Pazopanib, a novel multi-targeted kinase inhibitor, shows potent in vitro antitumor activity in gastric cancer cell lines with FGFR2 amplification

Seung Tae Kim1*, Hye-Lim Jang,1,3* Su Jin Lee,1 Jeeyun Lee,1 Yoon-La Choi,2 Kyoung-Mee Kim,2 Jeonghee Cho,4 Se Hoon Park,1 Young Suk Park,1 Ho Yeong Lim,1 Masakazu Yashiro,5 Won Ki Kang1 and Joon Oh Park1†

1Division of Hematology-Oncology, Departments of Medicine and 2Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea; 3Samsung Biomedical Research Institute, Seoul, Korea; 4Samsung Genome Institute, Samsung Medical Center, Seoul, Korea; 5Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Osaka, Japan

*S.T.K and H.L.J equally contributed to this work.

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† Correspondences to: Joon Oh Park, M.D., Ph.D.

Division of Hematology-Oncology, Department of Medicine
Samsung Medical Center, Sungkyunkwan University School of Medicine
50 Irwon-dong Gangnam-gu
Seoul 135-710 Korea
Tel: +82-2-3410-3457, Fax: +82-2-3410-1754
Email: oncopark@skku.edu
Disclosure of Potential Conflicts of Interest

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Abstract

Pazopanib is an orally bioavailable, ATP-competitive, multi-targeted kinase inhibitor mainly targeting VEGFR2 and PDGFR tyrosine kinases, but the biological sequences of pazopanib activities beyond anti-angiogenesis are poorly defined. We used a panel of 38 GC cell lines in order 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 GC cell lines harboring FGFR2 amplification with pazopanib resulted in marked decreases of cell survival with IC\textsubscript{50} in ranges of 0.1 - 2.0 μM, while 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 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 G\textsubscript{1}-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 GC patients and that pazopanib may provide genotype-correlated clinical benefits beyond the setting of highly vascular tumors.
INTRODUCTION

Gastric cancer (GC) is the leading cause of cancer death worldwide with an incidence of 18.9 deaths/100,000 people per year (1). There are approximately 934,000 new cases of GC each year worldwide, with 56% of the new cases occurring in East Asia (2). According to Central Tumor Registry data for 2002, GC accounts for 20.8% of all cancers in Korea (3). Although overall survival rates of GC have improved after the implementation of a national screening program in adults over 40 years old in Korea, a large proportion of patients are 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 GC remains a therapeutic challenge for medical oncologists. The role of molecularly targeted therapy has not been adequately explored in GC when compared to 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 comprised 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 \textit{FGFR1} has been reported in lung cancer and translocations involving \textit{FGFR3}, and activating in \textit{FGFR3} have been associated with multiple myeloma and bladder cancer. Missense mutations of \textit{FGFR2} has also identified 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 cell lines bearing such \textit{FGFR2} mutations, implicating \textit{FGFR2} as a novel therapeutic target (7). In GC, \textit{FGFR2} was first identified as the \textit{K-sam} gene amplification in the human GC cell line KATO-III, and is preferentially amplified or overexpressed in poorly differentiated or undifferentiated types of GCs (9, 10). Recent preclinical studies have demonstrated a critical role for \textit{FGFR2} amplification in GC cell proliferation and survival, and detected anti-tumor activities of multi-targeted TKIs and pan-FGFR inhibitors in \textit{FGFR2} amplified GC cell lines, which suggests that \textit{FGFR2} amplification may be a promising therapeutic target in GC (11-15). Therefore, the present preclinical study primarily focused on the antitumor activities of pazopanib on FGFR2 signaling in GC.
METHODS

Cell culture, cell growth assay, and colony formation assay

Thirty-eight genetically defined human GC 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 GC. Human GC cell lines AGS, KATO-III, MKN-1, MKN-28, MKN-45, MKN-74, 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 (Seoul, Korea) in 2008. MKN-7, NUGC-3 and NUGC-4 were purchased from 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, 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. Masakazu Yashiro (Osaka City University, Osaka, Japan) in 2008. FGFR2-expressing NIH 3T3 cells with retroviruses encoding isoform IIIb WT FGFR2 and FGFR2 S252W were kindly provided Dr. Matthew Meyerson (Dana-Farber Cancer Institute, Boston, MA) in 2010.
cell lines & NIH 3T3 lines were maintained in DMEM (Gibco-BRL, Carlsbad, CA, USA) and supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM glutamine. The other cell lines were cultured in RPMI-1640 medium (Gibco-BRL) and supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM glutamine. All cells were incubated in a humidified atmosphere containing 5% CO\(_2\) 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.

Growth and inhibition of growth were assessed by the 5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay according to previously established methods.(16) Pazopanib (GW786034) was kindly provided by GlaxoSmithKline (GSK) (Brentford, Middlesex, UK). Sorafenib and sunitinib were purchased from LC Laboratories (Woburn, MA, USA). Stock solution of each compound was reconstituted in DMSO at a concentration of 10 mM (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 six-well plates and treated with 0.1-1.0 μM of pazopanib for three days. Triplicate cultures of each cell type were maintained at 37°C for 14 days in an atmosphere of 5% CO\(_2\), with fresh medium added after seven 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, Hamburg, Germany). Colonies were photographed after two weeks and the percent 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, Hilden, Germany). One hundred nanograms of DNA were amplified in a 20 μL reaction solution containing 2 μL of 10X buffer (Roche, Mannheim, Germany), 1.7 to 2.5 mmol/L of MgCl₂, 0.3μM of each primer pairs, 250 μM of deoxynucleotide triphosphate, and 2.5 units 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 a QIAgen gel extraction kit (Qiagen). DNA templates were processed for the DNA sequencing reaction using the ABI-PRISM BigDye Terminator version 3.1 (Applied Biosystems,
Foster City, CA, USA) with both forward and reverse sequence-specific primers. Twenty nanograms of purified PCR products were used in a 20μL sequencing reaction solution containing 8 μL of BigDye Terminator v3.1 and 0.1 μM 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 \textit{FGFR2} sequencing were as follows: Exon 6, F: 5'-TCTGGCATGAGGTCACTG, R: 5'-CACTCTCTGCTGGCTAGT; exon 7, F: 5'-ATGTGTAAGGAGGTGAAGC, R: 5'-ATCTCACTGTGTGAAT; exon 8, F: 5'-TAGTGACAGTGAAGGC, R: 5'-ATCTCACTGTGTGAAT; exon 11, F: 5'-TAACAGTAGCTGCCCATG, R: 5'-CTTTGTGTTCATGGCTAGGA; exon 13, F: 5'-AACTACTGACCTCAAGTG, R: 5'-CGGTTGCTGATTATTCTG.

\textit{FGFR2 amplification with quantitative Real-Time PCR (qPCR) and FISH}

qPCR with TaqMan probes was performed to analyze \textit{FGFR2} copy number using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) The \textit{FGFR2} primers were F: CCCCCTCCACAATCATTCT, R: ACCGGCGGCCTAGAAAAC; the probe was labeled with the reporter dye 6-carboxyfluorescein (6-FAM-
TCGTCTAGCCTTTTCTTTT-MGBNFQ). 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 (Seoul, Korea) (Supplemental Data). Four micron tumor sections generated from TMA blocks were pretreated by deparaffinizing in xylene and dehydrating in ethanol. The sections were immersed in Tris-base and EDTA (TE), washed in phosphate-buffered saline (PBS), and then treated with Digest-All (Zymed, San Francisco, CA, USA). Sections were then fixed with formalin and dehydrated in ethanol. After co-denaturation of the tissue and the probe mixture (FGFR2 and CEP10) at 70°C for 3 minutes, the sections were hybridized at 37°C for 48-72 hours, washed with sodium citrate and Tween-20 containing buffers and counterstained with DAPI. One hundred cells from each TMA core were analyzed and the number of FGFR2 and CEP10 signals determined; tumors with FGFR2 to CEP10 ≥ 2 or presence of ≥ 10% gene cluster were defined as amplified.

*Immunoprecipitation (IP), western blot analysis and RTK array*

Cells grown under the previously specified conditions were lysed in the following lysis
buffer composition: 20 mM/L Tris (pH 7.4), 150 mM/L NaCl, 1% NP40, 10% glycerol, 1 mM/L EDTA, 1 mM/L EGTA, 5 mM/L sodium pyrophosphate, 50 mM/L NaF, 10 mM/L β-glycerophosphate, 1 mM/L sodium vanadate, 0.5 mM/L DTT, 4 µg/mL leupeptin, 4 µg/mL pepstatin, 4 µg/mL apoprotein, and 1 mM/L phenylmethylsulfonyl fluoride. Lysates were centrifuged at 16,000 × g for 5 min at 4°C. The supernatant was used for subsequent procedures.

For IPs, anti-FGFR2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the lysate and then incubated with protein A/G agarose beads (Santa Cruz Biotechnology). IPs 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 manufacturers' 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 (Santa Cruz, CA, USA). 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 RTKs on the same nitrocellulose membrane. Assays were performed in accordance with the manufacturer’s instructions.

Fluorescence activated cell sorting (FACS) for cell cycle analysis

The FACS analysis was performed using methods similar to those described previously. Briefly, 1–1.5 × 10⁶ cells were seeded into 10 cm² plates, and the drugs were added 24 h later. After 24 h of treatment, the cells were trypsinized and fixed overnight in ethanol at 4°C. Fixed cells were then resuspended in 0.5% RNase A (Sigma, St. Louis, MO, USA), centrifuged, resuspended in 5 μM propidium iodine (Sigma) and 38 mM sodium citrate, incubated at room temperature for 30 min, and analyzed by FACS Calibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) with a cell-cycle test software, ModFit LT™ (Verity Software House Inc., Topsham, ME, USA). All experiments were repeated three times.

Patients and tissue specimens

The outcomes of 544 stage II – IV (M0) GC patients 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 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 of any cause. Overall survival (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-square test or Fisher’s exact test. Differences in disease-free and overall survival were compared using log-rank tests and Cox proportional hazards analysis. \( P \) values less than 0.05 were considered statistically significant.
RESULTS

Correlation of sensitivity with multi-targeted kinase inhibitors (MTKIs) and FGFR2 amplification in human GC cell lines

The efficacy of MTKIs including pazopanib, sorafenib and sunitinib, whose clinical activity is not well recognized in GC, was first tested using the in vitro platform for modeling genotype-correlated drug sensitivity in a panel of 37 GC lines. Among the 37 GC cell lines tested, most cells were not sensitive to MTKIs and some cells showed growth inhibition based on the MTKI. However, four GC 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 (Figure 1A).

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

**Pazopanib blocks proliferation and survival of GC cell lines with FGFR2 amplification**

GC cell lines were first treated with pazopanib to investigate whether inhibition of FGFR2 kinase activity is effective against GC 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 GC cell lines harboring FGFR2 amplification with pazopanib resulted in marked decreases in cell survival with IC50 in ranges of 0.1 - 2.0 μM, whereas similar treatment of the cell lines without FGFR2 amplification had no effect (Figure 2A). In addition, treatment with either sorafenib or sunitinib similarly showed significant growth inhibition in the GC lines harboring FGFR2 amplifications (Figure 2B-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 to the NIH-3T3 cell with vector (Figure 3A-C).
Cell cycle analyses were performed to examine the mechanisms of growth inhibition of pazopanib in \textit{FGFR2}-amplified cells. All cell lines were treated for 24 hours in the presence and absence of 1 \( \mu \text{M} \) pazopanib. The \textit{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 \textit{FGFR2} amplification after treatment with pazopanib. In addition, pazopanib caused an increase in the substantial fraction of sub-G1 in \textit{FGFR2}-amplified cells only (Figure 4).

\textbf{Pazopanib selectively inhibits FGFR2 phosphorylation and downstream signaling molecules in \textit{FGFR2}-amplified GC cell lines}

The effects of drug treatment on FGFR2-dependent signaling were tested to address the mechanism by which pazopanib triggers cell death in \textit{FGFR2}-amplified cells. Pazopanib selectively suppressed the constitutive phospho-FGFR2 in \textit{FGFR2}-amplified KATO-III cells, whereas pazopanib did not suppress the phospho-MET and phospho-HER2 in MKN-45 and N87 cells, respectively (Figure 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 (Figure 5B-C). Sorafenib and sunitinib similarly down-regulated FGFR2 signaling in KATO-III and OCUM-2M cell lines (Figure 5A-
C). 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 (Figure 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 (Figure 4 and 5B). 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 GC cases, tumor specimens from 482 AGC patients were analyzed by qPCR using TaqMan probes and dual-color FISH (Figure 6). Of the 482 patients, 24 (5%) of the patients had FGFR2 copy numbers greater than 4.0 copies. FISH and qPCR analyses were also consistent in identifying FGFR2 amplification in tissue specimens, with higher-fold amplification apparent by FISH (Figure 6). This presumably reflects the effect
of low-level copy-number variability in the control locus used in qPCR analysis, resulting in underestimation of the true extent of FGFR2 amplification. However, FGFR2 amplification was not associated with clinicopathological parameters such as age, sex, histology, Bormann type, Lauren classification, tumor location or 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 GC and is closely associated with sensitivity to pazopanib. Pazopanib is an orally bioavailable, ATP-competitive, multi-targeted kinase inhibitor 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). Based on 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 biological sequences of multiple kinase activities beyond anti-angiogenesis 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 PDGFRα mutations that are largely confined to GISTs (24). In an in vitro kinase assay, pazopanib had modest activity against FGFR2 kinase with nanomolar concentrations expecting anti-tumor activity in tumors having dysregulated FGFR2 signaling (21).

In this preclinical study, pazopanib sensitivity was restricted to a small number of GC 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 GC cell lines, which argues in favor of amplification rather than mutation as the preferred mechanism of FGFR2 activation in a subset of GC. 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 GC 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 GC specimens might be a possible impediment in developing \(FGFR2\)-targeted therapeutics in GC. Moreover, because a large-scale analysis of \(FGFR2\) status has not been performed in gastric cancer tissues or DNA samples, the cut-off 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 (Figure 2A), it is presumed that the relatively lower level of amplification in SNU-16 & HSC-39 (Figure 1B) may be related to somewhat lower response to pazapainib. Therefore, a predictive role of \(FGFR2\) amplification for treatment response according the cut-off value should be further validated as a novel therapeutic target in prospective clinical studies. In addition, given that pazapainib is less potent against the FGFRs versus the VEGFRs, it is important whether an efficacious concentration of pazapainib can be achieved in gastric cancer patients. Although no pharmacokinetic (PK) data on pazapainib in GC patients are available, steady-state exposure plateaued at doses \(\geq 800\) mg/day and was \(\geq 15 \mu g/ml \approx 34 \mu M\) 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 $C_{\text{tough}}$ plasma pazopanib concentration of $\geq 15 \, \mu\text{g/ml}$ also appeared to correlate with clinical activity in patients with RCC, which could be effective for GC patients with $FGFR2$ amplification as well. A phase II trial is currently being conducted involving pazopanib combined with capecitabine and oxaliplatin (CapeOX) in AGC (ClinicalTrials.gov Identifier: NCT01130805). Of 66 patients enrolled in this investigator-sponsored trial as of July 2014, three 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 GC patients 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 GC 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.
References


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Figure legends

Figure 1. (A) Sensitivity of 38 gastric cancer cell lines to multi-targeted kinase inhibitors. Sensitivity is measured by MTS assay after treatment of multi-targeted agents for 72 hours. IC$_{50}$ value is calculated from at least three independent experiments. (B) Amplification of $FGFR2$ measured by qPCR in 38 gastric cancer cell lines. Each column represents the average of three independent experiments. Error bars indicate standard deviations. (C-H) $FGFR2$ amplification is also detected by dual-color FISH ($FGFR2$, red/CEP10, green) in gastric cancer cell lines; cells with $FGFR2$ amplification (C, KATO-III; D, SNU-16; E, OCUM-2M; F, HSC-39) and cells with no $FGFR2$ amplification (G, MKN-45; H, N87).

Figure 2. (A) Effect of pazopanib on cell growth in gastric cancer cell lines with various genetic alterations. Pazopanib has differential effects on survival of gastric cancer cell lines according to $FGFR2$ amplification status. Sensitivity to pazopanib is well correlated with $FGFR2$ amplification status. Sensitivity is measured by MTS assay after treatment of pazopanib for 72 hours and IC$_{50}$ value is calculated from at least three independent experiments. (B-C) Effect of either sunitinib or sorafenib on IC$_{50}$ value and cell growth in gastric cancer cell lines with various genetic alterations. Treatment with either sorafenib or sunitinib shows similar significant growth inhibition in the GC lines harboring $FGFR2$ amplifications, when compared to GC lines without $FGFR2$ amplifications. Sensitivity is measured by MTS assay after treatment of either sunitinib or sorafenib for 72 hours and IC$_{50}$ value is calculated from at least three independent experiments.

Figure 3. (A) Ectopic expression of $FGFR2$ WT and $FGFR2$ S252 mutation in NIH 3T3 cells is correlated with sensitivity to PD173074 and pazopanib. Treatment of the FGFR2-expressing NIH 3T3 cells with 1μM of PD173074 or pazopanib significantly inhibits cell growth as compared with the control. (B-C) Treatment with the indicated concentrations of PD173074 or pazopanib significantly inhibits colony formation by the $FGFR2$-expressing NIH 3T3 cells, when compared to the control cell. All experiments were repeated three times.

Figure 4. Pazopanib induces cell cycle arrest and apoptosis in the $FGFR2$-amplified cells in cell cycle analysis. KATO-III and OCUM-2M, representative of the $FGFR2$-amplified cells, undergo G1-S and G2-M arrest in response to pazopanib and show a sub-G1 peak following pazopanib exposure. However, there is no significant effect on cell cycle progression and sub-G1 peak in cells without
FGFR2 amplification after treatment of pazopanib. All experiments were repeated three times.

**Figure 5.** (A) Pazopanib effectively inhibits FGFR2 signaling and induces apoptosis in the FGFR2-amplified gastric cancer cell lines. A phospho-RTK array reveals that phospho-FGFR2 is selectively suppressed in the presence of pazopanib in FGFR2-amplified KATO-III cells, while phospho-MET or phospho-HER2 was not inhibited in MET-amplified or HER2-amplified cells. Each cell line was treated with 1 μM pazopanib, 2 μM sorafenib or 2 μM sunitinib for six hours. In the array, each RTK is spotted in duplicate and hybridization signals at the corners serve as controls. (B) Pazopanib effectively abrogates FGFR2 signaling and Akt phosphorylation in OCUM-2M cells. (C) There is a marked increase in the cleaved PARP (89 kDa) product in OCUM-2M after both pazopanib and sorafenib treatment. The cells were exposed to the indicated concentrations of either pazopanib, sorafenib or sunitinib for six hours. For PARP, the cells were treated with each drug for 24 hours. The cells were lysed and the proteins were separated by western blotting, transferred to a nitrocellulose membrane, and probed with the indicated antibodies. Cell extracts were also immunoprecipitated with an antibody to FGFR2. The precipitated proteins were determined by immunoblotting with the indicated antibodies.

**Figure 6.** (A-D) FGFR2 amplification is also detected by dual-color FISH in the specimens from gastric cancer patients; 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.
Figure 1. Kim et al.
Figure 2. Kim et al.
Figure 3. Kim et al.
Figure 4. Kim et al.
Figure 5. Kim et al.
Figure 6. Kim et al.
Pazopanib, a novel multi-targeted kinase inhibitor, shows potent in vitro antitumor activity in gastric cancer cell lines with FGFR2 amplification

Seung Tae Kim, Hye-Lim Jang, Su Jin Lee, et al.

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