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Antitumor Effect of AZD4547 in a Fibroblast Growth Factor Receptor 2–Amplified Gastric Cancer Patient–Derived Cell Model[1]

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Abstract

BACKGROUND: FGFR2 amplification is associated with aggressive gastric cancer (GC), and targeted drugs have been developed for treatment of GC. We evaluated the antitumor activity of an FGFR inhibitor in FGFR2-amplified GC patients with peritoneal carcinomatosis. *METHODS:* Two GC patients with FGFR2 amplification confirmed by fluorescence *in situ* hybridization showed peritoneal seeding and malignant ascites. We used the patient-derived xenograft model; patient-derived cells (PDCs) from malignant ascites were used to assess FGFR2 expression and its downstream pathway using immunofluorescence analysis and immunoblot assay *in vitro*. Apoptosis and cell cycle arrest after treatment of FGFR inhibitor were analyzed by Annexin V-FITC assay and cell cycle analysis. *RESULTS:* FGFR2 amplification was verified in both PDC lines. AZD4547 as an FGFR inhibitor decreased proliferation of PDCs, and the IC_{50} value was estimated to be 250 nM in PDC#1 and 210 nM in PDC#2. FGFR inhibitor also significantly decreased levels of phosphorylated FGFR2 and downstream signaling molecules in FGFR2-amplified PDC lines. In cell cycle analysis, apoptosis was significantly increased in AZD4547-treated cells compared with nontreated cells. The proportion of cells in the sub-G1 stage was significantly higher in AZD4547-treated PDCs than in control cells. *CONCLUSION:* Our findings suggest that FGFR2 amplification is a relevant therapeutic target in GC with peritoneal carcinomatosis.

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Introduction

Despite advances in targeted therapy for gastric cancer (GC) and an increase in early diagnoses, the prognosis of metastatic GC is still poor, and GC remains the second major cause of cancer death [1,2]. A majority of GC patients are admitted with advanced, inoperable, or metastatic tumors, and the 5-year survival rate of patients with such advanced GC is only 3.1% [3,4]. Peritoneal carcinomatosis (PC) accounts for 30% to 40% of the metastatic GC cases, and the peritoneum is the second most common tumor site after the liver [5,6]. PC can cause bowel obstruction or malignant ascites and thus decrease the quality of life. Furthermore, as PC in GC is difficult to treat with conventional therapy, its prognosis is grave, and the median survival is estimated to be 1 to 3 months [7,8].

Fibroblast growth factor receptors (FGFRs) are widely distributed transmembrane tyrosine kinase receptors that mediate development, differentiation, cell survival, migration and angiogenesis, as well as carcinogenesis [9,10]. FGFR2 mutation, gene fusion, and gene amplification can cause progression of several types of cancers and resistance for drugs that target other oncogenic pathways [11–13].

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According to recent study of FGFR aberration in 4835 solid tumors, FGFR2 mutations were found in 1.5% of cancers, and most aberrations were gene amplification [14]. These discoveries have spurred the development of anti-FGFR therapeutics for the treatment of various cancers [15].

In GC, multiple FGFR alterations also have been identified [16,17]. Overexpression of the FGFR2 protein was demonstrated in the diffuse type of GC and correlated with aggressive behavior [18]. Many preclinical studies have suggested that FGFR2 amplification was associated with increased sensitivity to FGFR inhibitors both *in vitro* and *in vivo*, which led to an increased application of FGFR inhibitors for GC treatment [18–20]. A randomized phase II trial that compared the efficiency of AZD4547 and paclitaxel as second-line treatment methods for advanced GC and gastroesophageal junction cancer with FGFR2 amplification or polysomy has been completed (NCT01457846). However, the results of the interim analysis did not show any increase in survival benefit compared to paclitaxel [21,22]. Recently, a monoclonal antibody against FGFR2b (FPA144) in FGFR2-amplified GC demonstrated a response rate of over 30% in salvage setting [23].

In this study, we established an FGFR2-amplified GC patient-derived cell (PDC) line and evaluated the antitumor efficacy of FGFR inhibitors.

Material and Methods

Patients

Malignant ascites were collected from patients after they signed the informed consent form provided by the SMC Institutional Review Board. The collected effusions (1-5 l) were distributed into 50-ml tubes, centrifuged at 1500 rpm for 10 minutes, and washed twice with PBS, as previously described [18]. Cell pellets were resuspended in the culture medium and were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% antibiotic-antimycotic (Gibco BRL, Paisley, UK), 0.5 g/ml of hydrocortisone (Sigma Aldrich), 5 mg/ml of insulin (PeproTech, Rocky Hill, NJ), and 5 ng of EGF (PeproTech). Cells were maintained at 37°C in a humidified 5% CO_2 incubator, and the medium was changed twice a week. PDCs were passaged using TrypLE Express (Gibco BRL, Paisley, UK) to detach cells when the culture achieved 80% to 90% confluence.

Fluorescence In Situ Hybridization (FISH)

Tumor sections were cut to 1-μm thickness and deparaffinized by incubation for 30 minutes with the pretreatment reagent (Abbott, 30-801250) at 80°C. Protease digestion was achieved by treatment with the protease reagent (Abbott, 30-801255) for 20 minutes at 37°C. *FGFR2* probes (LSI *FGFR2* Spectrum Orange Probe, 08N42-020) and CEP 10 (Spectrum Green Probe, 06J37-020) from Vysis (Abbott Molecular, IL) were hybridized at 73°C for 5 minutes and then at 37°C for 20 hours. After hybridization, slides were washed in 2× saline-sodium citrate/0.3% NP-40 at 72°C for 5 minutes, air dried, and counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) I and DAPI II (Abbott Molecular). Slides were then examined under a fluorescence microscope equipped with Spectrum Texas Red with isothiocyanate and DAPI filters. The *FGFR2*/CEP 10 ratio was estimated after counting at least 50 tumor cell nuclei. For all samples evaluated with FISH, IHC-negative stained areas in the tumors were also evaluated to determine the specificity of the IHC test. An *FGFR2*/CEP 10 ratio higher than 2.0 was interpreted as gene amplification positive. If the *FGFR2* copy number was greater than 4 in the absence of gene amplification, *FGFR2* polysomy was assumed.

Immunofluorescence

Cells were grown on four-chamber slides in an appropriate growth medium supplemented with 10% FBS for 5 days and then fixed with 4% paraformaldehyde for 20 minutes at room temperature. After washing three times with PBS, cells were treated with 0.25% Triton X-100 in PBS for 15 minutes at room temperature, blocked with 5% BSA in PBS for 30 minutes, and incubated overnight at 4°C with specific primary antibodies FGFR2 (1:25) from Cell Signaling Technology (Beverly, MA) and Phalloidin (1:100) from Thermo Fisher Scientific (Paisley, UK). After this incubation, samples were incubated for 1 hour at room temperature with the corresponding secondary antibodies (Alexa Fluor 488 donkey anti-rabbit, Thermo Fisher Scientific) and mounted. DAPI images were acquired with a confocal laser scanning microscope (LSM 780, Zeiss, Jena, Germany).

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Primers for amplifying *FGFR2* were constructed based on the PubMed sequence NM_000141.2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers obtained from Bioneer were used as the internal control. The reaction conditions were as follows: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. Real-time PCR amplifications were performed using an ABI PRISM 7000 cycler (Applied Biosystems), and threshold cycle numbers were transformed using the ΔCT comparative method. Each sample was analyzed in triplicate. Gene-specific expression values were normalized to the expression value of GAPDH within each sample. The amount of target, normalized to an endogenous control and relative to a calibrator, was determined by the comparative CT method (ΔΔCT). In brief, the ΔCT value was determined by subtracting the average of GAPDH CT values from the average of the target gene values for each sample. The ΔΔCT calculation involves subtraction of the ΔCT calibrator value.

Cell Proliferation Inhibition Assay

Cells (7×10^3 cells in 100 μl/well) were seeded on 96-well plates, incubated for 24 hours at 37°C, and treated with AZD4547 for 5 days at 37°C. After this drug treatment, 100 μl of the CTG reagent (CellTiter-Glo, Promega, Madison, WI) was added to each well. The luminescence signal was recorded using the Mithras^2 LB 943 Monochromator Multimode Reader (BERTHOLD TECHNOLOGIES GmbH & Co. KG, Germany).

Immunoblot Analysis

Total cell extracts were obtained using the RIPA buffer (Thermo Fisher Scientific, Waltham, MA) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) and a phosphatase inhibitor cocktail (Roche). Equal amounts (30 μg) of cell lysates were dissolved in 8% or 12% Bis-Tris gels with MOPS running buffer (Thermo Fisher Scientific), transferred onto nitrocellulose membranes, and incubated with antibodies against the following: pFGFR1/2 (Y463/Y466, cat # PA5-37816, ThermoFisher, USA), FGFR2 (cat # 11835, Cell

Signaling, MA, USA), pAkt (Ser473, cat # 9018, Cell Signaling), Akt (cat # 9272, Cell Signaling), pMEK (cat # 2338, Cell Signaling), MEK (cat # 9122, Cell Signaling, pERK (Thr202/Tyr204, cat # 4370, Cell Signaling), ERK (Thr202/Tyr204, cat # 9102, Cell Signaling), and beta actin (Sigma Aldrich). Primary antibody diluted 1:1000 in 3% BSA and beta-actin diluted 1:5000 in 3% nonfat dry milk. Immune complexes were visualized by enhanced chemiluminescence using the ECL Western Blotting Substrate (Thermo Fisher Scientific).

Annexin V Assay

Cells (1×10^6 cells/well) were seeded in a 60-mm dish and incubated for 24 hours before treatment with 1 μM AZD4547 for 5 days. After washing twice with PBS, the cells were stained using the Annexin V-FITC/Propidium iodide apoptosis kit (BD Bioscience, San Jose, CA) according to the manufacturer's instructions. Stained cells were detected and analyzed using FACSverse (Becton Dickinson, San Jose, CA).

Cell Cycle Analysis

For cell cycle analysis, cultured cells were removed with trypsin, fixed with 70% ethanol, and incubated for 20 minutes on ice. They were then stained with propidium iodide (20 μg/ml of propidium iodide, 200 μg/ml of DNase-free RNase A, and 0.1% triton X-100, prepared fresh in PBS). Cellular DNA complement was analyzed using FACSverse.

Statistical Analysis

The statistical significance of differences in cell growth, apoptosis, and cell cycle between different groups was calculated using Student's *t* test. All *P* values less than .05 were considered statistically significant. All statistical tests were two-sided.

Results

Case Presentation

Two GC patients had confirmed FGFR2-amplification. Patient #1 was a 40-year-old male who complained of epigastric soreness and dyspepsia. He was diagnosed with advanced GC without metastasis and underwent curative subtotal gastrectomy. Pathological diagnosis was poorly differentiated tubular adenocarcinoma, and the TNM stage was IIIA (pT2N3aM0) according to AJCC seventh edition. Eight cycles of XELOX (capecitabine plus oxaliplatin) as adjuvant therapy were treated. Four months after the last cycle of adjuvant therapy, he complained of back pain. Computed tomographic scan and blood tests showed multiple bone metastases, further complicated by disseminated intravascular coagulopathy and malignant ascites. The cancer was refractory to weekly paclitaxel as well as irinotecan monotherapy. The FGFR2/CEP 10 ratio was 36, which was verified using FISH (Figure 1). A PDC line was established from the malignant ascites after obtaining informed consent (Figure 2).

Patient #2 was a 63-year-old male who presented with an abnormal finding in annual esophagogastroduodenoscopy screening. Endoscopic biopsy showed tubular adenocarcinoma, and peritoneal seeding was detected at the surgical field leading to open and closure. The disease progressed rapidly and was refractory to capecitabine/oxaliplatin chemotherapy. After four cycles of XELOX, the patient developed medically intractable malignant ascites and died of the disease. The patient's tumor was confirmed to have FGFR2 amplification by FISH with FGFR2/CEP 10 ratio of 42 (Figure 1). The PDC line was established from malignant ascites after obtaining informed consent (Figure 2).

Confirmation of FGFR2 Amplification in PDCs from Malignant Ascites of GC

We performed immunofluorescence analysis for detection of FGFR2 expression in the cultured cell lines, as shown in Figure 3. Next, we tested the degree of FGFR2 gene amplification and protein expression in the established PDC lines. The FGFR2 expression values obtained from quantitative RT-PCR were normalized to GAPDH values within each sample (Figure 4*A*). We found that the FGFR2 gene was amplified in PDCs of GC in contrast to the negative control. To evaluate the antitumor effect of AZD4547, a novel selective FGFR inhibitor, FGFR2-amplified PDCs were treated for 5 days, after which cell viability was assessed using the CellTiter-Glo reagent. Figure 4*B* shows that AZD4547 inhibited the proliferation of PDCs, and the IC_{50} values were estimated to be 250 nM in PDC#1 and 210 nM in PDC#2.

To investigate the FGFR-signaling activity of AZD4547 in the PDCs, we examined the expression of FGFR2 and its downstream molecules in FGFR2-amplified PDCs using immunoblot assay. We performed the immunoblot assay after incubation with 1 μM of

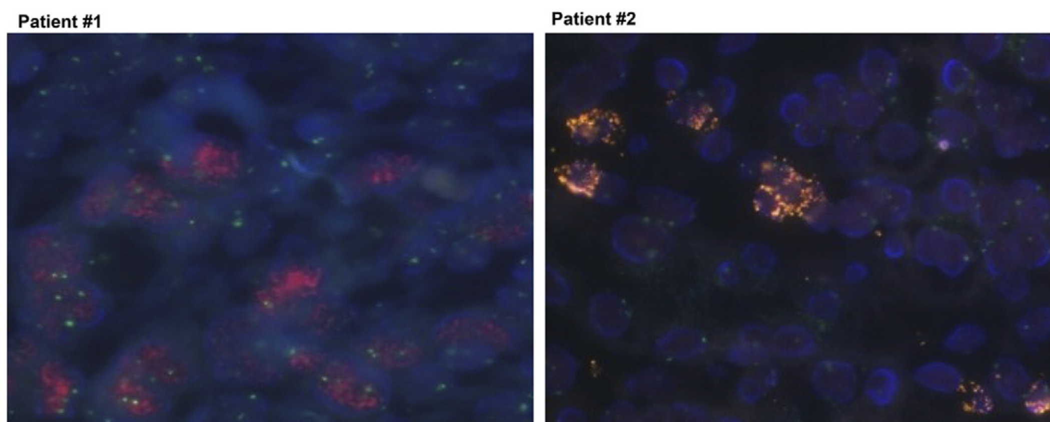

Figure 1. FGFR2 amplification in PDCs by FISH showing high-level amplification of target probe (red signal) to CEP-10 (green signal).

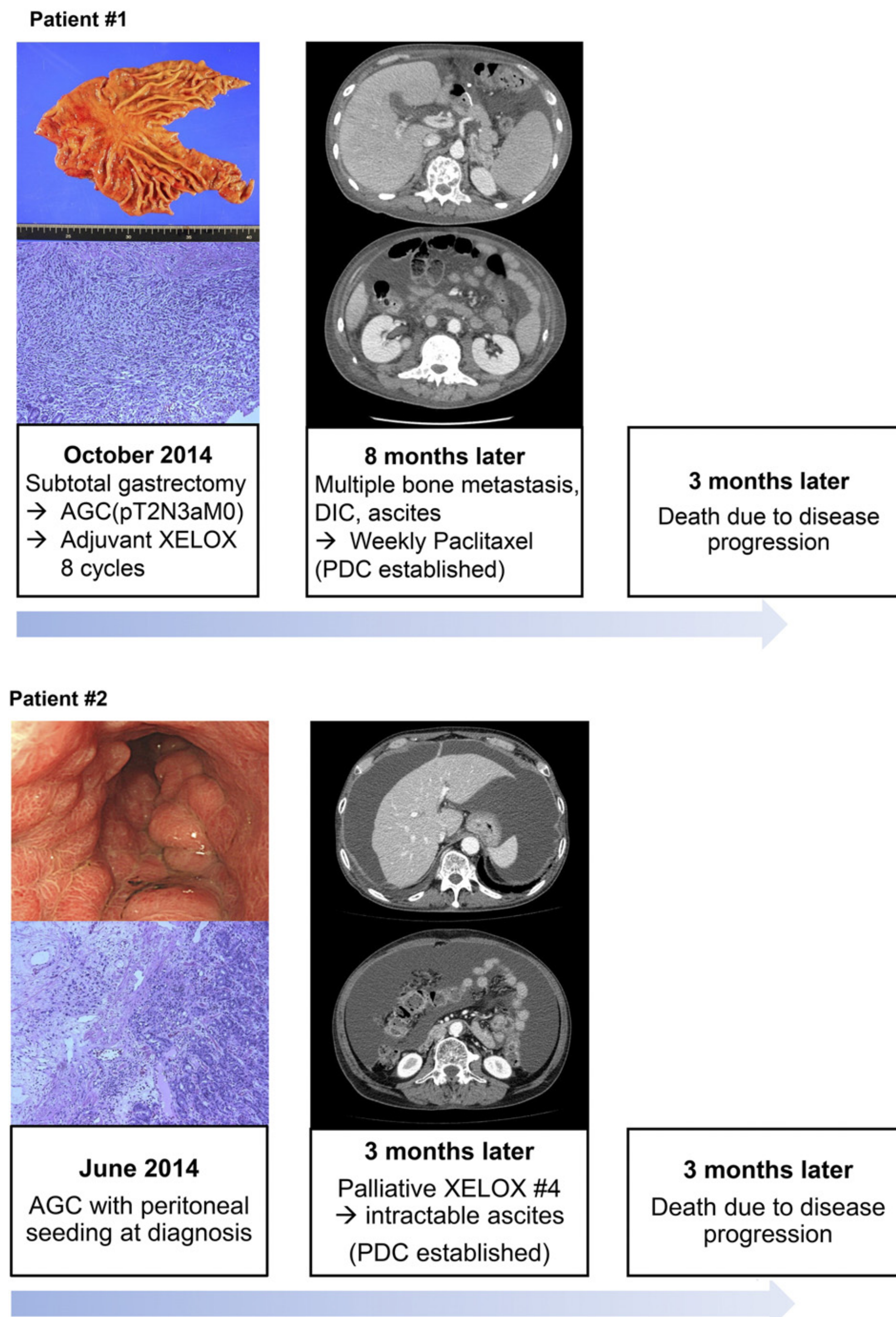

Figure 2. Clinical features of two cases showing illustrations of clinical courses. Left panel demonstrated gross and microscopic images, and computed tomographic scan showed malignant ascites (middle panel).

AZD4547. We evaluated levels of phosphorylated FGFR2 and its downstream signaling molecules including Akt, MEK, and ERK1/2. Treatment of the PDCs with AZD4547 attenuated the phosphorylation of downstream proteins, as levels in the treated cells were reduced compared with those in the control (Figure 4*C*).

Apoptosis and Cell Cycle Arrest Were Increased by AZD4547

We also assessed the effect of AZD4547 on apoptosis of PDCs using Annexin V-FITC assay. Annexin V-FITC/PI labeling in cells was quantitatively measured by flow cytometry. Compared with nontreated cells, AZD4547-treated cells showed a consistent and statistically significant ($P < .0001$) increase in apoptosis (PDC#1: nontreated versus AZD4547-treated, mean apoptosis rate = 2.33 ± 0.41% and 13.94 ± 0.217%, respectively, $P < .0001$; PDC#2: nontreated versus AZD4547-treated, mean apoptosis rate = 0.71 ± 0.08% and 1.36 ± 0.12%, respectively, $P < .0001$) (Figure 5*A*). We then investigated the effect of AZD4547 on cell cycle progression of FGFR2-amplified PDCs. Cell cycle progression was analyzed using propidium iodide and FACSverse after treatment with AZD4547 for 5 days. As shown in Figure 5*B*, the proportion of cells in the sub-G1 stage was significantly higher in AZD4547-treated PDCs than in control cells (PDC#1: nontreated versus AZD4547-treated, mean proportion of cells in the sub-G1 stage =34.45 ± 0.52% and 42.94 ± 0.63%, $P = .0002$; PDC#2: nontreated versus AZD4547-treated, mean proportion of cells in the sub-G1 stage =22.73 ± 0.57% and 40.30 ± 0.72%, $P < .0001$).

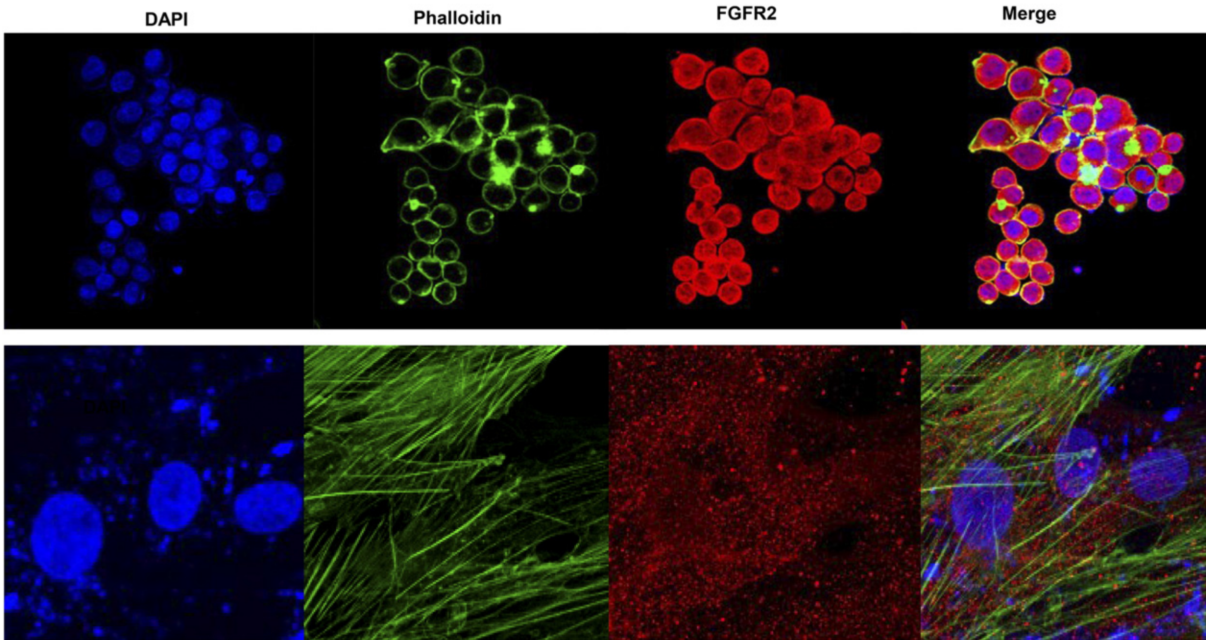

Figure 3. The blue color in the nucleus shows DAPI staining, the green color in the cytoskeleton shows phalloidin staining, and the red color is the FGFR signal. FGFR2 protein expression was observed in the cytoplasm.

Discussion

Variations in the FGFR gene have been investigated in several solid tumors including breast cancer [24], non–small cell lung cancer [25], bladder cancer [26], and glioblastomas [27]. In particular, FGFR2 amplification has been correlated with lung cancer [28], colon cancer, and GC [11]. These findings have led to the development of FGFR inhibitors such as dovitinib, BGJ398, ponatinib, LY2874455, and AZD4547. Among these agents, AZD4547 has shown great promise in studies on FGFR-dysregulated cancers [29,30]. AZD4547 also showed favorable therapeutic activity against GC with FGFR2

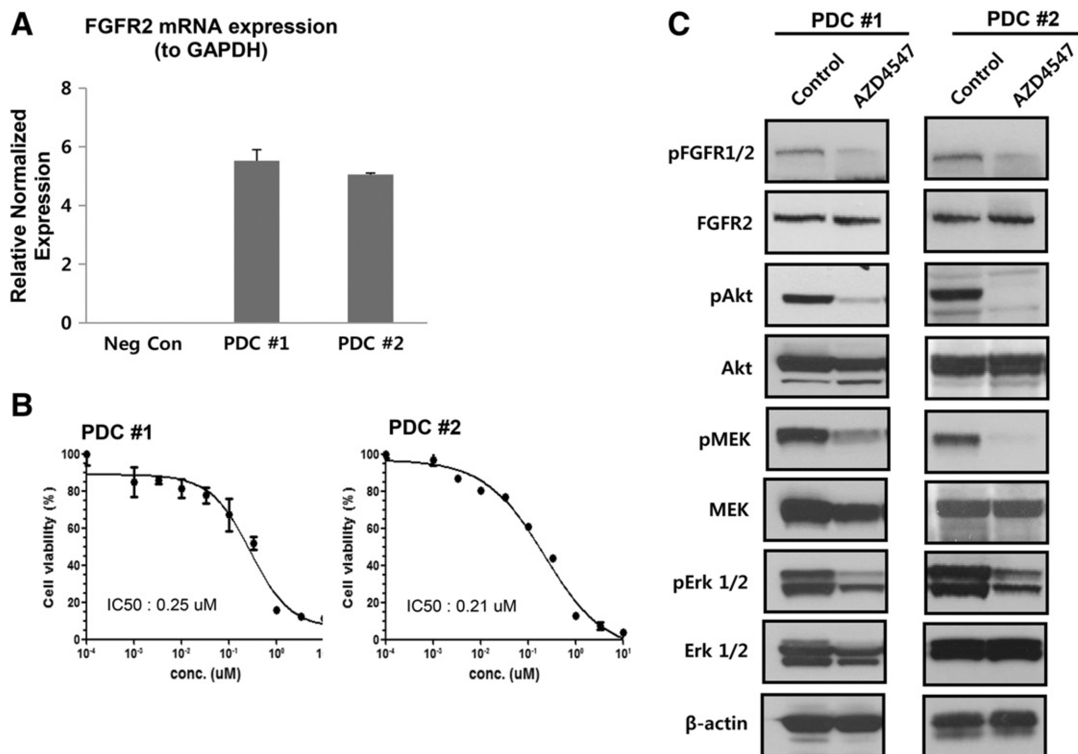

Figure 4. (A) Detection of FGFR2 gene amplification in FGFR2-amplified PDCs by quantitative RT-PCR analysis. (B) The viability of FGFR2-amplified PDCs was measured by CTG assay after treatment with various concentrations of AZD4547 for 5 days. Cell viability (%) represents the percentage of growth compared to the control (no treatment). IC_{50} values are 250 nM and 210 nM for PDC#1 and PDC#2, respectively. (C) Immunoblot assay for determining FGFR phosphorylation and targeted downstream pathways. Cells were treated with 1 μM AZD4547 for 5 days. Control cells were treated with DMSO.

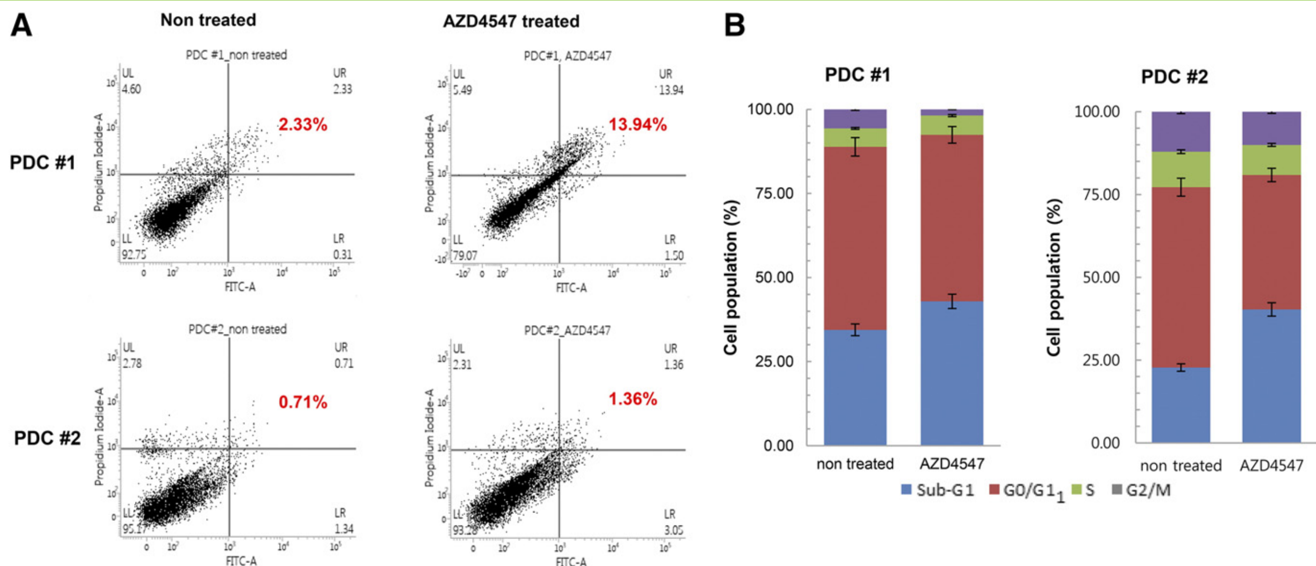

Figure 5. (A) Effect of AZD4547 on apoptosis of FGFR2-amplified PDCs. The AZD4547-treated PDC culture showed a higher apoptotic cell population (11.3%) than the control cell culture (0.65%) ($P < .001$). (B) Flow cytometry analysis of cell cycle of PDCs treated with 1 μM AZD4547 for 5 days. Cell cycle progression was analyzed using propidium iodide.

amplification *in vitro* and *in vivo* [20], and a phase II study on the efficacy of AZD4547 versus paclitaxel in advanced GC has been conducted (SHINE study, NCT01457846). However, the proportion of patients with PC was not provided in that study, and subgroup analysis of patients with PC was not performed [22]. Our results indicate that patients with advanced GC with PC might benefit from treatment with FGFR inhibitors.

Previous studies have reported that the prevalence of FGFR2 amplification in GC was 4% to 7% and that it was correlated with aggressive behavior [31,32]. However, some intratumor heterogeneity of FGFR was reported in an exploratory biomarker analysis as part of the SHINE study [22]. In our study, FGFR was shown to be markedly overexpressed in PDCs from malignant ascites of GC. This finding suggests a possible correlation between FGFR2 amplification and peritoneal metastasis. FGFR2 amplification could be one of the key signals involved in peritoneal metastasis. In addition, a low concordance between elevated FGFR2 expression and FGFR2 amplification was reported in the SHINE study. Our study also showed a discordant result for FGFR2 expression when estimated using IHC and FISH. Tissue from PDC#2 was FGFR2 negative according to IHC but was found to be FGFR2 amplified according to FISH analysis.

Peritoneal dissemination is a common cause (30%-40%) of GC recurrence after curative resection, and more than 10% of patients with *de novo* GC exhibit PC at diagnosis [33–35]. The survival period of GC patients with PC is less than 6 months because effective therapy for these patients has not yet been developed [8]. To understand the therapeutic activity of FGFR inhibitors against GC with PC, we treated FGFR2-amplified PDCs from GC with AZD4547. Patient-derived xenograft model was used in our study; this model is preferable than traditional cell lines. Patient-derived xenograft model has more similarities to the parental tumors and shows improved predictive value for preclinical studies [36,37]. We demonstrated that AZD4547 effectively blocked the phosphorylation of FGFR and its subsequent downstream proteins, induced cell-cycle arrest at the G1-S transition phase, and induced apoptosis in PDCs from malignant ascites of GC. These results imply that AZD4547 could be part of a novel therapeutic strategy for GC patients with PC.

In conclusion, our results suggest that FGFR2 amplification is a key signal in patients with PC in GC and that these patients could benefit from treatment with FGFR inhibitors such as AZD4547.

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