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# Upregulation of EphB3 in gastric cancer with acquired resistance to a FGFR inhibitor

Suk-young Lee[a,1], Yoo Jin Na[a,1], Yoon A Jeong[a], Jung Lim Kim[a], Sang Cheul Oh[a,**], Dae-Hee Lee[a,b,*]

[a] Division of Oncology/Hematology, Department of Internal Medicine, College of Medicine, Korea University, 148 Gurodong-ro, Guro-gu, Seoul 08308, Republic of Korea
[b] Brain Korea 21 Program for Biomedicine Science, College of Medicine, Korea University, 148 Gurodong-ro, Guro-gu, Seoul 08308, Republic of Korea

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ABSTRACT

Amplification of *fibroblast growth factor receptor2 (FGFR2)* has been regarded as a druggable target in gastric cancer (GC). Despite known potential of AZD4547, a selective inhibitor of FGFR 1–3, to suppress tumorigenic effects of activated FGFR2, resistance to the targeted agent has been an unresolved issue. This study was performed to elucidate the mechanism of AZD4547 resistance in GC cells. SNU-16 cells were used to establish an AZD4547-resistant GC cell line, SNU-16R. Elevated phosphorylation of EphB3 was confirmed using the Human Phospho-Receptor Tyrosine Kinase Array kit. A tyrosine kinase inhibitor (TKI) of EphB3 was used to investigate the effects of suppressed EphB3 activity in the SNU-16R cell line. SNU-16R cells exhibited upregulated phosphorylation of EphB3. Treatment of SNU-16R cells with the EphB3 TKI resulted in induction of apoptosis, decreased cellular viability, and cell cycle arrest at sub-G1 phase. SNU-16R cells expressed upregulated levels of N-cadherin, vimentin, Snail, matrix metalloproteinase 2 (MMP-2), and MMP-9, and reduced levels of E-cadherin, characteristic of epithelial to mesenchymal transition (EMT). Matrigel invasion assay also demonstrated the increased invasiveness of SNU-16R cells. EphB3 TKI treatment inhibited EMT of SNU-16R cells. Activation of mammalian target of rapamycin (mTOR) through the Ras-ERK1/2 pathway was suggested as the signal transduction mechanism downstream EphB3 by showing enhanced phosphorylation of Raf-1, MEK1/2, ERK1/2, mTOR and its downstream substrates in SNU-16R cells. As expected, EphB3 TKI decreased phosphorylation of these proteins. Our data suggest phosphorylation of mTOR through signaling by EphB3 is a potential mechanism of AZD4547 resistance in GC cells.

## 1. Introduction

Gastric cancer (GC) is one of difficult cancers to treat in terms of its poor prognosis and limited applicability of anti-cancer therapeutic agents. Amplification of *fibroblast growth factor receptor2 (FGFR2)* has been one of genomic aberrations to be targeted, for its role as a driver of tumorigenesis and its relatively frequent incidence in GC (Cancer Genome Atlas Research, 2014; Hierro et al., 2017; Touat et al., 2015). FGFR is a receptor tyrosine kinase (RTK) family comprising four members, FGFR1, FGFR2, FGFR3, and FGFR4 (Eswarakumar et al., 2005). FGFR signaling is initiated by interaction with 23 different FGF ligands, and it is involved in many cellular processes, including proliferation, differentiation, migration, angiogenesis, and embryonic development (Eswarakumar et al., 2005; Klint and Claesson-Welsh, 1999; Touat et al., 2015).

Several *in vitro* and *in vivo* studies have shown a critical role of amplified *FGFR2* in the carcinogenesis of GC. A previous study reported that suppression of FGFR2 kinase activity by a specific small-molecule inhibitor resulted in growth inhibition and induction of apoptosis in a series of *FGFR2*-amplified GC cell lines (Kunii et al., 2008). Suppression of tumor growth by oral administration of a TKI to GC mouse xenograft models overexpressing FGFR2 supported the role of FGFR2 signaling in tumorigenesis (Takeda et al., 2007). In addition, tumor growth of xenografts of SNU-16 GC cells harboring *FGFR2* amplification was inhibited by intraperitoneal injection of a monoclonal antibody against FGFR2 (Zhao et al., 2010). Analyses of clinical data again suggested the

* Corresponding author at: Graduate School of Medicine, Korea University Guro Hospital, 148 Gurodong-gil, Guro-gu, Seoul 08308, Republic of Korea.
** Corresponding author.
*E-mail addresses:* totokee777@naver.com (S.-y. Lee), wing1278@naver.com (Y.J. Na), leomi2614@naver.com (Y.A. Jeong), clickkjl@naver.com (J.L. Kim), sachoh@korea.ac.kr (S.C. Oh), humyam@daum.net, neogene@korea.ac.kr (D.-H. Lee).
[1] These authors equally contributed to this work.

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potential of FGFR2 as a therapeutic target in treatment of GC by showing poor prognosis in patients with the genomic aberrance of *FGFR2* (Betts et al., 2014; Su et al., 2014).

Given the considerable devotion of deregulated FGF/FGFR signaling to the oncogenesis of GC, many anticancer agents targeting the signaling pathway have been developed. AZD4547, one of selective FGFR inhibitors that inhibits tyrosine kinase activity of FGFR 1–3, has demonstrated efficacy in tumor control of FGFR-deregulated *in vivo* models and cell lines (Gavine et al., 2012; Xie et al., 2013). Although the potential antitumor activity of AZD4547 has been supported by preclinical data and has culminated into clinical trials, development of resistance to small-molecule inhibitors by cancers remains an issue (Hierro et al., 2017; Van Cutsem et al., 2017). Several previous studies have tried to clarify the mechanisms of resistance against FGFR inhibitors in FGFR-dependent cell lines. Development of mutations in the FGFR2 kinase domain resistant to FGFR inhibitors is one of suggested resistance mechanisms. A previous study reported that 14 mutations resistant to dovitinib were identified in FGFR2-overexpressing BaF3 cells after culture with the pan-FGFR inhibitor (Byron et al., 2013). Chell et al. identified a heterozygous mutation, V555M, at the gatekeeper residue of FGFR3, cross-resistant to AZD4547 in KMS-11 myeloma cells (Chell et al., 2013). In addition to acquiring somatic mutations, activation of alternative RTKs and subsequent intracellular signal transduction leading to resistance to FGFR inhibitors is another often reported theory. *in vitro* studies of *FGFR2*-amplified GC cells showed that cells with multiple activated RTKs were hyposensitive to AZD4547 (Chang et al., 2015). A high-throughput platform assay for secreted proteins also demonstrated bypass signaling pathways turned on by ligand-mediated activation of alternative RTKs (Harbinski et al., 2012).

The Eph receptor, a RTK family comprising 16 receptors, is known to play a crucial role in embryonic development by directing cell migration and positioning, axon guidance, and tissue morphogenesis (Park and Lee, 2015). Although the role of Eph receptor-ephrin signaling in oncogenesis is still controversial, several studies report aberrant expression of Eph receptor in various cancers and implicate its involvement in cancer progression (Ji et al., 2011; Miyazaki et al., 2013; Zelinski et al., 2001). A previous study reported overexpression of EphB3 in non-small cell lung cancer (NSCLC) and its contribution to cell growth and migration using the EphB3 overexpressing *in vivo* model, indicating the involvement of EphB3 signaling in promoting epithelial to mesenchymal transition (EMT) (Ji et al., 2011). EMT is a biological process by which epithelial cells with polarity gain the phenotype of mesenchymal cells through multiple biochemical transformations, rendering those cells to obtain the capacity of migration and invasiveness (Kalluri and Weinberg, 2009). EMT has been shown to play a function in development of resistance to various TKI in cancer cells (Rho et al., 2009; Yauch et al., 2005). In fact, a previous *in vitro* study reported EMT-mediated resistance in AZD4547-treated *FGFR2*-amplified GC cells, although definitive alternative signaling pathways that induced EMT were not elucidated (Grygielewicz et al., 2016).

Acquiring resistance to the FGFR inhibitor may determine treatment outcomes in patients with GC dependent on *FGFR2* amplification, and we investigated the mechanism of AZD4547 resistance in *FGFR2*-amplified GC cells. We established a *FGFR2*-amplified GC cell line resistant to the selective FGFR inhibitor, AZD4547. We observed elevated phosphorylation of EphB3 and a pattern of expression levels of various proteins characteristic of EMT. Treatment with an EphB3 TKI abrogated the expression pattern, suggesting the EMT-mediated resistance to AZD4547 was due to activation of EphB3. Signaling through EphB3 was observed to be transmitted to mTOR via the Ras-ERK1/2 pathway, supporting use of mTOR inhibitors as one of therapeutic strategies to overcome resistance to the selective FGFR inhibitor.

## 2. Material and methods

### 2.1. Cell culture

AGS, MKN28 and MKN45, human GC cell lines, and COLO 205, a human colon cancer cell line, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and SNU638, SNU-16 and KATOIII were purchased from the Korea Cell Line Bank (Seoul, Korea). All cell lines were grown in RPMI 1640 (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS) and l-glutamine at 37 °C in a humidified chamber with 5% $CO_2$.

### 2.2. Reagents and antibodies

AZD4547 was provided by AstraZeneca (Luton, UK). Everolimus was purchased from Selleckchem (Houston, TX, USA). LDN-211904 was purchased from Calbiochem (San Diego, CA, USA). LDN-211904 is a potent and reversible EphB3 TKI profiled for inhibitory activity against a panel of 288 kinases but quite selective for tyrosine kinases, among them EphB3 (Qiao et al., 2009). Anti-FGFR2, anti-phospho-FGFR2, anti-PTEN, anti-phospho-PTEN, anti-Akt, anti-phospho-Akt, anti-ERK1/2, anti-phospho-ERK1/2, anti-mTOR, anti-phospho-mTOR, anti-phospho-p70-S6K1, anti-phospho-4E-BP1, anti-cleaved PARP, anti-MMP-2, and anti-MMP-9 were obtained from Cell Signaling (Beverly, MA, USA). Anti-pan-Ras, anti-Raf-1, anti-phospho-Raf-1, anti-MEK1/2, anti-phospho-MEK1/2, and anti-Snail were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-E-cadherin and anti-*N*-cadherin were obtained from BD Biosciences (San Diego, CA, USA). Anti-actin, anti-EphB3 and anti-phospho-EphB3, and anti-vimentin were purchased from Sigma (St. Louis, MO, USA), Abcam (Cambridge, MA, USA), and Dako (Kyoto, Japan), respectively.

### 2.3. Generation of the AZD4547-resistant SNU-16 cell line, SNU-16R

SNU-16 GC cells were maintained in liquid cultures and exposed to gradually increasing concentrations of AZD4547, starting with a concentration of 10 nM to a final concentration of 1 μM. The parental cell line was maintained without AZD4547 to be used as a control.

### 2.4. Human phospho-RTK array kit

Phospho-RTK Assay Kit (ARY001B) was obtained from R&D System (Minneapolis, MN, USA). Levels of tyrosine phosphorylation of human RTKs were detected by X-ray film using the kit according to the manufacturer's instructions. Briefly, cell lysates (100 μg total protein) were incubated with nitrocellulose array membranes at 4 °C overnight. Next, each array was washed and incubated with a cocktail of anti-phosphotyrosine antibody conjugated to horseradish peroxidase (HRP) for 2 h. Levels of phosphorylated tyrosine on activated receptors were detected by chemiluminescent reaction.

### 2.5. RNA preparation and real-time polymerase chain reaction (PCR)

Total RNA was extracted using TRIzol reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. Amplification of transcripts was performed by reverse transcriptase polymerase chain reaction (Life Technologies, Gaithersburg, MD). Real time-PCR was performed on an Applied Biosystems QuantStudio 6 Flex using Taqman probes (Applied Biosystems). Taqman probes were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), FGFR2 (Hs01552918_m1), MMP-2 (Hs01548727_m1), and MMP-9 (Hs00234579_m1). For expression of mRNA, gene expression was normalized to GAPDH.