Inhibition of FGF/FGFR-dependent Angiogenesis in Urothelial Carcinoma

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Directed by Professor Sun Young Rha

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Seungtaek Lim

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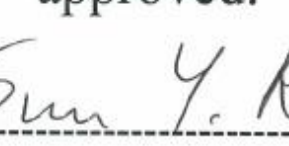
This certifies that the Doctoral Dissertation of Seungtaek Lim is approved.



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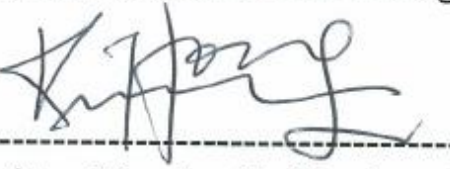

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ABSTRACT

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Purpose: Fibroblast growth factors (FGFs) and fibroblast growth factor receptors (FGFRs) are implicated in tumorigenesis of urothelial carcinoma (UC) including angiogenesis. Recently, FGFR specific inhibitor, erdafitinib showed its efficacy in UC patients harboring FGFR aberration. Herein, we investigated the feasibility of suppression of UC growth by inhibiting angiogenesis using erdafitinib.

Materials and Methods: We evaluated the inhibitory effect of erdafitinib on UC cells using wound healing assay, transwell invasion assay, and zymography assay. Next, we performed tubule formation assay and transwell migration assay to assess inhibitory effect of erdafitinib on endothelial cells (EC). We also assessed crosstalk of EC co-cultured with UC cell using transwell migration assay. For *in vivo* study, the athymic nude mice bearing RT4 cells were randomly divided into a control group and erdafitinib group. Tumors were then extracted for immunohistochemistry and further analysis.

Results: The results of wound healing assay and transwell invasion assay

demonstrated that erdafitinib has inhibitory actions on the migratory and invasive potential of UC cells. Erdafitinib also suppressed tube formation and migratory potential of ECs. Transwell migration assay using EC co-cultured with RT4 showed that migration of EC was significantly inhibited by erdafitinib. *In vivo* model showed that erdafitinib dramatically suppressed tumor volumes and weight. The decrease of both CD31-positive and Ki-67-positive cells indicated that erdafitinib exerted anti-angiogenic activity as well as anti-proliferative effect.

Conclusions: Our study showed that targeting angiogenesis by inhibiting FGFR pathway is feasible therapeutic strategy, and warrants further investigation.

Key words: carcinoma, transitional cell; angiogenesis inhibitors; receptors, fibroblast growth factor; erdafitinib

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I. INTRODUCTION

Urothelial carcinoma (UC), the 6th most common malignancy in men worldwide, accounts for 549,393 of new cases and 199,922 cancer related deaths in 2018[1]. Previously, there was no standard treatment for patients with metastatic urothelial carcinoma (mUC) after failure with platinum-based chemotherapy in the first line setting. Recently, various immune checkpoint inhibitors (ICIs) targeting PD-1 or PDL-1 demonstrated clinical efficacy in clinical trials[2-6] and were approved for patients who progressed after 1st line chemotherapy. In addition, erdafitinib, a selective fibroblast growth factor receptor (FGFR) inhibitor, received an accelerated approval by Food and Drug Administration (FDA), based on the anti-tumor activity shown in a phase II study conducted in UC patients harboring FGFR alterations[7]. However, both ICIs and erdafitinib have limited efficacy since many patients treated with ICIs have progressive disease as their best response, and only a small number of patients have FGFR alterations eligible for treatment of erdafitinib. Hence, there is still unmet need for more effective treatment strategy in mUC.

Fibroblast growth factors (FGFs) and FGFRs have been known to be important regulators in carcinogenesis of various malignancies including UC[8]. In non-muscle invasive UC, FGFR3 mutation and overexpression is frequently found[9]. Also, FGFR1 overexpression is commonly found in UC of all grades and

stages, and it is predictive of worse survival in UC patients who underwent radical cystectomy[10]. In addition, FGFs and FGFRs are also involved in the process of angiogenesis[11-12]. FGFs are known to exert their angiogenic effect by modulating proliferation and migration of endothelial cells, production of proteases, and promotion of integrin and cadherin receptor expression[13]. Several FGFR inhibitors have been investigated for their anti-angiogenic effect as a therapeutic strategy[14-17]. This finding suggests that targeting angiogenesis by inhibiting FGFR pathway could be considered a relevant therapeutic strategy for the mUC patients.

Therefore, we evaluated whether erdafitinib has antiangiogenic activity on UC cell *in vitro* and *in vivo*.

II. MATERIALS AND METHODS

1. Cell lines and cell culture

Urothelial cancer (UC) cell lines (UM-UC-3, HT-1197, J82, T24, 5637, 253J, RT4, and HT-1379), human umbilical vein endothelial cells (HUVEC), and human dermal microvascular endothelial cells (HMVEC) were purchased from American Type Culture Collection (ATCC). Cancer cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS; Lonza, Basel, Switzerland), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Lonza). Endothelial cells were cultured in Endothelial Growth Medium-2 (EGM-2) (Lonza) and cells below passage 3 were used for experiments. Cultured cells were maintained at 37°C in an atmosphere with 5% CO_2.

2. Drugs

Erdafitinib (JNJ-42756493) was purchased from Selleckchem (S8401) and dissolved in DMSO (Sigma-Aldrich, St Louis, MO, USA) as a 50mM stock solution for use *in vitro* or dissolved in normal saline containing 5% (v/v) DMSO and 10% (w/v) Kleptose HPB (Roquette, Lestrem, France) for the *in vivo* experiments.

3. Enzyme-linked immunoassay (ELISA)

Cancer cells and endothelial cells (1 X 10^6 cells/well) were plated into 6-well plates. After 24h, the medium was changed to serum-free medium with or without erdafitinib. Conditioned media were collected at 24h. Cell debris was removed by centrifugation and supernatants were stored at -80°C until analysis. The human FGF-2 (DFB50, R&D systems, Minneapolis, Minnesota, USA), human VEGF (R&D systems), and mouse VEGF (R&D systems) ELISA assays were performed according to the manufacturer`s protocol with the standard curve. Absorbance values were detected at 450 nm using a microplate reader and values were calculated using the formula obtained from

the trendline.

4. Western blot immunoblotting

Total protein was extracted from each UC cells and ECs with M-PER™ mammalian protein extraction reagents (ThermoFisher, Waltham, Massachusetts, USA) containing protease and phosphatase inhibitors, and 20 μg of the protein extract was used for western blot analysis with the following primary antibodies: p-FGFR1 (Y654) #ab59194, p-FGFR3 (Y724) #ab155960 (Abcam, Cambridge, UK), FGFR1 #9740, FGFR3 #4574, VEGFR2 #2472, p-VEGFR2 #2478, p-Akt (S473) #4058, Akt #9272, p-Erk1/2 #4695 (Cell Signaling Technology, Danvers, Massachusetts, USA), Erk1 #sc-94 (Santa Cruz Biotech), and α-tubulin #T6199 (Sigma-Aldrich). Peroxidase-conjugated anti-mouse or anti-rabbit antibody was used as secondary antibody. Immunoreactive proteins were visualized using a ChemiDoc XRS+ System (Bio-rad, Hercules, California, USA).

5. Cell viability assay

The UC cells (5×10^3 cells/well) were seeded in 96-well plates overnight at 37°C and exposed to specific doses of erdafitinib (0, 0.19, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, and 50 μM) at 37°C for 72 h. MTT solution (Sigma-Aldrich, St Louis, MO, USA) was added, and the plates were incubated at 37 °C. After incubating for 4 h, the medium was removed, and DMSO was added. The absorbance was measured at 570 nm and IC_{50} values were determined using the CalcuSyn software (Biosoft, Cambridge, UK). HUVEC cell viability was assayed by using a cell counting kit-8 (CCK-8, Dojindo, Japan). Cells were seeded in 96-well plates at density of 8,000 cells into each well and incubated at 37 °C, 5% CO_2 overnight. Each well was treated half diluted concentration of erdafitinib (0, 0.19, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, and 50 μM) at 37 °C, 5% CO_2 for incubation 72 h. CCK-8 solution (BioMAX) was 10μL added and incubated for 2 h at 37 °C. Using a Microplate Reader (Tecan, Switzerland) the 96-well plate of

absorbance at 450 nm and IC_{50} values were determined using the CalcuSyn software (Biosoft).

6. Tube formation assay

Matrigel (BD Biosciences) was added to 96-well plate. After polymerizing at 37 °C for 1 hr, HUVECs (2 X 10^4 cells/well) were plated onto the Matrigel with or without erdafitinib. After 18h incubation at 37 °C, images of capillary-like tube structures were obtained by microscope (Axiovert 200, Carl Zeiss, Oberkochen, Germany). The number of intact tubes per field was counted for each experimental condition.

7. Migration and invasion assay

Invasion ability of UC cells was assessed using Transwell (Corning, Corning, New York, USA) invasion assay. Cells (5 X 10^4 cells/well) were plated into top chambers with 8 μm-pore membranes with Matrigel-coated membrane. Serum-free medium with or without erdafitinib was added into the upper chamber, and the medium containing 10 % FBS was added into the lower chamber. After 24h incubation at 37 °C, cells were fixed in 4% paraformaldehyde for 10 min, and stained with 0.5% crystal violet solution for 5 min. Migration ability of HUVECs to interact with UC cells was assessed using Transwell migration assay. UC cells (2 X 10^5 cells/well) were seeded into bottom chamber and when cells reached confluent, medium was changed to 1 % FBS containing medium with or without erdafitinib. These UC cells were cultured in the lower chamber for 24 h at 37 °C; then, HUVECs (5 X 10^4 cells/well) were placed in the upper chamber containing serum-free medium. After 48 h incubation at 37 °C, cells were fixed in 4 % paraformaldehyde for 10 min, and stained with 0.5 % crystal violet solution for 5 min. The migrated or invaded cells on the lower side of the membrane were counted using image J software. Five random fields (x 200) were selected and the mean number of cells were identified as the invaded cell number.

8. Wound healing assay

The cells (2 X 10^5 cells/well) were seeded into 24-well plate. After 24 h, when cells reached confluent, the cells were wounded by scratching with a sterile P200 pipette tip and washed by phosphate buffered saline (PBS) subsequently to eliminate detached cells. The medium was changed to serum-free medium with or without erdafitinib. After 16 h, wound width was measured using Image-J software (NIH, Bethesda, MD, USA).

9. Zymography

Conditioned medium of UC cells and ECs was collected, and samples mixed with an equal volume of sample buffer (Novex® Tris–Glycine SDS sample Buffer 2×, Invitrogen, Carlsbad, California, USA) were electrophoresed on 10 % Tris-Glycine poli-acrylamide gels with 0.1 % gelatin (Novex® Zymogram Gels, Invitrogen). Electrophoresis was performed under non-reducing conditions at a constant voltage of 125 V for 120 min; activated HT-1080 by concanavalin (12 μg/ml) treatment was run together as marker. The gels were washed for 30 min in Novex® Zymogram Buffer (Invitrogen), equilibrated at room temperature for 30 min in developing buffer (Novex® Zymogram Developing Buffer, Invitrogen) and then incubated with a fresh aliquot of the same buffer at 37 °C for 18 h. Band of gelatinolytic activity was developed by staining gels for 2 h with 0.5 % Coomassie Blue stain solution, destained with 5 % methanol and 7.5 % acetic acid solution. Gelatinase activity was visualized using a ChemiDoc XRS+ System (Bio-rad, Hercules, California, USA).

10. *In vivo* experiments

Athymic nude mice (female, 5.5-weeks old, 18~20 g) were purchased from Central Lab Animal (Seoul, Korea). Animal protocols were approved by the “Guide for the Care and Use of Laboratory Animals” (National Research Council, USA) and Institutional Animal Care and Use Committee (AAALAC

International) of Yonsei University College of Medicine. RT4 and J82 cells (1×10^7), in a 1:1 mixture of PBS and Matrigel (BD Bioscience, Franklin Lakes, New Jersey, USA), were subcutaneously injected into the right flank of each mouse. Tumor size was monitored by measuring the length (L) and width (W) using calipers, and tumor volume was calculated using the following formula: $(L \times W^2) \times 1/2$. When the average tumor volume reached 100-200 mm^3, the mice were randomly divided into a control group treated with vehicle (n = 8 for RT4 cells, n = 6 for J82 cells), and a treatment group treated with 40 mg/kg erdafitinib via oral gavage (PO) three times a week for 21 days (n = 7 for RT4 cells, n = 6 for J82 cells). The mice were sacrificed the day after treatment completion. The extracted tumors were paraffin-embedded for immunohistochemistry (IHC) or frozen at -80 °C for western blot analysis.

11. Immunohistochemistry and micro-vessel density (MVD) calculation

The paraffin-embedded tumor tissues (4-μm thick) were deparaffinized and rehydrated using xylene and graded ethanol solutions, respectively. Antigens were retrieved using the heat-induced method by placing slides in citrate buffer (pH 6.0). The sections were incubated with Ki-67 (#M7240, Dako, Glostrup, Denmark) and CD31 (#77699, Cell Signaling Technology) antibodies (1:100 dilution) with diaminobenzidine (DAB; #K5007, Dako,) as a chromogen. The sections were counterstained with hematoxylin and mounted using a glass cover slip. For calculation of micro-vessel density (MVD), ImageJ (v1.47, Wayne Rasband, 64bits) program was used to perform morphometric image analysis to detect and measure the micro-vessels. The total percentage of CD31-positive pixels per tissue area analyzed is used as measure for the average microvessel density in that whole tumor section.

12. Statistical analysis

Data were presented as the mean ± standard deviation (SD) accordingly.

Statistical analysis was performed using the SPSS 25.0 software (SPSS Inc., Chicago, IL, USA). Values of $P<0.05$ were considered to be statistically significant.

III. RESULTS

1. Baseline Characteristics of Urothelial Carcinoma (UC) cells

First, we investigated the baseline characteristics of UC cell lines. Previous reports showed that UC cells are categorized as mesenchymal or epithelial cell lines based on the overexpression of FGFR1 or FGFR3, respectively[18]. We evaluated the expression of FGFR1, FGFR3 and vasculo-endothelial growth factor receptor (VEGFR)2 of each UC cell (Figure 1A, top). Overexpression of FGFR1 was observed in UM-UC3, HT-1197, J82 and 253J cell line. The highest expression of FGFR3 was observed in RT4, followed by HT 1197, J82, and HT-1376.

We also evaluated the amount of fibroblast growth factor 2 (FGF2) (pg/mL) and VEGF (pg/mL) secreted by each UC cell line using ELISA method. A large amount of FGF2 was detected in J82 and T24 cells. In contrast, a large amount of VEGF was detected in RT4 (Figure 1A, bottom).

Matrix metalloproteinases (MMPs) are involved in cancer invasion, metastasis, and angiogenesis. Hence, we analyzed the baseline level of MMPs (MMP-2 and MMP-9) in pro-form and active form using zymography. Relatively large amount of active-form of MMP-2 was detected in UM-UC3, J82, HT-1197, and 5637 (Figure 1B). In contrast, active form of MMP-9 was not detected in any of the cell lines.

Next, we examined the invasiveness of each UC cell line using transwell invasion assay. J82 and T24 were characterized as the most invasive cell lines, and followed by UM-UC3, 5637 and HT1376 (Figure 1C). RT4 was the least invasive cell among the UC cell lines analyzed.

The expression of FGFR1, FGFR3, and VEGFR2 in endothelial cell lines (HUVEC-C and HMEC) were also examined. Western blot analysis showed that overexpression of both FGFR1 and VEGFR2 were observed in HUVEC-C and HMEC (Figure 1A). In addition, a large amount of FGF2 secretion was detected in both ECs.

Based on these results, RT4, J82 and UM-UC-3 were selected for further analysis. RT4 and both J82 and UM-UC-3, expressed FGFR3 and FGFR1, respectively. The epithelial type cell line (RT4) was characterized by the least invasive feature, and the mesenchymal type cell lines (J82 UM-UC-3) were characterized by the most invasive cell lines.

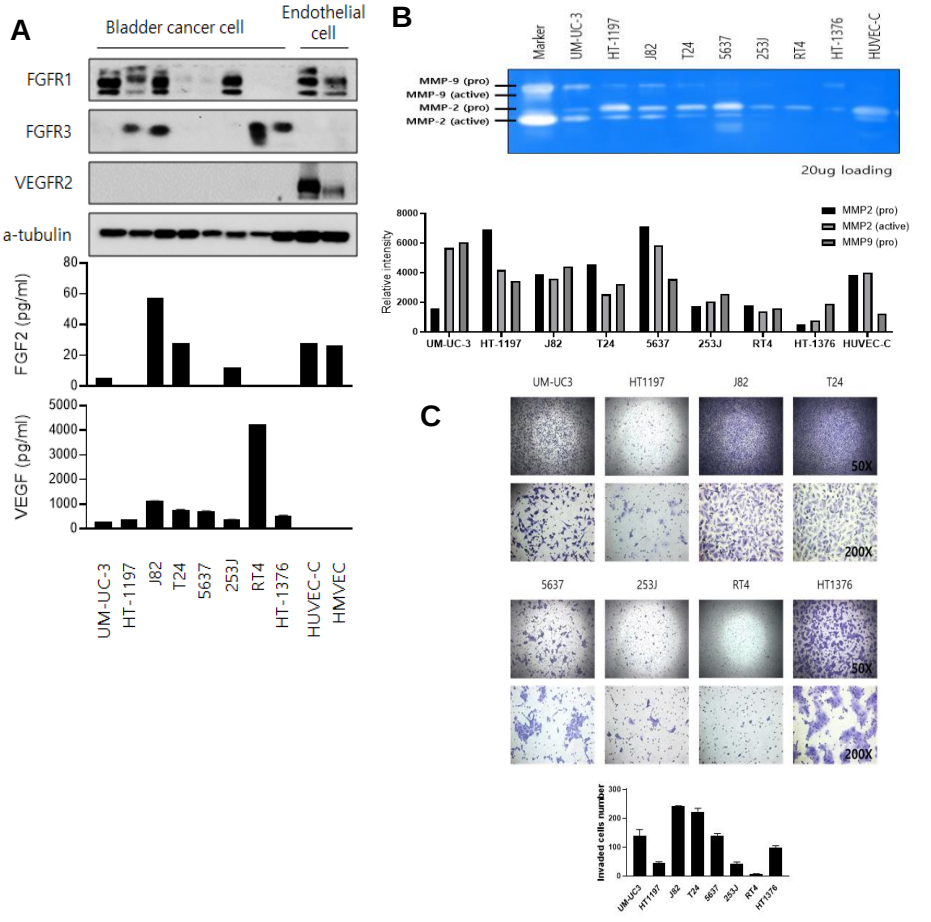


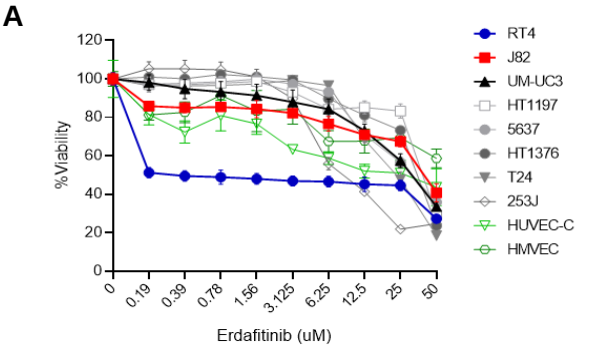
Figure 1. Baseline characteristics of UC cell line and endothelial cells

A. Expression of FGFR1, FGFR3 and VEGFR2 of UC cells and ECs were analyzed using Western Blot immunostaining (upper) and FGF2 and VEGF were measured using ELISA assay (lower) B. Baseline activity of MMP-2 and 9 of each UC and EC cell line was evaluated by zymography (upper), and its quantitation (lower) C. Invasiveness of each UC cells were examined by Transwell invasion assay (x50 in the first row, x200 in the second row) (upper), and its quantitation (lower)

2. The effect of erdafitinib on UC cells

We then investigated the effect of erdafitinib on UC cells. First, we peformed MTT assay to examine the anti-proliferative effect of erdafitinib on each UC cell. The result showed that RT4 was the most sensitive to erdafitinib with IC_{50} of 0.362uM (Figure 2A). This finding was consistent with the previous study which showed erdafitinib was effective in cells with FGFR aberration including *FGFR3-TACC* fusion, the genetic aberration of RT4 harbors[19]. Other UC cells were not sensitive to erdafitinib. Next, we examined inhibitory effect of erdafitinib on the amount of FGF and VEGF secreted by each UC cell using ELISA assay. Erdafitinib increased the amount of FGF2 in J82 cell, and it has relatively no effect on UM-UC-3 cell (Figure 2B). Of note, FGF2 was not measured in RT4 cell even before erdafitinib treatment. By contrast, VEGF level was significantly reduced by erdafitinib in RT4 cell, but there was no change in VEGF level before and after erdafitinib treatment in J82 and UM-UC-3 cell.

We then investigated the downstream molecular changes by erdafitinib in each UC cells. In RT4, pFGFR3, p-Akt and p-Erk were down-regulated by erdafitinib (Figure 2C). On the contrary, p-FGFR1 was down-regulated by erdafitinib in J82 and UM-UC-3, whereas the expression of p-Akt and p-Erk was not decreased by erdafitinib. This finding was consistent with previous studies which showed that robust antitumor activity of erdafitinib was seen in patient- derived-xenograft model with down-regulation of p-Erk[19]



Cell line	IC_{50} (uM)
RT4	0.36187
253J	11.83608
T24	20.51195
HT1376	29.541
5637	34.04269
HT1197	34.75404
UM-UC3	36.71671
J82	45.51463

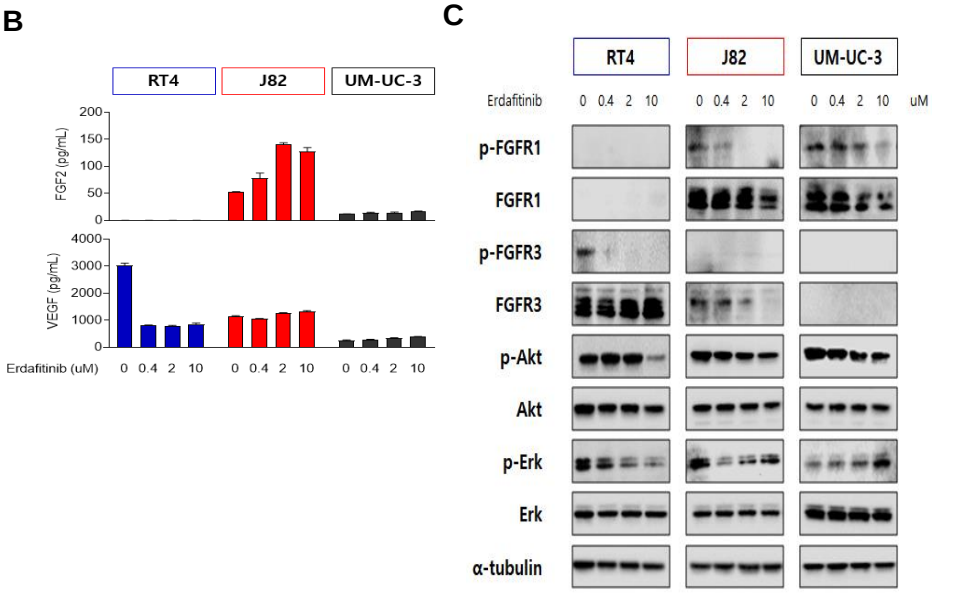


Figure 2. The effect of erdafitinib on UC cells

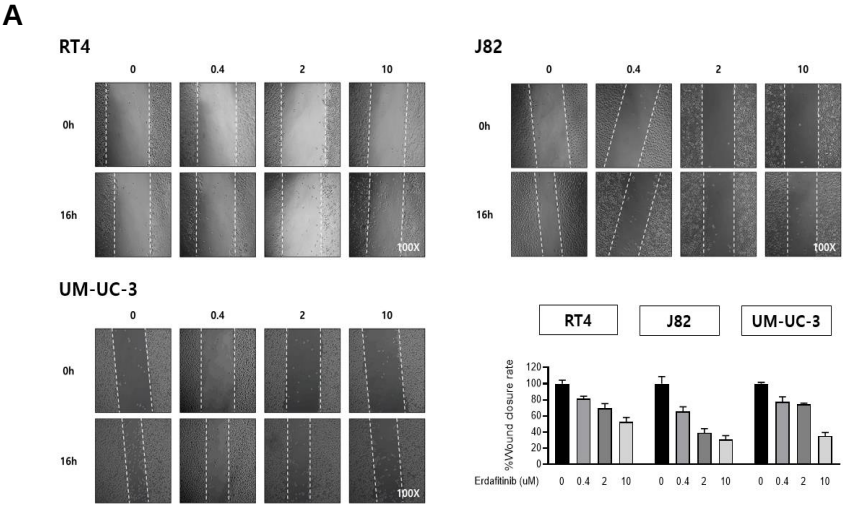
A. Anti-proliferative activity of erdafitinib on each UC cell line was investigated by MTT assay (left panel). RT4 was shown to be most sensitive cell line with IC_{50} of 0.362 μM to erdafitinib treatment (right panel). B. Effect of erdafitinib on FGF and VEGF level of each UC cell line measured by ELISA. VEGF level was decreased by erdafitinib at a dose of 0.4uM in RT4 cell. C. Western blot analysis showed the downregulation of pFGFR3, AKT, and pERK by erdafitinib in RT4 cell. In J82 and UM-UC3, pFGFR1 was also downregulated, but pAKT and pERK were not reduced by erdafitinib.

3. Erdafitinib inhibits the migratory and invasive potential of UC cells

One of characteristics of malignant cell including UC cells, is the ability to migrate to or invade adjacent tissues. Hence, we used wound-healing assay to investigate whether erdafitinib could inhibit the migratory potential of UC cells. Migratory closure in all UC cells examined was significantly inhibited by erdafitinib in a dose-dependent manner (Figure 3A).

We then investigated the effect of erdafitinib on the invasiveness of UC cells. We performed transwell invasion assay using UC cell line J82 and UM-UC-3. RT4 was not used because it lacked invasiveness. Erdafitinib significantly suppressed the invasiveness of UM-UC-3 cell at a dose of 10 μM, but had no effect on J82 cells (Figure 3B).

Aberrant expression of MMPs has close association with invasiveness of UC cells[12]. Thus, we investigated whether erdafitinib inhibited the activities of MMPs in UC cells using gelatin zymography. The active form of MMP-2 was reduced by erdafitinib in a dose-dependent manner in J82 cell, although it was not reduced by erdafitinib in RT4 and UM-UC-3 cells (Figure 3C).



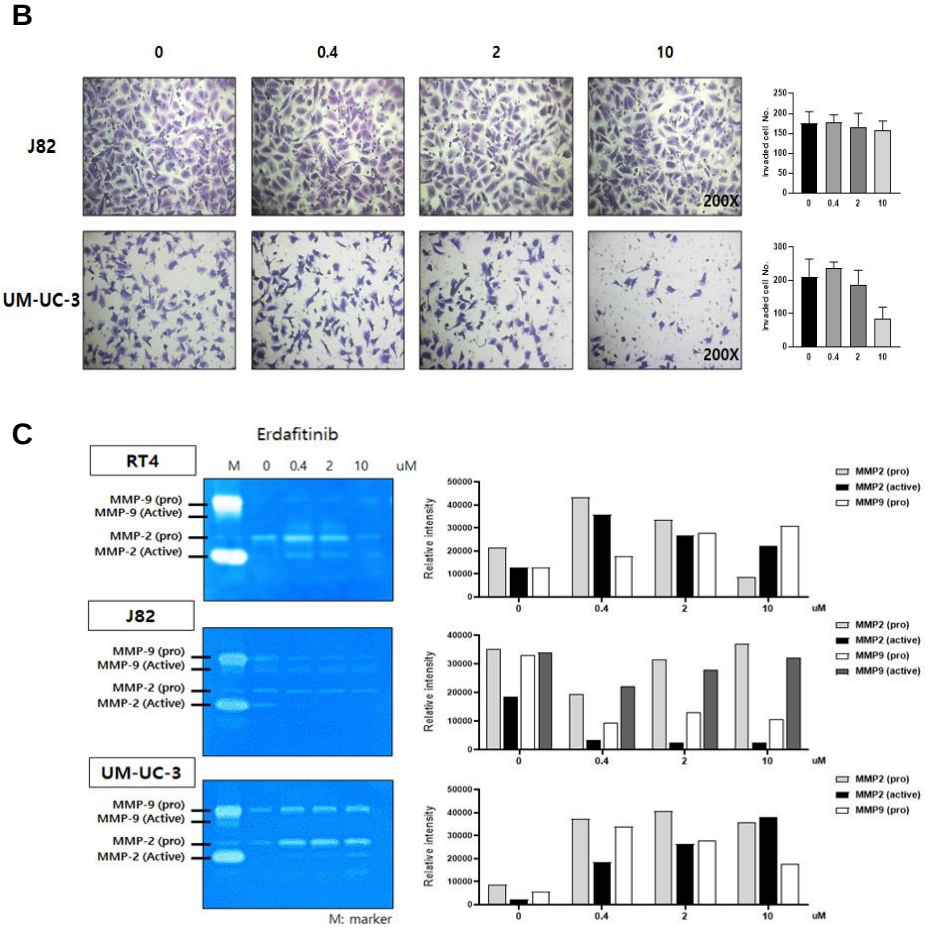


Figure 3. Erdafitinib has inhibitory effect on the migratory potential of UC cells

A. Wound healing assay and its quantitation (right lower panel) showed that erdafitinib has inhibitory activity on the migration of UC cells B. Inhibition of invasiveness of J82 and UM-UC-3 by erdafitinib was shown in transwell invasion assay (left, x200), and its quantitation (right). C. Effect of erdafitinib on pro-form and active form of MMP2 and MMP9 level of each UC cell line measured using zymography (left panel), and its quantitation (right panel). The active form of MMP2 was significantly decreased by erdafitinib in J82 cell.

4. Erdafitinib inhibits tube formation, migration and invasion of HUVECs

FGFRs have been reported to be responsible for tumor angiogenesis, which is a complex process that includes the proliferation, migration, and tube formation of endothelial cells[13]. Therefore, we evaluated the inhibitory activity of erdaftinib on tube forming capacity and migratory potential of ECs. We first investigated the anti-proliferative effect of erdafitinib on ECs using CCK assay, and found that erdafitinib inhibited the growth of ECs (Figure 4A). Next, we investigated whether erdafitinib can inhibit the formation of tubular structure of HUVECs using tube formation assay. We found that the tubule formation by HUVEC was significantly suppressed by erdafitinib in a dose-dependent manner (Figure 4B).

Then we examined the effect of erdafitinib on the invasiveness of HUVECs which is an essential step in the angiogenic process[20]. The invasiveness of HUVECs was significantly suppressed by erdafitinib in a concentration dependent manner (Figure 4C).

Finally, we investigated molecular changes in the anti-angiogenic effect on ECs. Western-Blot assay showed that FGFR1β and FGFR1α was downregulated and upregulated by erdafitinib, respectively (Figure 4D). Since FGFR1β has higher binding affinity to FGF2 than FGFR1α[21-24], this finding suggests that the overall binding affinity of FGFR1 to FGF2 was decreased by erdafitinib.

Collectively, these results indicated that erdafitinb was able to inhibit both the HUVEC's tube formation and invasive activity via the suppression of binding activity of FGFR1 to FGF2 in HUVECs.

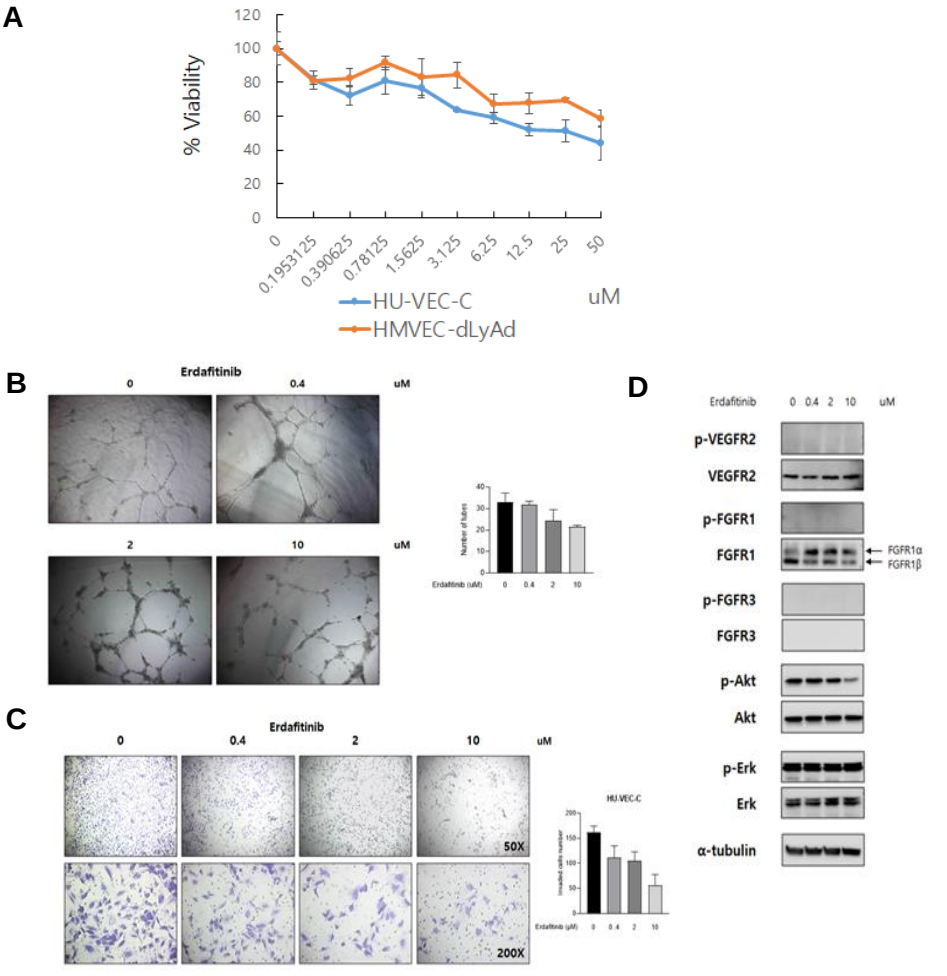


Figure 4. The inhibitory effect of erdafitinib on endothelial cells

A. Erdafitinib has anti-proliferative effect on endothelial cells. B. Tubule formation of HUVECs was suppressed by erdafitinib (left panel), and its quantitation (right panel) C. Migratory potential of HUVECs was inhibited by erdafitinib (x50 in upper panel and x200 in lower panel), and its quantitation (right panel) D. Western blot assay showed FGFR1β was dowregulated and FGFR1α was upregulated by erdafitinib. Considering higher affinity of FGFR1β than FGFR1α to FGF2, this result indicated that overall binding affinity of FGFR1 to FGF2 was decreased by erdafitinib.

5. Crosstalk of urothelial carcinoma cells and endothelial cells

Tumor and stromal cells release FGFs, thereby exerting angiogenic action via autocrine and paracrine signaling[13]. Thus, we investigated whether paracrine factors from UC cells (RT4 and J82) secreted into co-culture supernatants would promote HUVEC migration *in vitro*. HUVEC transwell migration assay was performed to assess the crosstalk between HUVECs and UC cells. The migration of HUVECs induced by the supernatants from co-cultured RT4 cell was significantly decreased by erdafitinib (Figure 5A, upper panel). In contrast, HUVEC co-cultured with J82 did not show migratory potential irrespective of erdafitinib treatment (Figure 5A, lower panel). Although the exact inhibitory mechanism of erdafitinib remains uncertain, the stimulation of VEGF secreted by RT4 (Figure 1A) may be a potential mechanism for the migration of HUVECs, and its suppression by erdafitinib (Figure 2B) could inhibit the migratory potential of HUVECs (Figure 5B). The possible explanation for the lack of migratory potential in HUVECs co-cultured with J82 is attributed to the relatively small amount of endogenous FGF secreted by J82 cell.

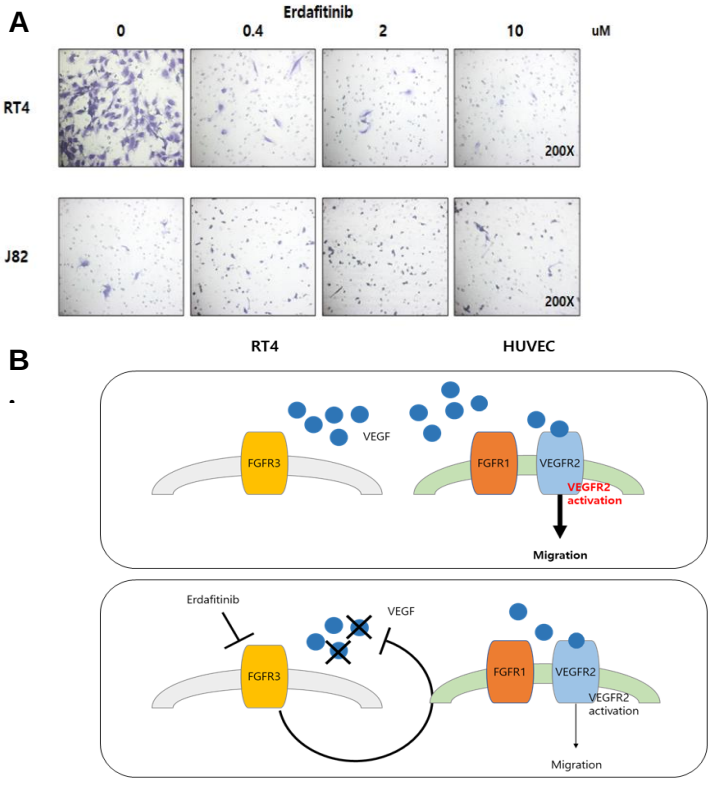


Figure 5. Effect of erdafitinib on crosstalk of UC and endothelial cell

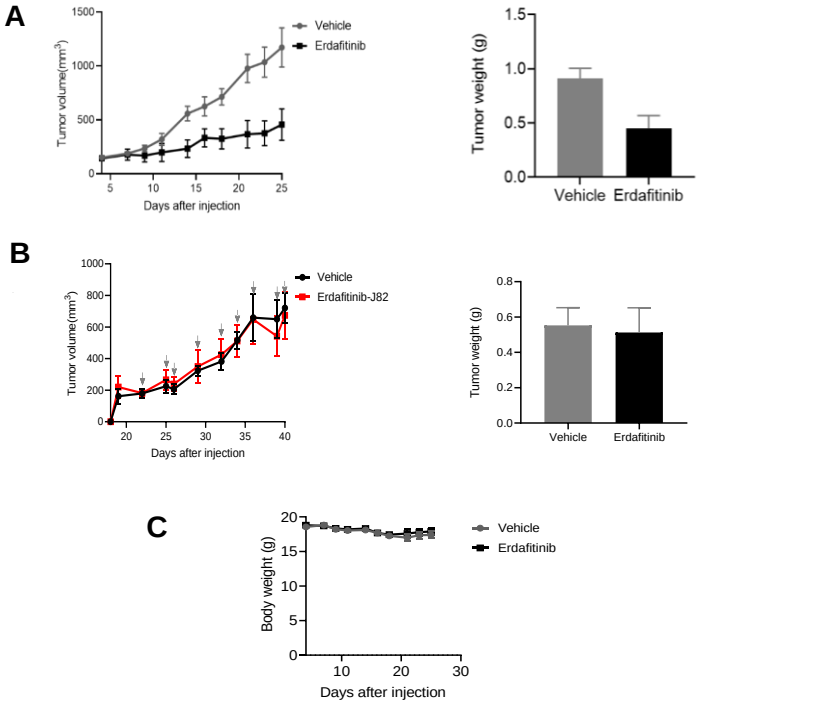
A. Migration of HUVECs induced by supernatants from co-cultured RT4 cell was decreased by erdafitinib (upper panel, x200). HUVEC co-cultured with J82 did not show the migratory potential (lower panel, x200) B. Potential mechanism of HUVEC co-cultured with RT4

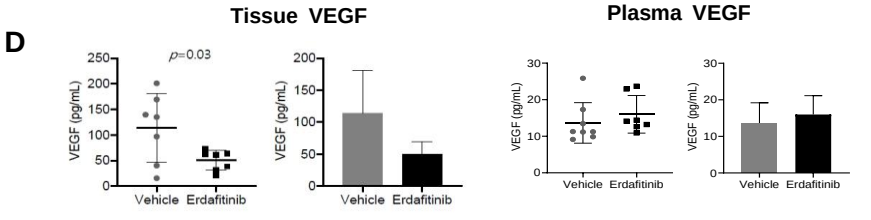
6. Erdafitinib inhibits angiogenesis *in vivo*

Based on the results from the *in vitro* experiments regarding the anti-angiogenic ability of erdafitinib, we further investigated the effect of erdafitinib on angiogenesis using *in vivo* models. Immuno-deficient mice bearing RT4 and J82 xenografts were treated with or without erdafitinib (40mg/kg) orally three times a week for three weeks. The mice were then sacrificed, and tumor tissues were taken out for further analysis. Erdafitinib dramatically suppressed tumor volumes (Figure 6A, left) and tumor weight (Figure 6A, right) of RT4 bearing xenografts. In contrast, there was no significant difference in tumor volume (Figure 6B, left) and weight (Figure 6B, right) between erdafitinib group and control group in J82 bearing xenografts.

Moreover, tumor (both RT4 and J82) bearing mice did not exhibit significant weigh loss during administration of erdafitinib compared to control, suggesting that treatment was well tolerated (Figure 6C).

To further investigate underlying mechanism of tumor growth suppression by erdafitinib in RT4 bearing xenograft, we measured the expression of VEGF in tumor and plasma using ELISA methods. The result showed that VEGF expression in tumor tissue, not in plasma was significantly reduced by erdafitinib (Figure 6D). In addition, tumor tissues were stained with specific antibodies against Ki-67 and CD31 before and after erdafitinib treatment. Ki-67 expression was significantly reduced after erdafitinib treatment, indicating that erdafitinib has anti-proliferative effect on RT4 xenograft model (upper panel of Figure 6E). Furthermore, angiogenesis in tumor tissue could be measured via microvessel density (MVD) through CD31 immunostaining, and we measured MVD to evaluate the anti-angiogenic activity of erdafitinib. MVD was also significantly decreased by erdafitinib treatment in RT4 xenograft model (lower panel of Figure 6E). Taken together, these results indicated that erdafitinib significantly inhibited the growth of FGFR-dependent xenografts by exerting anti-angiogenic activity as well as anti-proliferative activity on RT4 bearing xenograft model.





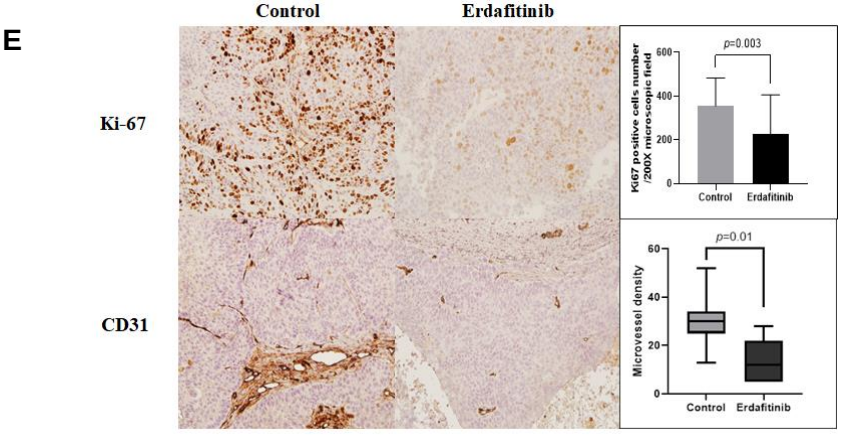


Figure 6. Erdafitinib inhibited tumor growth and angiogenesis on RT4 and J82 urothelial cancer xenografts

A. Treatment with erdafitinib resulted in significant reduction of tumor volume (Left) and tumor weight (Right) on RT4 bearing xenograft. B. Treatment with erdafitinib did not result in reduction of tumor volume (Left) and weight (Right) on J82 bearing xenograft. C. There was no difference in body weight between erdafitinib and vehicle treatment, indicating erdafitinib was well tolerated. D. In RT4 bearing xenograft, VEGF expression measured by ELISA method showed that VEGF was lower in erdafitinib group compared to vehicle group in tissue (Left), not in plasma (Right). E. Representative photography of immunohistochemical analysis (x200 magnification) was presented. (Upper panel) Expression of Ki-67 (Left) and its quantitation (Right) showed that erdafitinib has anti-proliferative effect on RT4 bearing xenograft. (Lower panel) Expression of CD-31 (left) and microvessel density (right) was significantly decreased by erdafitinib indicating antiangiogenic activity of erdafitinib on RT4 bearing xenograft.

IV. DISCUSSION

Tumor angiogenesis is one of the hallmarks for carcinogenesis and is considered as the therapeutic target for numerous malignancies including UC[12]. Among several agents targeting angiogenesis, ramucirumab, a human IgG1 VEGFR-2 antagonist, in combination with docetaxel, significantly prolonged progressive-free survival for patients with platinum-refractory advanced UC[25]. Although VEGF and VEGFR2 has been the most extensively studied and considered established factors in angiogenesis, other angiogenic factors including FGF2 are crucial in angiogenesis. FGFs are known to exert their angiogenic effect by modulating proliferation and migration of endothelial cells, production of proteases, and promotion of integrin and cadherin receptor expression[13]. Hence, studies have attempted to delineate the role of anti-angiogenesis in FGFR inhibitors as a therapeutic strategy[14-17].

Erdafitinib, a potent and selective tyrosine kinase inhibitor of FGFR1-4, demonstrated anti-tumor activity in treatment of advanced UC patients with FGFR genetic alterations[7]. Based on the result, it was granted accelerated approval by FDA for use in patients with advanced UC harboring FGFR alteration who have progressed after platinum-based chemotherapy. However, erdafitinib has not been studied regarding its anti-angiogenic activity. Therefore, we intended to determine the anti-angiogenic activity of erdafitinib using *in vitro* and *in vivo* UC models.

In this study, we examined the baseline characteristics of UC cell lines and investigated the effect of erdafitinib on UC cells. Treatment with erdafitnib significantly inhibited the proliferation of RT4 cells with IC_{50} value of 0.362uM (Figure 2A). This is consistent with previous studies in that erdafitinib showed the anti-proliferative activity in RT4 cell harboring FGFR3 gene aberration (*FGFR3-TACC* fusion). The downregulation of pERK was also observed only in RT4 (Figure 2C) as the downstream signaling change of FGFR as previously reported[19]. Therefore, our study confirms that erdafitinib has anti-proliferative effect on the UC cell line with FGFR aberration.

Since the migratory potential of cancer cells is crucial for cancer metastasis

and progression, we determined the anti-migratory effect of erdafitinib on UC cells. The results of wound healing assay showed that erdafitinib significantly inhibit the migratory potential of UC cells (Figure 3A). Furthermore, erdafitinib inhibited the invasive potential of UC cell (UM-UC-3) using transwell assay (Figure 3B). Since MMPs have been known as critical molecules involved in several aspects of carcinogenesis such as angiogenesis, tumor growth, and metastasis, we have investigated whether the inhibitory activity of erdafitinib on invasiveness of UC cells are associated with MMPs. The result of zymography showed that erdafitinib suppress the expression of active form MMP-2 of J82 cell in a dose-dependent manner (Figure 3C). In contrast, the expression of MMPs have not been reduced by erdafitinib treatment in RT4 and UM-UC-3 cells. This discrepancy between cell lines needs to be further clarified. Collectively, the results of wound healing assay, transwell assay, and zymography assay indicated that erdafitinib has inhibitory effect on the migratory potential of UC cells.

Endothelial cells (ECs) are crucial component for angiogenesis because they play important roles by releasing protease to degrade the basement membrane and migration potentials toward angiogenic signals[12]. ECs also have proliferative capacity to shape new blood vessels and form three-dimensional capillary-like tube structures. Therefore, we examined whether EC's migratory and invasive potential during angiogenesis can be inhibited with erdafitinib. Erdafitinib exhibited potent inhibitory action against capillary-like tube formation in HUVECs (Figure 4B) and their invasive potential (Figure 4C). The result of western blot analysis revealed that these inhibitory effect of erdafitinib was potentially due to downregulation of pFGFR1 β (Figure 4D) which has been known to have more potent binding affinity to FGF2 than FGFR1α[21-24].

Furthermore, in transwell migration assay using HUVEC co-cultured with UC cells (RT4 and J82), migration of HUVEC co-cultured with RT4 was significantly inhibited by erdafitinib (Figure 5A, upper panel). The inhibitory potential of erdafitinib on VEGF secreted by RT4 could be possible

explanation of this finding (Figure 5B). In contrast, HUVECs co-cultured with J82 did not show the migratory potential (Figure 5B, lower panel). This result was unexpected in that the activity of active form MMP2 was reduced by erdafitinib in J82 cell (Figure 3C). The relative paucity of endogenous FGF levels secreted by J82 cell might explain the result. Taken together, erdafitinib has inhibitory effect on the ability of HUVEC to form capillary change and migrate toward angiogenic signals which are essential for angiogenesis.

Based on the results of *in vitro* studies, we evaluated the anti-angiogenic effect of erdafitinib *in vivo* models using RT4 and J82 bearing xenografts. In RT4 bearing xenografts, both tumor volume (Figure 6A, left) and weight (Figure 6A, right) were significantly decreased by erdafitinib, and it was also well tolerated in that body weight of the mice was not reduced (Figure 6C). On the contrary, in J82 bearing xenografts, both tumor volume (Figure 6B, left) and weight (Figure 6B, right) were not reduced by eardafitinib. Since RT4 showed sensitivity to erdafitinib *in vitro* studies, the reduction of tumor volume and weight could be attributed to the anti-proliferative effect of the drug. To investigate whether erdafitinib exert anti-angiogenic activity as well as anti-proliferative effect, we conducted immune-histochemical staining using antibody against Ki-67 and CD31 (Figure 6E). The reduced expression of Ki-67 by erdafitinib indicated that erdafitinib exerted the anti-proliferate activity on xenografts bearing RT4 harboring FGFR aberration. In addition, decrease in MVD shown as reduced CD31 expression by erdafitinib treatment in the RT4 xenografted tumors suggested that erdafitinib also exhibited anti-angiogenic potential in RT4 cells.

The major limitation of this study is that anti-angiogenetic effect of erdafitinib was found only in RT4 cell. Hence, it is hard to generalize the conclusion of this study to all UC cell lines. For generalization, subsequent studies need to conducted using different cell lines

V. CONCLUSION

In conclusion, our study result showed that erdafitinib has anti-angiogenic activities both *in vitro* and *in vivo.* Our study demonstrated that targeting angiogenesis by inhibiting FGFR pathway is feasible therapeutic strategy, and warrants further investigation.

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ABSTRACT (IN KOREAN)

요로상피암에서 FGF/FGFR 의존적 혈관 생성 억제 효과

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임승택

목적: 섬유아세포 성장인자(Fibroblast growth factors, FGFs)와 그 수용체(fibroblast growth factor receptors, FGFRs)는 상피 간엽 이행 및 혈관 생성 같은 종양 생성과 연관되어 있습니다. FGFR의 선택적 억제제인 erdafitinib은 FGFR 경로에 변이를 가지고 있는 요로상피암에서 효과를 보여주고 있습니다. 따라서, 우리는 erdafitinib을 이용하여 EMT와 혈관생성을 억제함으로써 요로상피암을 억제하는 전략의 유효성을 연구하였습니다.

재료 및 방법: 우리는 요로상피암 세포에 대한 erdafitinib의 억제 효과에 대해서 wound healing assay, transwell invasion assay, 및 zymography assay를 시행하였습니다. 또한 tubule formation assay와 transwell migration assay를 시행하여 erdafitinib의 혈관 내피세포에 억제 효과를 확인하였고. transwell migration assay를 시행하여 요로상피암 세포와 공동 배양한 혈관 내피세포 사이의 crosstalk을 평가하였습니다. 생체 내에서 erdafitinib의 효과를 확인하기 위해서 RT4세포를 주입한 nude mice를 이용, 위약군과 erdafitinib 군으로 나누어 실험을 진행하였고, 종양을 제거한 후 면역형광염색과 추가 실험을 진행하였습니다.

결과: Wound healing assay 및 transwell invasion assay 결과는 erdafitinib이 요로상피암 세포의 이동 및 침습 능력을 저해하는 것을 보여주었습니다. Erdafitinib은 또한 혈관 내피세포의 tube formation 및 이동 능력을 저해하였습니다. RT4 세포와 혈관 내피세포를 공동배양하여 시행한 transwell migration assay 결과 혈관 내피 세포의 이동이 erdafitinib에 의해 현저히 저해되었습니다. 생체 모델에서 erdafitinib은 종양의 부피와 무게를 현저히 억제하였고, Ki-67과 CD31 양성 세포가 동시에 감소한 것을 볼 때 erdafitinib은 항 종양 성장 억제 효과와 동시에 혈관 생성 억제 효과가 있음을 확인할 수 있었습니다.

결론: 우리의 연구 결과는 FGFR경로를 억제함으로써 혈관 생성을 표적으로 하는 치료 전략의 유효함을 보여주었으며, 후속 연구가 필요할 것으로 판단됩니다.

핵심 되는 말: carcinoma, transitional cell; angiogenesis inhibitors; receptors, fibroblast growth factor; erdafitinib

PUBLICATION LIST

1. Lim S, Koh MJ, Jeong HJ, Cho NH, Choi YD, Cho do Y, et al. Fibroblast Growth Factor Receptor 1 Overexpression Is Associated with Poor Survival in Patients with Resected Muscle Invasive Urothelial Carcinoma. Yonsei Med J 2016;57:831-9.