석 사 학 위 논 문

Anti-Tumor Effect of FGFR Inhibitor AZD4547 in Ovarian Cancer Cells

계 명 대 학 교 대 학 원

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이 논문을 석사학위 논문으로 제출함

2020년 2월

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나유란의 석사학위 논문을 인준함

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Acknowledgment

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마지막으로 멀리 떨어져있어도 늘 믿어주시고 격려해주신 부모님께 감사드립니다. 아직 철부지 없는 딸이지만 깊고 넓은 사랑으로 보듬어주신 부모님께 이 논문을 바칩니다.

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나 유 란

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1. Introduction

Ovarian cancer is the most lethal gynecologic malignancy and the 5th leading cause of cancer deaths among women in the world (1,2). Unfortunately, 70% of women with ovarian cancer are not diagnosed until advanced-stage, leading to the highest mortality (3). Cisplatin and paclitaxel are used as a first-line chemotherapeutic agents against ovarian cancer (4). However, most patients with high response to initial treatment develop recurrent tumors within 2 yr and finally acquire resistance to chemotherapy (5). Therefore, the development of new therapeutics is needed.

Fibroblast growth factor/fibroblast growth factor receptor (FGF/FGFR) is a tyrosine kinase signaling pathway. FGF family consists of twenty-two secreted proteins that bind to four FGFRs (6). FGF/FGFR signaling regulates a wide range of biological processes, including tissue regeneration, angiogenesis and embryonic development (7,8).

Recent studies have reported that FGF/FGFR signaling is also involved in tumor development. FGF/FGFR signaling regulates proliferation, differentiation, migration and apoptosis of cancer cells (9-11). FGFR aberrations are common in many types of cancer, with the majority being gene amplifications or activating mutations (10). For example, FGFR1 and FGFR2 amplification occurs in lung and breast cancer (12,13). In gastric cancer, FGFR2 amplification is highly correlated with lymphatic metastasis and poor prognosis (14).

Thus, inhibition of FGF/FGFR signaling has been considered as a new therapeutic approach to cancer, and currently a number of FGF/FGFR inhibitors were developed (15). Several studies have investigated the anti-tumor potential of FGFR inhibitors in a number of cancer models.

SU5402, FGFR1 inhibitor, inhibited cell survival in breast, lung and urothelial carcinoma cells (16-18). Similarly, PD173074, FGFR1 and FGFR3 dual inhibitor, blocked cell cycle progression and caused apoptosis in lung, breast, urothelial, head and neck carcinoma cells (17,19-21). BGJ398, FGFR1-3 inhibitor, reduced migration of urothelial and bladder cancer cells (22,23).

A previous study showed that FGFR aberrations were found in 7.1% of cancers using next-generation sequencing. Among the various types of cancer malignancies, ovarian cancer is the fifth frequently altered cancer (~9%) in FGFR activity (11). FGFR4 expression was elevated in serous ovarian tumor tissues compared to adjacent non-tumor tissues. Moreover, higher FGFR4 expression was known to be highly associated with a poorer overall survival rate of ovarian cancer patients (24). These results suggest that inhibition of FGF/FGFR signaling could be a novel therapeutic target against ovarian cancer.

Recently developed AZD4547 is an inhibitor of FGFR1-4 with potential as a targeted anti-tumor therapy (25). AZD4547 was demonstrated to promote beneficial therapeutic outcomes against diverse types of FGFR-dysregulated cancer, including glioblastoma, endometrial, lung and gastric cancer (26-29). However, little is known about the efficacy of AZD4547 against ovarian cancer. In this study, I examined the anti-tumor activity of AZD4547 in ovarian cancer cells. AZD4547 suppressed proliferation, survival, migration and stemness of ovarian cancer cells. AZD4547 also inhibited angiogenesis of human umbilical vein endothelial cells (HUVECs). These data suggest that AZD4547 is a potent anti-cancer agent for ovarian cancer treatment.

2. Materials and Methods

2.1. Cell culture and reagents:

Human ovary cancer cell lines, ovcar3, ovcar8, ES2 and A2780, were obtained from the ATCC. All cells were maintained in RPMI 1640 culture media with 10% FBS, 1% antibiotic-antimycotic at 37 °C in an incubator with 5% CO_2 atmosphere. AZD4547 was purchased from APExBIO (Houston, TX, USA).

2.2. Western blot analysis:

Harvested cells were lysed in RIPA buffer (Thermo Scientific, Boston, MA, USA) containing protease inhibitors and phenylmethylsulfonyl fluoride (Thermo Scientific, Boston, MA, USA). The lysates were centrifuged for 20 min at 7500 rpm at 4 °C and the supernatant was collected.

The lysates were quantified using BCA Protein Assay Kit (Thermo Scientific, Boston, MA, USA). Thirty μg of protein was separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). The membranes were blocked in 5% skim milk in TBS-T (10 mM Tris-HCl, 50 mM NaCl, and 0.25% Tween-20) for 1 hr at room temperature. The membranes were incubated with anti-phospho-FGFR, anti-phospho-FRS2, anti-phospho-ERK1/2, anti-pan ERK1/2 (Cell Signaling Technology, Danvers, MA, USA), anti-FGFR1 (Proteintech, Rosemont, IL, USA), anti-FGFR2, anti-FGFR3, anti-cleaved

caspase-3, anti-cleaved PARP (Abcam, Cambridge, UK), anti-FRS2 and anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies for overnight at 4 °C. The membranes were washed in TBS-T and incubated with a horseradish peroxidase-conjugated (HRP) secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). Antibody-bound proteins were detected using LAS-3000 (Fujifilm, Tokyo, Japan) with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Boston, MA, USA).

2.3. Cell cytotoxicity assay:

Cells were seeded in 96-well plates (1×10^4 cells/well). Cells were treated with AZD4547 for 48 hr. Cell viability was measured using Cell Counting Kit 8 (CCK-8, Dogindo, Nagasaki, Japan) according to the manufacturer's instructions. Briefly, CCK-8 reagent was added to the cells for 1 hr, then absorbance was measured at 450 nm using a microplate reader (BioTek, Männedorf, Switzerland).

2.4. Colony formation and soft agar colony formation assay:

Ovarian cancer cells were seeded in 6-well plates (1×10^4 cells/well). Cells were treated with AZD4547 for 1 wk. Cells were fixed in 4% paraformaldehyde (PFA) and stained with 0.1% crystal violet (in 75% ethanol). Colonies for each sample were counted and images of the stained colonies were visualized with microscope.

Soft agar colony assay was performed in 6-well plates. The bottom layer and top layer contain 0.6% agarose and 0.3% agarose, respectively.

1 x 10^4 cells were seeded to top layer. Cells were treated with 500 μM thymidine and AZD4547 for 3 wk. Colonies were stained using nitro-tetrazolium blue chloride (Sigma Aldrich, St. Louis, MO, USA) for 24 hr. Colonies were visualized using a microscope and counted using Image J software (NIH, Bethesda, MD, USA).

2.5. Immunofluorescence staining:

Ovarian cancer cells were seeded in 8-well chamber (1 x 10^4 cells/well). The cells were fixed with 4% PFA for 30 min at room temperature. Permeabilization was conducted with 0.1% Triton X-100, and non-specific binding was blocked with 1% bovine serum albumin (BSA). Staining was performed using the primary antibody for Ki-67 (Abcam, Cambridge, UK). Alexa Fluor 488-conjugated rabbit IgG (Invitrogen, Carlsbad, CA, USA) was used as a secondary antibody. Ki-67 expression was analyzed using Carl Zeiss LSM5 EXCITER fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2 .6. Fluorescence-activated cell sorting (FACS):

Cells were seeded in 60 mm plates (1 × 10^5 cells/plates). Cells were treated with AZD4547 for 48 hr. Harvested cells were labeled using FITC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. Population of dead cells were analyzed by FACSCanto Ⅱ flow cytometer (BD, Franklin, NJ, USA).

2.7. Wound healing assay:

For wound healing assay, cells were seeded in 6-well plates (1 x 10^5 cells/well). When cells were confluent, cells were wounded with sterile pipette tips, followed by treatment of 500 μM thymidine and AZD4547 for 30 hr. The migrated cells were visualized using microscope and analyzed by Image J software.

2.8. Migration and invasion assay:

Cell migration assays were carried out in transwell with 8 μm chamber (Corning, Tewksbury, MA, USA). For invasion assays, the transwell chamber filters were coated with 1 μg/ml Matrigel (Corning, Tewksbury, MA, USA). Cells were resuspended with serum-free media, added to the upper chamber, and complete media was added to the lower chambers. After 24 hr incubation, filters were fixed in 4% PFA for 10 min and stained with 0.1% crystal violet for 30 min. Migrated cells were visualized and counted using a microscope.

2.9. Reverse transcription polymerase chain reaction (RT-PCR):

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized with M-MLV reverse transcriptase (Promega, Madison, WI, USA) using 2 μg RNA. PCR amplification was performed using specific primers (Tabel 1) for

target genes and Go Taq Flexi DNA Polymerase (Promega, Madison, WI, USA). The amplified PCR product was assessed by electrophoresis on 1.2% agarose gel and visualized by ethidium bromide (Sigma Aldrich, Saint Louis, MO, USA).

2.10. Tumor sphere formation and self-renewal capacity assay:

Ovarian cancer cells were resuspended in serum free B-27/Neurobasal media (Thermo Fisher Scientific, Waltham, MA, USA) containing 20 ng/ml EGF, 10 ng/ml bFGF (R&D system, Minneapolis, MN, USA), 2 mM L-glutamine, 20 mM HEPES (Sigma Aldrich, Saint Louis, MO, USA), 2.5 μg/ml amphotericin B (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin. For sphere formation assay, 1×10^4 cells were seeded in ultra-low attachment 6-well plates (Corning, Tewksbury, MA, USA) and treated with AZD4547 for 7 days.

For self-renewal capacity assay, sphere forming cells were harvested. And 1×10^3 cells were seeded in ultra-low attachment 6-well plates and grown without AZD4547 for 7 days. The number and size of spheres were visualized and calculated using a microscope.

2.11. Matirgel tube formation assay:

Tube formation was performed in 96-well plates with pre-coated Matrigel (Corning, Tewksbury, MA, USA). Cells were plated at a seeding density of 5×10^3 cells/well with pre-treatment of AZD4547. After 2 hr incubation, serum was added to the cells. After 4 hr, tube formation was quantified by counting the number of tube branches by

using microscope.

2.12. Statistical analysis:

All data were executed with at least three independent experiments. The data were presented as the mean ± S.D. ($n \geq 3$). Student's t-test was performed for statistical analysis. p value of < 0.05 (*), < 0.01 (**), < 0.001 (***) were considered to indicate statistical significance.

Table 1. Details of PCR Primer Pair Sequences

Name	Sequences of primers	℃	bp
CD133	Forward : 5 '- GCCACCGCTCTAGATACTGC - 3' Reverse : 5' - ACAGGAAGGGAGGGAGTCAT - 3'	60	175
FGFR1	Forward : 5' - ATATGTCTGCCCCCTCTATG - 3' Reverse : 5' - AAGCCAAGATCTGCGACAGT - 3'	60	188
FGFR2	Forward : 5' - CACAGACCAACGTTCAAGCA - 3' Reverse : 5' - TTCTCCTCCTGGGGAAGATT - 3'	60	425
FGFR3	Forward : 5' - CACCGACAAGGAGCTAGAGG - 3' Reverse : 5' - TCAGTGGCATCGTCTTTCAG - 3'	60	600
FGFR4	Forward : 5' - AGCACCCTACTGGACACACC - 3' Reverse : 5' - ACGCTCTCCATCACGAGACT - 3'	60	207
GAPDH	Forward : 5' - GGCCTCCAAGGAGTAAGACC - 3' Reverse : 5' - AGGGGTCTACATGGCAACTG - 3'	60	147
NANOG	Forward : 5' - GATTTGTGGGCCTGAAGAAA - 3' Reverse : 5' - AAGTGGGTTGTTTGCCTTTG - 3'	60	155
OCT4	Forward : 5' - GTACTCCTCGGTCCCTTTCC - 3' Reverse : 5' - CAAAAACCCTGGCACAAACT - 3'	60	168
SOX2	Forward : 5' - ACACCAATCCCATCCACACT - 3' Reverse : 5' - GCAAACTTCCTGCAAAGCTC - 3	60	224

FGFR1: fibroblast growth factor receptor 1; *FGFR2*: fibroblast growth factor receptor 2; *FGFR3*: fibroblast growth factor receptor 3; *FGFR4*: fibroblast growth factor receptor 4; *NANOG*: nanog homeobox ; *OCT4*: octamer-binding transcription factor 4; *SOX2*: sex determining region Y box 2; bp: base pair.

3. Results

3.1. AZD4547 downregulates FGF/FGFR signaling in ovarian cancer cells:

Inhibitory effect of AZD4547 on FGF/FGFR signaling was examined in ovarian cancer cells. FGF/FGFR signaling was activated by serum, then effect of AZD4547 on phospho-FGFR and phospho-FRS were analyzed by western blot. As shown in Figure 1A&B, AZD4547 reduced phospho-FGFR and phospho-FRS in a dose-dependent manner in ovarian cancer cells.

ERK1/2 is known as FGF/FGFR signaling downstream molecule and plays an important role in tumor growth and survival (30,31). To investigate whether AZD4547 inactivates ERK1/2, I also checked phospho-ERK1/2 by western blot. As shown in Figure 1A&B, AZD4547 significantly reduced phospho-ERK1/2 in a dose-dependent manner in ovcar3 and ovcar8 cells. These results indicate that AZD4547 downregulates FGF/FGFRs signaling in ovarian cancer cells.

3.2. AZD4547 induces cytotoxicity in ovarian cancer cells:

Uncontrolled cell growth is a hallmark of cancer (32), and FGF/FGFR signaling is known to be involved in the growth of cancer cells (9).

To determine whether AZD4547 inhibits cell growth, cytotoxic effect of AZD4547 was measured in ovarian cancer cells. Cells were treated with AZD4547 for 48 hr, then cell viability was analyzed by CCK-8

assay. As shown in Figure 2, AZD4547 decreased ovarian cancer cell viability in a dose-dependent manner. These results suggest that AZD4547 induces cytotoxicity in ovarian cancer cells.

3.3. AZD4547 inhibits proliferation of ovarian cancer cells:

To confirm whether AZD4547 inhibits cell proliferation, colony formation assay and Ki-67 staining were performed.

For anchorage-dependent colony formation assay, ovcar3 and ovcar8 cells were treated with AZD4547 for 7 days. As shown in Figure 3A, the number of colonies was significantly reduced by AZD4547 treatment in a dose-dependent manner.

Cancer cells have the capability to grow without attachment to a substrate (33). To assess the anchorage-independent growth ability of cancer cells, soft agar colony formation assay was conducted. Ovcar3 and ovcar8 cells were treated with AZD4547 for 3 wk. As shown in Figure 3B, AZD4547 notably reduced the number of colonies in soft agar in a dose-dependent manner.

Immunofluorescence staining was performed to analyze expression level of Ki-67, a marker for cell proliferation. As shown in Figure 3E, Ki-67 expression was significantly decreased in AZD4547-treated group compared to the control group. These results indicate that AZD4547 inhibits ovarian cancer cell proliferation.

3.4. AZD4547 induces apoptosis in ovarian cancer cells:

Since FGF/FGFR signaling are known to be associated with apoptosis

of cancer cells (9), I investigated whether AZD4547 activates apoptosis signaling in ovarian cancer cells. Both ovcar8 and ES2 cells were treated with AZD4547 for 48 hr, then cleaved caspase-3 and cleaved PARP were analyzed by western blot. As shown in Figure 4A, AZD4547 induced cleavage of caspase-3 and PARP in a dose-dependent manner.

For FACS analysis, ovcar8 and ES2 cells were treated with AZD4547 for 48 hr and stained with Annexin V antibody and 7-AAD. As shown in Figure 4B&C, AZD4547 significantly increased the population of Annexin V and 7-AAD-positive ovcar8 and ES2 cells in a dose-dependent manner. These results demonstrate that AZD4547 induces apoptosis in ovarian cancer cells.

3.5. AZD4547 prevents migration and invasion of ovarian cancer cells:

Previous studies have shown that the FGF/FGFR pathway plays an important role in the migration of cancer cells (9). Therefore, I evaluated whether AZD4547 inhibits the migration and invasion of ovarian cancer cells.

For wound healing assay, ovcar8 and ES2 cells were wounded, followed by treatment with thymidine and AZD4547 for 30 hr. As shown in Figure 5A, AZD4547 significantly inhibited the migration of ovarian cancer cells.

Inhibitory effect of AZD4547 on cell migration and invasion was also confirmed by transwell assay. As shown in Figure 5B&C, AZD4547

significantly inhibited migration and invasion of ovarian cancer cells in a dose-dependent manner.

3.6. The expression of FGFRs increases in sphere-forming ovarian cancer cells:

Cancer stem cells (CSCs) are a subpopulation of cells within tumor with various capabilities, including self-renewal, differentiation and tumorigenicity (34). Sphere forming cultivation is known to enrich the characteristic of CSCs (34).

To investigate the relationship of FGF/FGFR signaling with stemness of ovarian cancer cells, sphere formation assay was performed. As shown in Figure 6A, expression of CSCs markers was increased in sphere forming culture condition compared to adherent culture condition. Intriguingly, expression of FGFRs was also upregulated in the sphere forming cells compared to the adherent cells (Figure. 6B). These results suggest that FGF/FGFR signaling may be related to the stemness of ovarian cancer cells.

3.7. AZD4547 prohibits stemness of ovarian cancer cells:

To investigate whether AZD4547 inhibits the stemness of ovarian cancer cells, sphere forming and self-renewal capacities of CSCs were analyzed. For sphere formation assay, ovcar8 and A2780 cells were seeded in ultra-low attachment 6-well plates and treated with AZD4547 for 7 days. As shown in Figure 7A&B, AZD4547 markedly reduced the number and size of spheres in a dose-dependent manner.

To analyze self-renewal capacity of CSCs, the spheres were collected and dissociated into single cells. Single cells were seeded in ultra-low attachment 6-well plates and grown without AZD4547 for 7 days. As shown in Figure 7C, AZD4547 reduced the self-renewal capacity of ovcar8 cells in a dose-dependent manner. These results suggest that AZD4547 prohibits stemness of ovarian cancer cells.

3.8. AZD4547 inhibits angiogenesis:

To overcome limitation of oxygen and nutrients as tumor grows, tumor secretes several chemical substrates to attract nearby blood vessels. Process of the formation of new blood vessels is called angiogenesis, which is essential for tumor growth and survival (36).

Since FGFs are also known as a representative angiogenic factor (9), anti-angiogenic effect of AZD4547 was investigated in HUVECs. HUVECs were pretreated with AZD4547 for 2 hr, followed by serum treatment to stimulate tube formation. As shown in Figure 8, the number of tube bridges was reduced in the AZD4547-treated group compared to control group. These results suggest that AZD4547 has an anti-angiogenic effect on HUVECs.

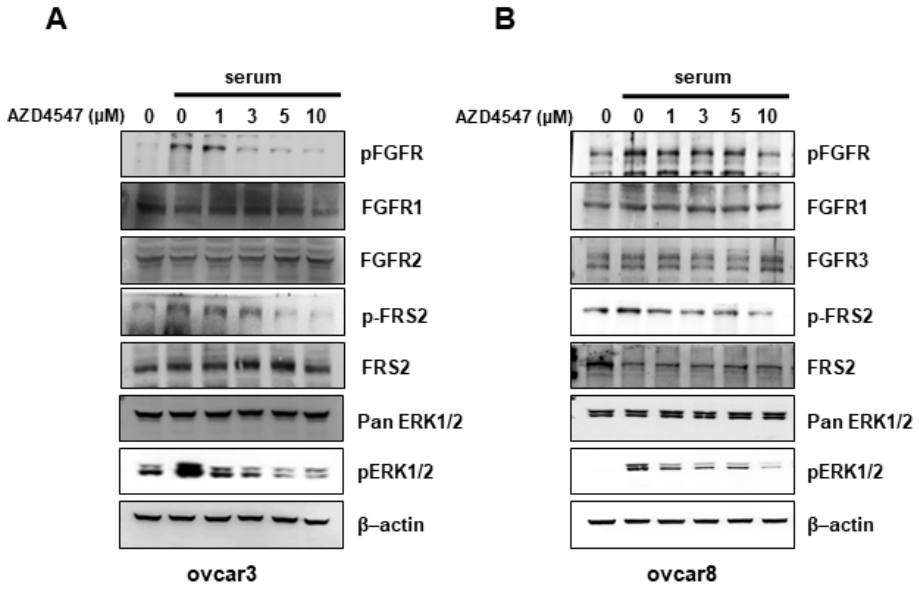


Figure 1. AZD4547 reduces FGF/FGFR signaling in ovarian cancer cells. (A&B) AZD4547 suppressed phosphorylation of FGFR, FRS2 and ERK1/2 in ovcar3 (A) and ovcar8 (B) cells. Ovcar3 and ovcar8 cells were pretreated with AZD4547 (0, 1, 3, 5 and 10 μM) for 2 hr, followed by serum treatment to activate the FGF/FGFR signaling. Phosphorylation of FGFR, FRS2 and ERK1/2 was analyzed by western blot. The results are representative of three independent experiments.

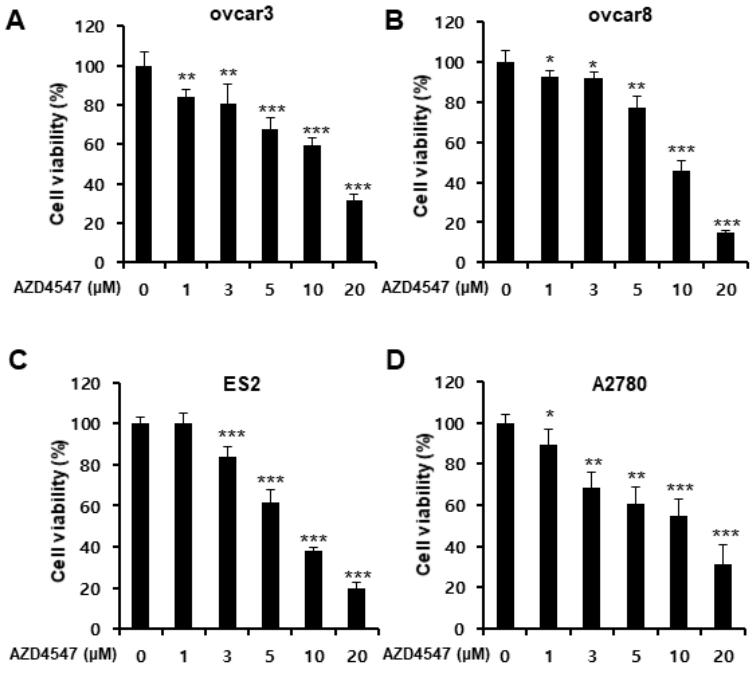


Figure 2. AZD4547 induces cytotoxicity in ovarian cancer cells. (A-D) AZD4547 treatment decreased cell viability of ovarian cancer cells. Ovcar3 (A), ovcar8 (B), ES2 (C) and A2780 (D) cells were treated with AZD4547 (0, 1, 3, 5, 10 and 20 μM) for 48 hr. Cell viability was determined using CCK-8 assay. Graph represents the average of five independent experimental groups. All values are presented as the mean ± S.D (n = 5). Statistical significance was determined by comparisons between AZD4547-treated and control groups. Significant differences are indicated; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

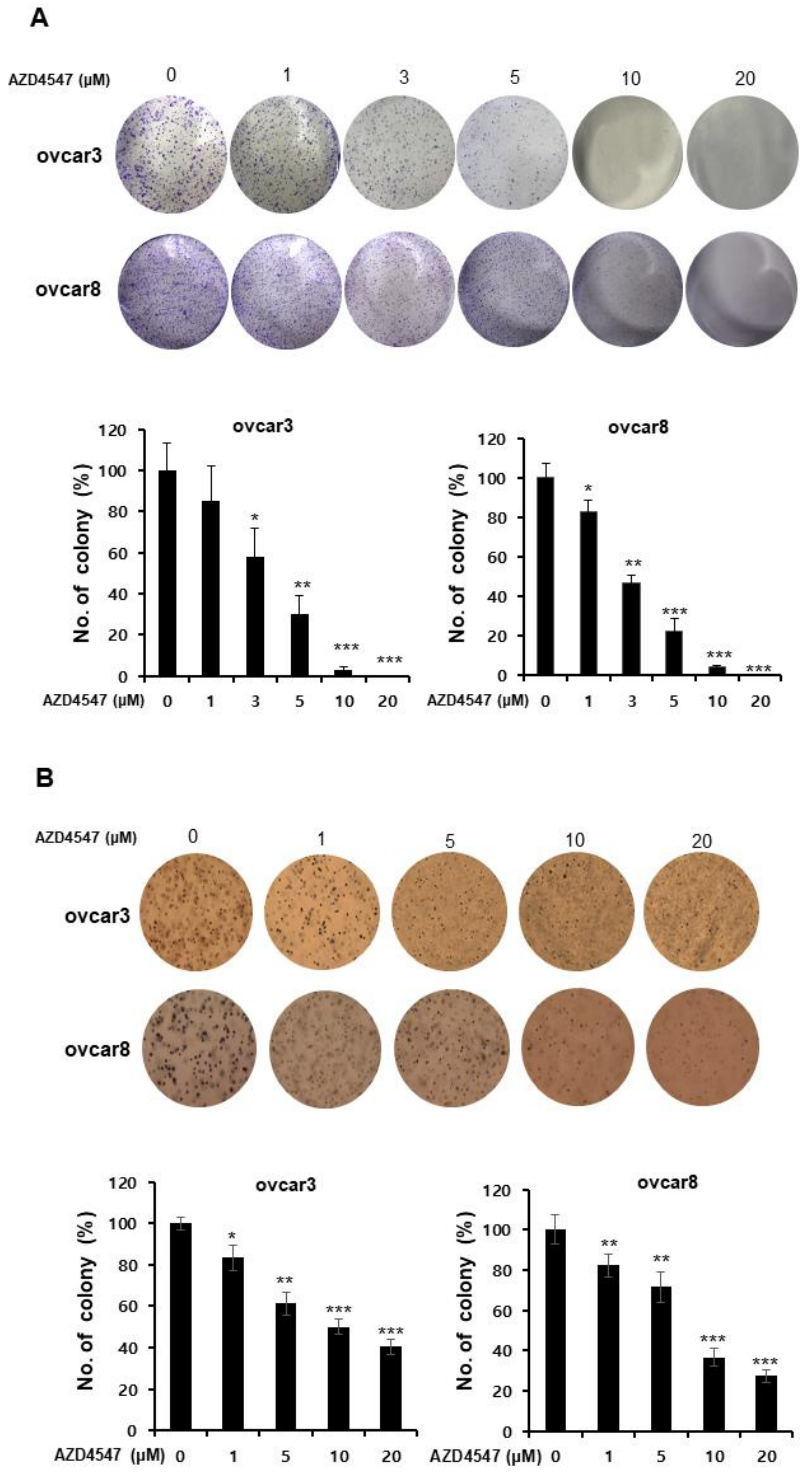


Figure 3A. AZD4547 inhibits proliferation of ovarian cancer cells. (A) AZD4547 treatment reduced anchorage-dependent growth of ovarian cancer cells. Ovcar3 and ovcar8 cells were treated with AZD4547 (0, 1, 3, 5, 10 and 20 μM) for 7 days. (B) AZD4547 treatment inhibited anchorage-independent growth of ovarian cancer cells. Ovcar3 and ovcar8 cells were seeded to soft agar and treated with AZD4547 for 3 wk. Representative images of colonies from the indicated assays are shown. Graph represents the average of three independent experimental groups. All values are presented as the mean ± S.D (n = 3). Statistical significance was determined by comparisons between AZD4547-treated and control groups. Significant differences are indicated; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

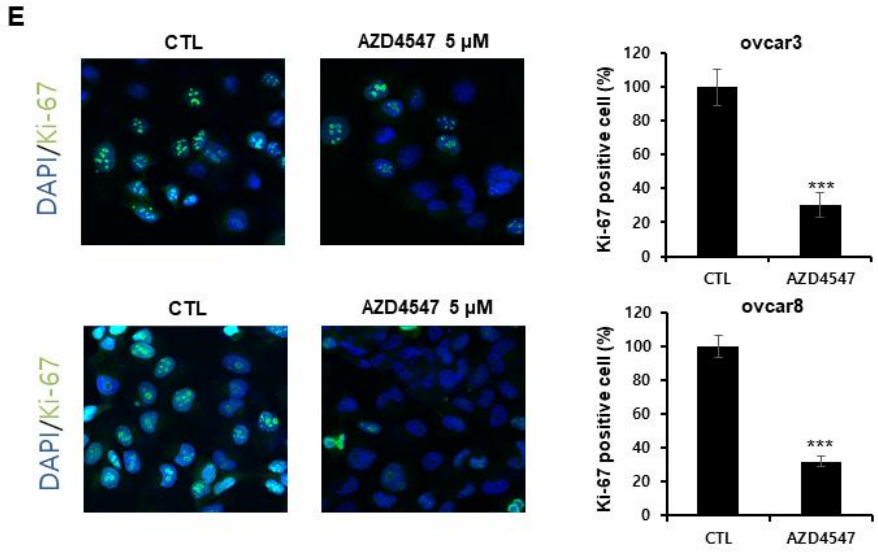


Figure 3B. AZD4547 inhibits proliferation of ovarian cancer cells (continued). (E) AZD4547 treatment decreased Ki-67 expression in ovarian cancer cells. Ovcar3 and ovcar8 cells were treated with AZD4547 (5 μM) for 24 hr. Green color directs positive fluorescence staining for Ki-67, while blue color represents counterstaining of cell nuclei with DAPI. Representative images are shown. Graph represents the average of three independent experimental groups. All values are presented as the mean ± S.D (n = 3). Statistical significance was determined by comparisons between AZD4547-treated and control groups. Significant differences are indicated; ***$p < 0.001$.

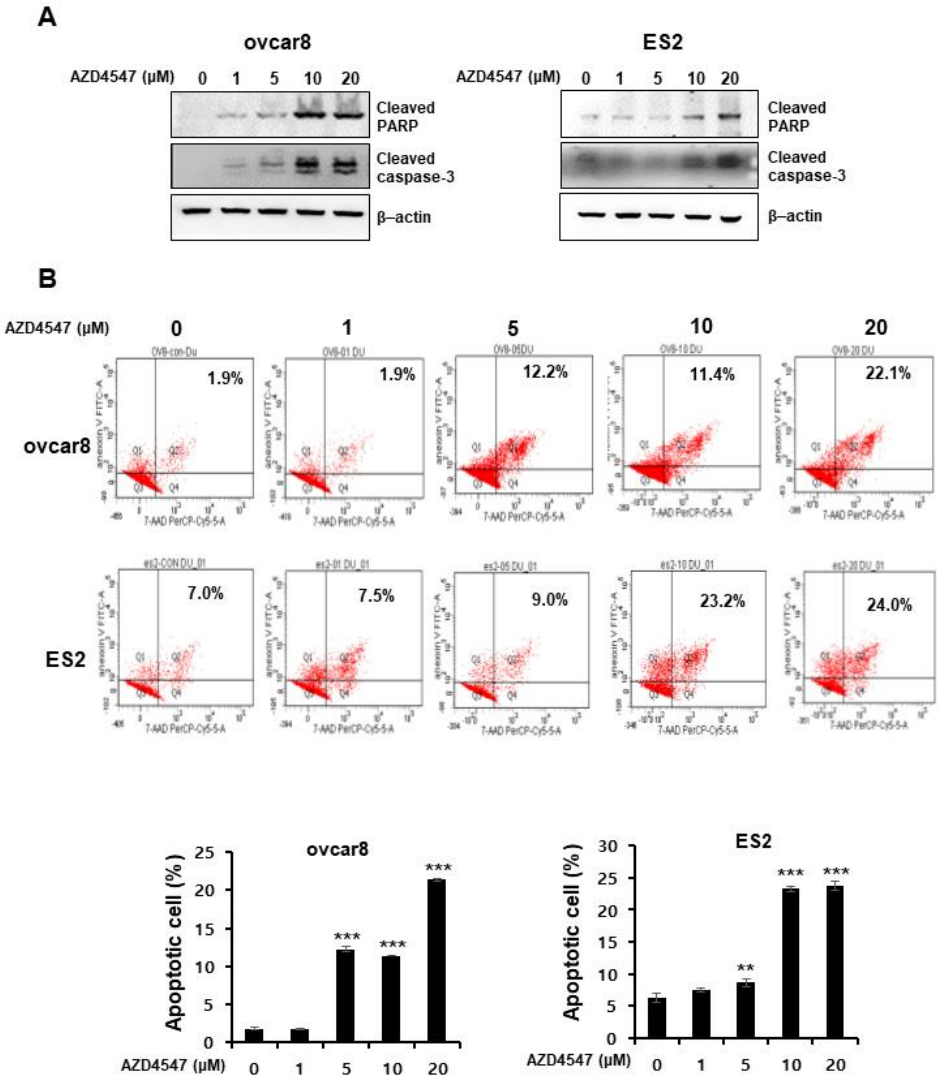


Figure 4. AZD4547 induces apoptosis in ovarian cancer cells. (A) AZD4547 treatment induced cleavage of caspase-3 and PARP in ovarian cancer cells. Ovcar8 and ES2 cells were treated with AZD4547 (0, 1, 5, 10 and 20 μM) for 48 hr. Apoptotic markers were analyzed by western blot. (B) AZD4547 treatment increased the population of apoptotic cells. Ovcar8

and ES2 cells were treated with AZD4547 for 48 hr and stained with Annexin V antibody and 7-AAD. (C) Graph represents the average of three independent experimental groups. All values are presented as the mean ± S.D (n = 3). Statistical significance was determined by comparisons between AZD4547-treated and control groups. Significant differences are indicated; $^{**}p < 0.01$, $^{***}p < 0.001$.

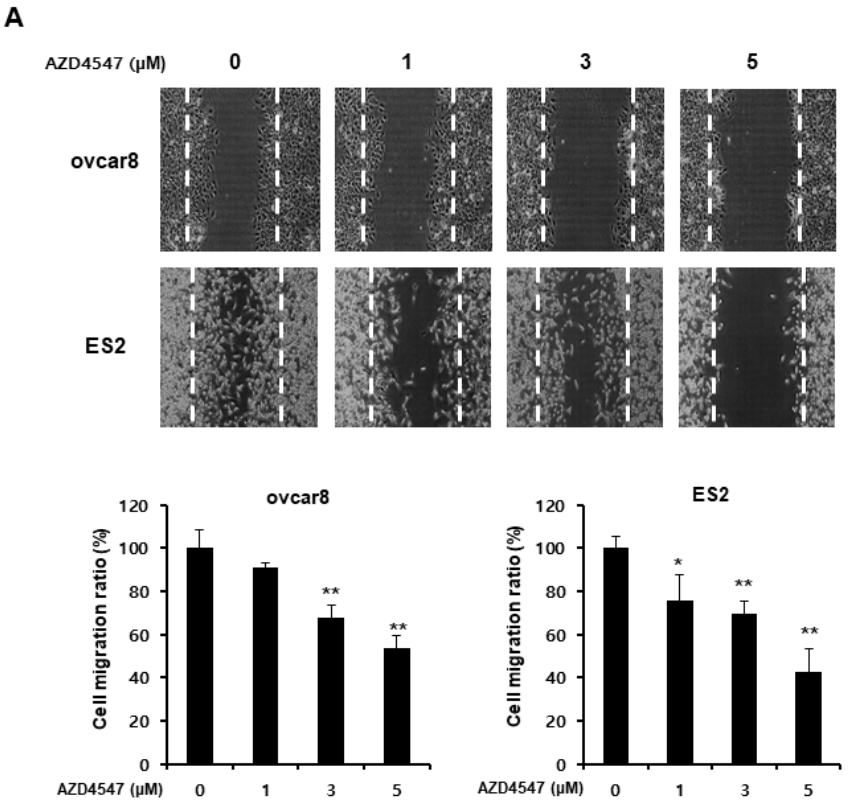


Figure 5A. AZD4547 prevents migration and invasion of ovarian cancer cells. (A) AZD4547 treatment significantly inhibited the migration of ovarian cancer cells. Ovcar8 and ES2 cells were treated with thymidine and AZD4547 (0, 1, 3 and 5 μM) for 30 hr. White lines point to the wound borders at the beginning of the assay. Representative images are shown. The percentage of cell-covered area are shown in the bar diagram as mean ± S.D (n = 3) from three independent experimental groups. Statistical significance was determined by comparisons between AZD4547-treated and control groups. Significant differences are indicated; $*p < 0.05$, $**p < 0.01$.

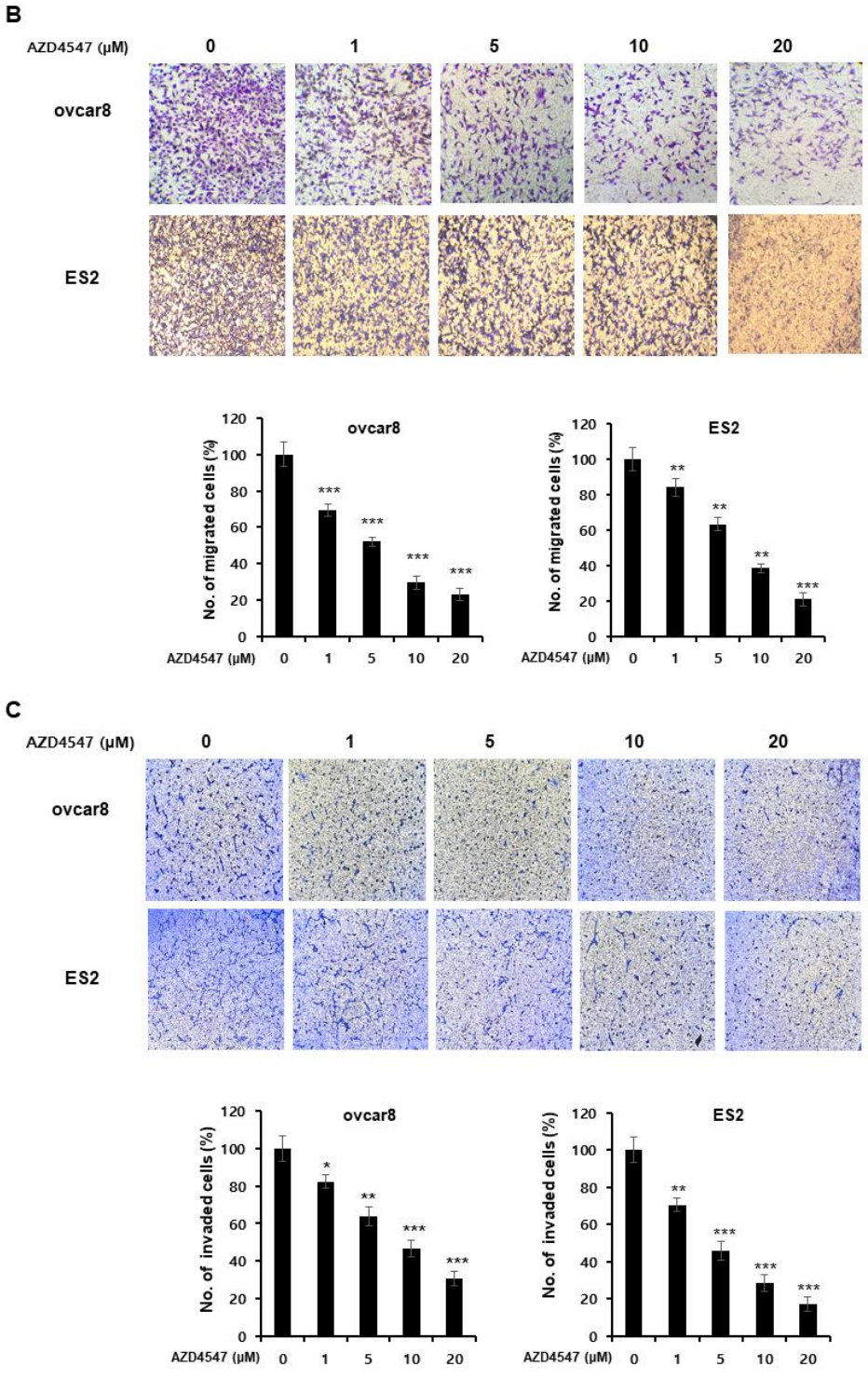


Figure 5B. AZD4547 prevents migration and invasion of ovarian cancer cells (continued). (B&C) AZD4547 treatment inhibited migration (B) and invasion (C) of ovarian cancer cells. In transwell assay, ovcar8 and ES2 cells were treated with AZD4547 (0, 1, 5, 10 and 20 μM) for 24 hr. Representative images of migrated cells are shown. Graph represents the average of three independent experimental groups. All values are presented as the mean ± S.D (n = 3). Statistical significance was determined by comparisons between AZD4547-treated and control groups. Significant differences are indicated; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

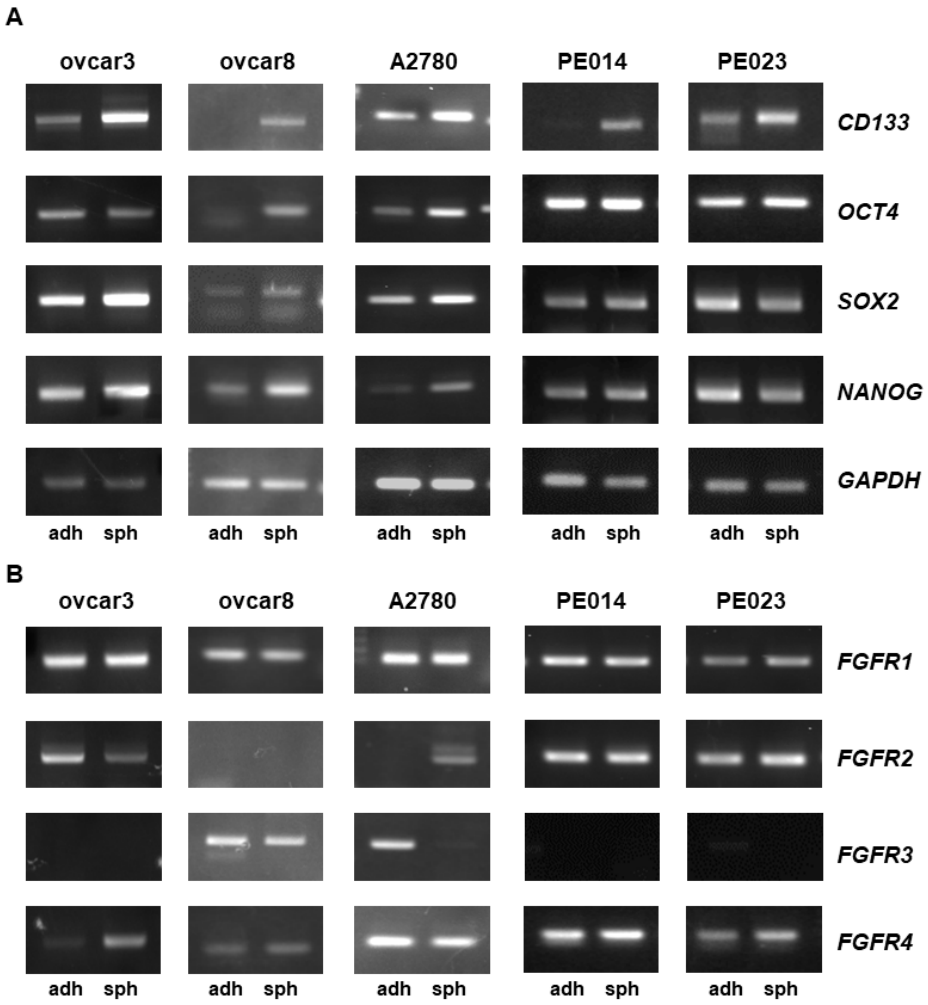
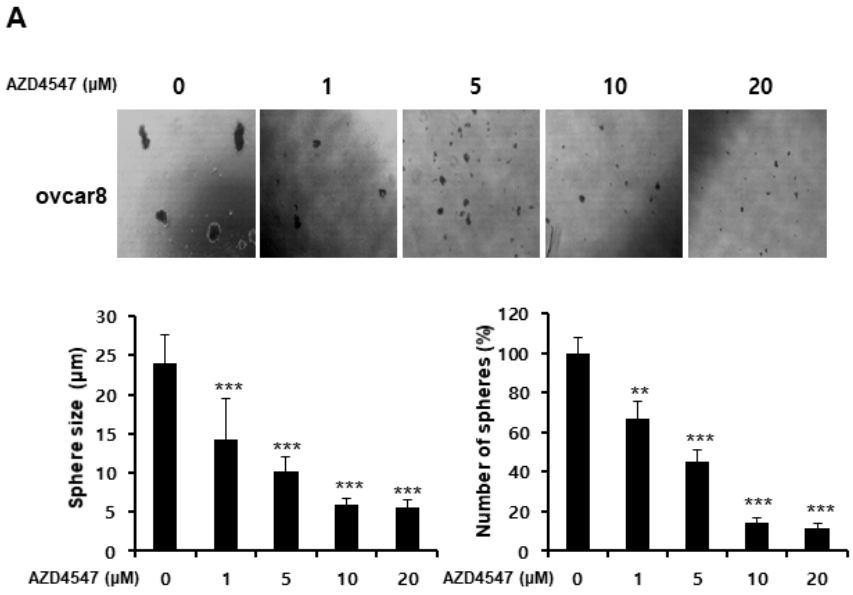
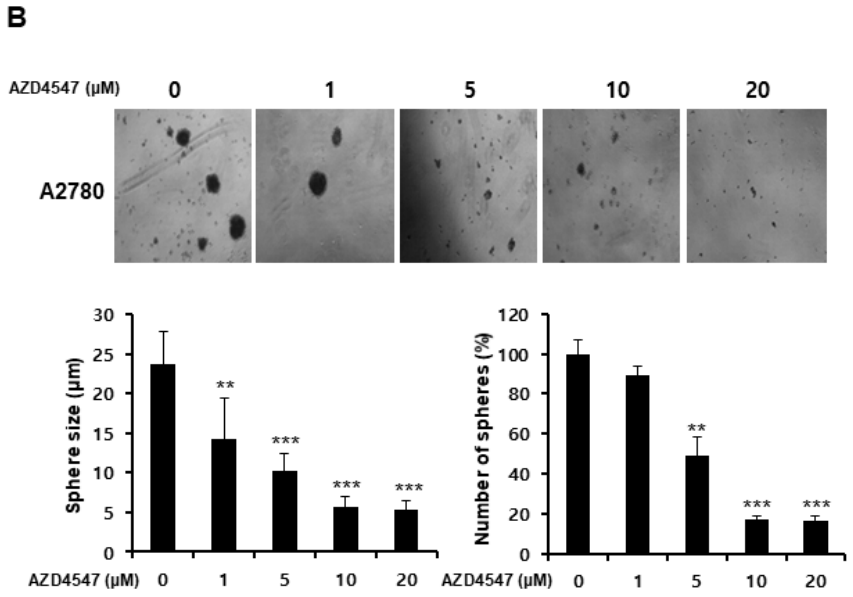


Figure 6. The expression of FGFRs increases in sphere-forming ovarian cancer cells. (A) The expression of CSCs markers was increased under sphere forming culture condition. (B) The expression of FGFRs was upregulated in sphere forming cells compared to adherent cells. Ovarian cancer cells were seeded in ultra-low attachment 6-well plates and grown for 7 days. Expressions of FGFRs and CSCs markers were analyzed by RT-PCR. adh: adherent cell; sph: sphere forming cell.





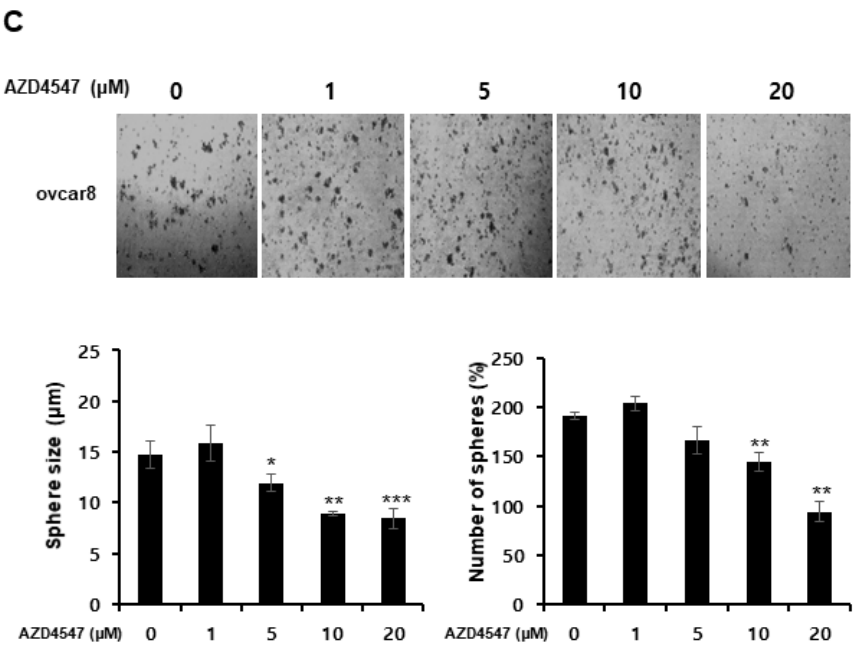


Figure 7. AZD4547 prohibits stemness of ovarian cancer cells. (A&B) AZD4547 treatment reduced sphere forming ability of ovar8 (A) and A2780 (B) cells. Cells were seeded in ultra-low attachment 6-well plates and treated with AZD4547 for 7 days. (C) AZD4547 treatment inhibited the self-renewal capacity of ovcar8 cells. Spheres were collected and dissociated into single cells. Single cells were seeded in ultra-low attachment 6-well plates and grown without AZD4547 for 7 days. Representative images of sphere forming cells are shown. Graph represents the average of three independent experimental groups. All values are presented as the mean ± S.D (n = 3). Statistical significance was determined by comparisons between AZD4547-treated and control groups. Significant differences are indicated; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

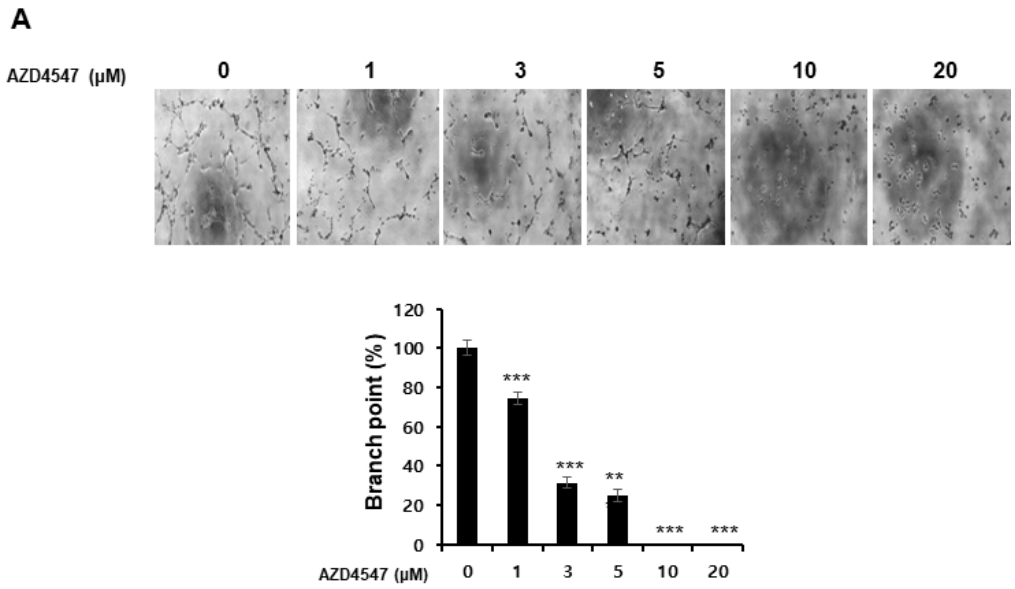


Figure 8. AZD4547 inhibits angiogenesis. (A) AZD4547 treatment prevented tube formation of HUVECs. HUVECs were plated in Matrigel-covered wells and pretreated with AZD4547 for 2 hr, followed by serum treatment to stimulate tube formation. Representative images of tube formation are shown. Graph represents the average of three independent experimental groups. All values are presented as the mean ± S.D (n = 3). Statistical significance was determined by comparisons between AZD4547-treated and control groups. Significant differences are indicated; ***$p < 0.001$.

4. Discussion

FGF/FGFR signaling plays an important role in the regulation of diverse biological events, such as growth, survival, differentiation, embryonic development and angiogenesis. In cancer, FGF/FGFR signaling is considered as an oncogenic signaling pathway. Dysregulation of FGFRs has been reported in various types of cancer, including urothelial, hepatocellular carcinoma (HCC), lung and ovarian cancer (37). In ovarian cancer, FGFR1 and FGFR2 are known to be highly amplified (38,39) and related to in chemotherapy sensitivity (40). Moreover, higher FGFR4 expression in serous ovarian cancer was correlated to poorer overall survival rate of patients (24). Based on these previous reports, this study examined the anti-tumor effect of FGFR inhibitor on ovarian cancer cells.

Recently, diverse small molecule inhibitors targeting FGFRs have been developed. In this study, I used AZD4547, which is an orally available and highly selective small molecule inhibitor of FGFR1-4 (25). AZD4547 has an advantage of targeting a wide range of FGFRs, minimizing the risk of drug resistance due to other FGF/FGFR signaling (41). In previous studies, AZD4547 showed potent anti-proliferative effect in breast, endometial and lung cancer (26,29,42). In addition, AZD4547 is evaluated in phase II clinical trials for gastric, lung and breast cancer (26-28). However, anti-cancer activity of AZD4547 has not been evaluated in ovarian cancer.

This study showed that AZD4547 has a potent anti-tumor effect in ovarian cancer cells. The results revealed that AZD4547 inhibits proliferation and survival of ovarian cancer cells by downregulation of FGF/FGFR signaling (Figures 1-4). AZD4547 also reduces migration and

invasion of ovarian cancer cells and suppresses angiogenesis (Figures 5&8). Interestingly, AZD4547 reduces the stemness of ovarian cancer cells. Sphere forming and self-renewal capacities of ovarian CSCs were markedly inhibited by AZD4547 treatment (Figures 6&7), suggesting that AZD4547 is a promising drug that targets CSCs as well as cancer cells. Based on these results, this study demonstrated *in vitro* effect of AZD4547 in ovarian cancer cells, however further study is warranted to explore inhibitory effect of AZD4547 on tumor growth, metastasis and recurrence *in vivo*.

In conclusion, I propose that FGF/FGFR signaling is a promising target in ovarian cancer. Inhibition of FGFRs using AZD4547 could be an effective way to simultaneously suppress multiple cellular mechanisms essential for tumor development, such as proliferation, migration, invasion, stemness and angiogenesis. Taken together, this study suggests the possibility of AZD4547 for the application to ovarian cancer treatment.

5. Summary

The purpose of this study is to investigate the anti-tumor effect of AZD4547, an FGFR1-4 inhibitor, in ovarian cancer cells. AZD4547 effectively inhibited FGF/FGFR signaling and suppressed proliferation and survival of ovarian cancer cells in a dose-dependent manner. In non-toxic condition, AZD4547 also reduced migration and invasion of ovarian cacner cells. In addition, AZD4547 inhibited sphere forming and self-renewal capacities of ovarian CSCs. Furthemore, AZD4547 showed anti-angiogenic effect in HUVECs. Taken together, this study suggests that AZD4547 is a potent anti-tumor agent for ovarian cancer treatment.

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FGFR inhibitor, AZD4547, impedes the stemness of mammary epithelial cells in the premalignant tissues of MMTV-ErbB2 transgenic mice. Sci Rep 2017; 1: 11306.

Anti-Tumor Effect of FGFR Inhibitor AZD4547 in Ovarian Cancer Cells

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(Abstract)

Ovarian cancer is the most fatal malignancy of gynecological cancer and has a strong resistance to chemotherapeutic agents. This raises the need for new therapeutic target. Fibroblast growth factors (FGFs) have been implicated in malignant transformation, tumor mitogenesis, angiogenesis and chemoresistance. Recently, fibroblast growth factor receptor (FGFR) inhibitors are being assessed in clinical trials for several FGFR-driven cancers. Although FGFRs is known to be highly dysregulated in ovarian cancer, the effect of FGFR inhibition has not been evaluated in ovarian cancer. In this study, I examined the anti-tumor effects of AZD4547, a FGFR1-4 inhibitor, in ovarian cancer cells. AZD4547 markedly inhibited proliferation and induced apoptosis of ovarian cancer cells in a dose-dependent manner. In addition, AZD4547

reduced cell migration and invasion under non-toxic condition. Moreover, AZD4547 prohibited the ability of sphere forming and self-renewal capacities of ovarian cancer stem cells AZD4547 also showed anti-angiogenic effect on human umbilical vein endothelial cells (HUVECs). Taken together, this study demonstrates anti-tumor effects of AZD4547 on ovarian cancer cells and suggests that AZD4547 is a promising agent for ovarian cancer therapy.

난소암 세포주에서 FGFR 억제제인 AZD4547의 항암효과

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(초록)

난소암은 부인암 중 가장 치명적인 악성 종양으로 화학 약물 요법에 대해 강한 내성을 가지고 있다. 이러한 문제점은 새로운 항암 치료 타겟의 필요성을 대두시킨다. FGF/FGFR (fibroblast growth factor/fibroblast growth factor receptor) 신호전달은 종양의 생성과 발달, 혈관 신생 및 항암제 내성 등에 관련되어 있다고 알려져 있으며, 최근에는 FGFR 발현이 높은 일부 암종에 대해 FGFR 억제제에 대한 임상시험들이 진행 중이다. 난소암은 FGFR의 발현이 비정상적으로 높은 암으로 알려져 있음에도 불구하고 난소암에 대한 FGFR 억제제의 효능에 대해서는 아직 알려진 바가 없다. 본 연구에서는 다양한 종류의 난소암 세포주에서 FGFR1-4 저해제인 AZD4547의 항암 효과를 조사하였다. 그 결과, AZD4547은 난소암 세포의 성장을 효과적으로 억제하였으며, 농도 의존적으로 세포 사멸사를 일으켰다. 또한 독성을 나타내지 않는 조건에서도 난소암 세포의 이동 및 침윤이 AZD4547에 의해 현저히 감소하였다. 뿐만 아니라 AZD4547은 난소암 세포

외에도 난소암 줄기 세포의 구체 형성 및 자가 재생 능력을 효과적으로 억제하였다. 이 외에도 AZD4547은 암의 성장에 필수적인 혈관신생 작용을 억제하는 효과를 보였다. 결과적으로, 본 연구는 난소암 세포에 대한 AZD4547의 항암 효과를 입증하였고, 이를 통해 AZD4547의 난소암 치료제로서 활용 가능성을 제시한다.