cancer.
pazopanib. Pazopanib is an orally bioavailable, ATP-competitive, MTKI that mainly targets VEGFR2, PDGFR, and c-Kit tyrosine kinases in a low-nanomolar concentration, resulting in selective inhibition of VEGF-induced endothelial cell proliferation and the growth of a broad range of human tumor xenografts in mice (20, 21). On the basis of these preclinical results, pazopanib is under clinical development for the treatment of multiple tumor types and has demonstrated significant clinical activity as monotherapy in patients with renal cell carcinoma (RCC) in recent phase III trial (22). Like other MTKIs, including sunitinib and sorafenib, the clinical success of pazopanib in RCC may indicate the role of pazopanib as a VEGFR inhibitor and its consequent effects on angiogenesis (23). Notably, RCC tumors are typically highly vascularized, suggesting a potential critical requirement for angiogenesis in that disease setting.

MTKIs are a relatively new class of therapeutics with a propensity to inhibit multiple kinases, but the biologic sequences of multiple kinase activities beyond antiangiogenesis are poorly defined (21). Moreover, although the ability of MTKIs to target additional kinases may contribute to clinical activities in other solid tumors, this possibility has not been well investigated beyond PDGFRA mutations that are largely confined to gastrointestinal stromal tumor (GIST; ref. 24). In an in vitro kinase assay,
Pazopanib had modest activity against FGFR2 kinase with nanomolar concentrations expecting antitumor activity in tumors having dysregulated FGFR2 signaling (21).

In this preclinical study, pazopanib sensitivity was restricted to a small number of gastric cancer cell lines harboring FGFR2 amplification. This study also showed...
that FGFR2 activation is coupled to critical downstream effectors, such as Erk and Akt, and that disrupting these pathways seems to mediate the inhibitory effects of pazopanib on proliferation and cell survival in the FGFR2-addicted cells in vitro. Hence, these findings suggest that activated FGFR2 signaling by amplification may be a critical target, and that pazopanib might be useful as an FGFR2 inhibitor in the clinical management of the subset of tumors that exhibit FGFR2 activation. Interestingly, there was no evidence of FGFR2 mutations in 38 gastric cancer cell lines, which argues in favor of amplification rather than mutation as the preferred mechanism of FGFR2 activation in a subset of gastric cancer. In contrast, FGFR2 activation is mainly associated with oncogenic mutations in endometrial and breast cancers (7).

We found that FGFR2 amplification was also observed in a relatively small subset (5%) of gastric cancer clinical samples in this study, which is consistent with recent Japanese and Chinese study on genomic alterations (25, 26). Although the frequency of FGFR2 amplification has not been well investigated in a large cohort, relatively low incidence of FGFR2 amplification in gastric cancer specimens might be a possible impediment in developing FGFR2-targeted therapeutics in gastric cancer. Moreover, because a large-scale analysis of FGFR2 status has not been performed in gastric cancer tissues or DNA samples, the cutoff value for FGFR2 amplification or FGFR2 expression has not yet been determined. Therefore, companion diagnostics to reliably detect FGFR2 activation measured by FGFR2 amplification or
FGFR2 protein expression needs to be standardized. As the growth curves for HSC-39 and SNU-16 only reach a lower limit of 40% survival in this preclinical study (Fig. 2A), it is presumed that the relatively lower level of amplification in SNU-16 and HSC-39 (Fig. 1B) may be related to somewhat lower response to pazopanib. Therefore, a predictive role of FGFR2 amplification for treatment response according the cutoff value should be further validated as a novel therapeutic target in prospective clinical studies. In addition, given that pazopanib is less potent against the FGFRs versus the VEGFRs, it is important whether an efficacious concentration of pazopanib can be achieved in patients with gastric cancer. Although no pharmacokinetic (PK) data on pazopanib in patients with gastric cancer are available, steady-state exposure plateaued at doses ≥800 mg/day and was ≥15 μg/mL (≈34 μmol/L) in 93% of patients receiving a dose of 800 mg daily from PK data in a phase I study (27), which plasma concentration can be clinically achieved might be enough to block FGFRs. This C_{trough} plasma pazopanib concentration of ≥15 μg/mL also appeared to correlate with clinical activity in patients with RCC, which could be effective for patients with gastric cancer with FGFR2 amplification as well. A phase II trial is currently being conducted involving pazopanib combined with capcitabine and oxaliplatin (CapeOX) in AGC (ClinicalTrials.gov Identifier: NCT01130805). Of 66 patients enrolled in this investigator-sponsored trial as of July 2014, 3 cases (7.1%) of 42 patients tested were found to have FGFR2 amplification by dual-color FISH. The complete clinical data of this trial will be available in the late 2014, and the significance of FGFR2 amplification and other biomarkers on clinical outcomes in this context of the trial will be also investigated. As several FGFR kinase inhibitors are now in clinical trials, including brivanib, dovitinib, BIBF 1120, and SU-6668, it may be useful to test these inhibitors on patients with gastric cancer harboring focal FGFR2 amplifications (28–31).

In conclusion, the findings of this study show that activation of FGFR2 signaling by amplification may be a critical mediator of cell proliferation in a small subset of gastric cancer patients and may sensitize these cancer cells to pazopanib. These findings suggest that pazopanib as well as other MTKIs may provide genotype-associated clinical benefits beyond the setting of highly vascular tumors such as RCCs. Although molecular targeted therapy has been studied less extensively in AGC than in other solid tumor types, future research should focus on novel therapeutic targets in clinical trials.

Disclosure of Potential Conflicts of Interest
J.O. Park received a commercial research grant from GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

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Figure 6. A–D, FGFR2 amplification is also detected by dual-color FISH in the specimens from patients with gastric cancer; no FGFR2 amplification (A) and FGFR2 amplification (B–D). Tumors with FGFR2 to CEP10 ≥2 or presence of ≥10% gene cluster were defined as amplified.
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