Anti-tumor activity and acquired resistance mechanism of Dovitinib (TKI258) in RET rearranged lung adenocarcinoma

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Anti-tumor activity and acquired resistance mechanism of Dovitinib (TKI258) in RET rearranged lung adenocarcinoma

Directed by Professor Byoung Chul Cho

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Chan Woo Kang

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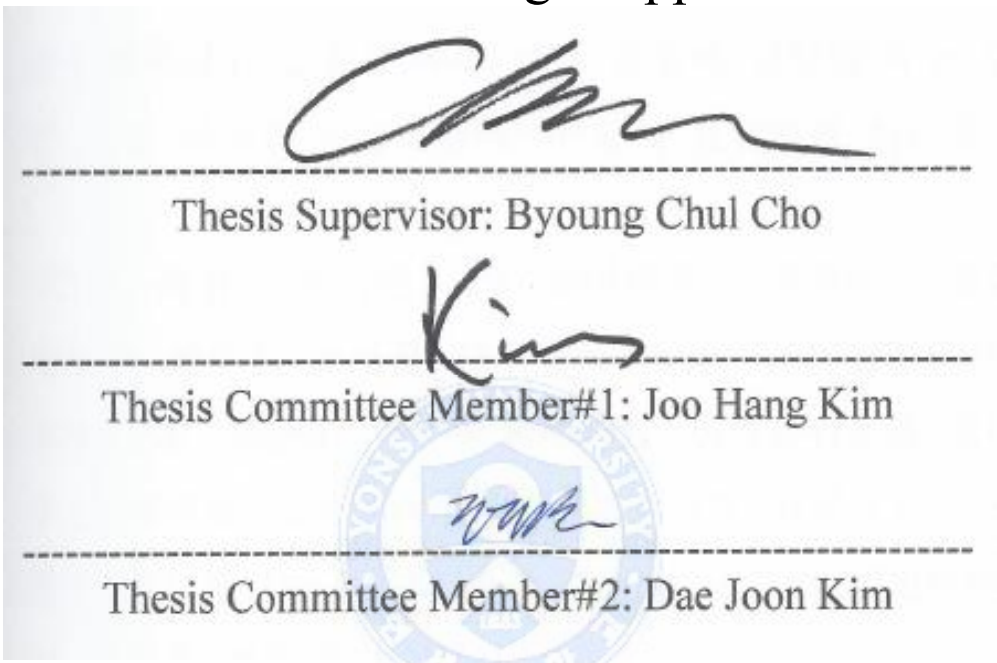
This certifies that the Master's Thesis of
Chan Woo Kang is approved.



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The Graduate School
Yonsei University

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ACKNOWLEGEMENTS

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록 가르쳐 주셔서 감사합니다.

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Anti-tumor activity and acquired resistance mechanism of Dovitinib (TKI258) in *RET* rearranged lung adenocarcinoma

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RET rearrangement is a newly identified oncogenic mutation in lung adenocarcinoma (LADC). Activity of dovitinib (TKI258), a potent inhibitor of FGFR, VEGFR, and PDGFR, on RET-rearranged LADC has not been known. The aims of the study are to explore antitumor effects and mechanism of acquired resistance of dovitinib in RET-rearranged LADC. *In silico* analysis results indicated that dovitinib strongly interacts with the surrounding residues in RET kinase domain, dovitinib potently induced cell cycle arrest in G1 phase and does-dependent suppression of phosphorylation of RET and ERK in LC-2/ad LADC cells harboring CCDC6-RET rearrangement. The therapeutic effects of dovitinib were photocopied by siRNA knockdown of RET. Selective inhibition of dovitinib on autophosphorylation of RET kinases was confirmed in HEK293 cells expressing

KIF5B-RET or CCDC6-RET. A phospho-RTK array reveals that LC-2/ad cells maintain phosphorylation of FGFRs and VEGFRs in the presence of dovitinib. Dovitinib inhibited ERK phosphorylation more efficiently than vandetanib, sunitinib and sorafenib. The effects on ERK phosphorylation were correlated with the results of cell viability assays. Antitumor effect of dovitinib was observed in tumor xenograft model. To identify the acquired resistance mechanism, dovitinib-resistant cells (LC-2/ad DR) were established by exposure of LC-2/ad to stepwise increasing concentration of dovitinib. Knockdown of RET or dovitinib treatment did not inhibit ERK phosphorylation in LC-2/ad DR cells. Saracatinib, a src kinase inhibitor, suppressed ERK phosphorylation and growth of LC-2/ad DR cells. Taken together, dovitinib can be a potential therapeutic option for RET-rearranged LADC, and acquired resistance to dovitinib can be overcome by targeting SRC.

Key Words: lung adenocarcinoma, non-small cell lung cancer, RET rearrangement, dovitinib (TKI258), Src, acquired resistance

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I. INTRODUCTION

Lung cancer is the most common cause of cancer death worldwide.[1] Although survival of lung cancer patients still remains poor, molecular targeted therapies, which block important oncogenic pathways, have made remarkable progress over the years, particularly in lung adenocarcinoma (LADC), which is the most common histological subtype of lung cancer.[2-4] Indeed, treatment with gefitinib or crizotinib in *EGFR* mutation-positive or *ALK*-rearranged advanced LADC, respectively, has led to unprecedented improvements in objective response rate and progression-free survival over cytotoxic chemotherapy.[5] The prevailing concept of genomics-driven medicine based on remarkable efficacy of these targeted therapies and the expanding genomic landscape of LADC have led to a significant surge in the

number of genotype-directed lung cancer trials in molecular subtypes defined by oncogenic driver mutations in *PIK3CA*, *HER2, BRAF*, and *ROS1*. [1]

RET encodes the tyrosine kinase receptor of growth factors belonging to the glial-derived neurotrophic factor family. Recently, *RET* rearrangement, specifically fusion, has emerged as a new molecular subtype in LADC.[6, 7] The prevalence of *RET* fusions has been estimated as approximately 1.2% to 2.0% in LADC.[6-11] Several *RET* fusions have been identified in LADC, including kinesin family member 5B (*KIF5B*)-*RET*, coiled-coil domain containing 6 (*CCDC6*)-*RET*, tripartite motif-containing 33 (*TRIM33*)-*RET* and nuclear receptor coactivator 4 (*NCOA4)-RET*. Among all fusion variants, *KIF5B-RET* is the most common type of fusion involving *RET*, present in approximately 90% of cases reported to date.[9] *RET* fusion was mutually exclusive with other oncogenic driver mutations, such as *EGFR*, *HER2*, *BRAF* or *EML4-ALK* fusion.[9, 10, 12, 13] Furthermore, expression of exogenous *KIF5B-RET* induced morphological transformation and anchorage-independent growth of NIH3T3 fibroblasts. [7] These findings strongly suggest that targeted inhibition of *RET* oncogene might be a potential strategy in the treatment of *RET* fusion-positive LADC.

Small molecule tyrosine kinase inhibitors (TKIs), including vandetanib, sunitinib and sorafenib, effectively inhibited *RET* fusion-positive LADC in preclinical models.[7, 10, 11, 14, 15] Therefore, several clinical trials of RET inhibitors are ongoing in RET fusion-positive LADC. Recently, treatment of cabozantinib and

vandetanib, which have RET inhibitory activity, resulted in dramatic responses in patients with *RET* fusion-positive LADC.[12, 16]

Dovitinib (TKI258) is a potent inhibitor of receptor tyrosine kinases, including fibroblast growth factor receptor (FGFR) 1-3, vascular endothelial growth factor (VEGFR) 1-3, platelet derived growth factor receptor (PDGFR)-β, which has shown anti-tumor and anti-angiogenic effects in preclinical models of colon, breast, bladder, pancreatic, and renal cell cancers.[17-19] However, anti-tumor activity of dovitinib in *RET*-rearranged LADC has not been reported, except for the fact that dovitinib inhibits the RET kinase at a range of 7nM in *in vitro* kinase assay (Ref: dovitinib investigator's brochure).

Herein, we show potent anti-tumor activity of dovitinib in preclinical models of *RET*-rearranged LADC. Additionally, we report the mechanism of acquired resistance to dovitinib in *RET*-rearranged LADC.

II. MATERIALS AND METHODS

1. Compounds, antibodies, and cell lines

Dovitinib was kindly provided by Novartis Institutes for Biomedical Research (Basel, Switzerland). Gefitinib, vandetanib, sorafenib, sunitinib and saracatinib were purchased from Selleck Chemical. Antibodies against EGFR, p-EGFR, RET, p-RET (Y905), AKT, p-AKT (S473), ERK1/2, p-ERK1/2 (T202/Y204), Cyclin D1, p21, p27, FAK, p-FAK (Y397), Src, p-Src (Y416), Paxillin, p-Paxillin (Y118) and Integrin β1, were purchased from Cell Signaling Technology. Rabbit polyclonal antibody to RET (p-Y1062) (ab51103) was purchased from Abcam. Anti-β-actin antibody was purchased from Sigma Aldrich. LC-2/ad cells, a human LADC cell line harboring *CCDC6-RET* fusion, were purchased from RIKEN. H1299 cells and HEK293 cells were purchased from American Type Culture Collection. Each cell line was cultured using the medium recommended by the suppliers.

2. Plasmid constructs and transfection

The plasmids that express KIF5B-RET, CCDC6-RET, and KRAS G12V were purchased from Addgene. HEK293 cells were plated at a density of 0.5×10^5 per 60 mm dish 1 day before transfection. HEK293 cells were transfected with the appropriate expression plasmids using Polyjet transfection reagent (SignaGenat), according to the manufacturer's instructions. Transfected HEK293 cells were cultured at 37 °C for 24 hr and then treated with or without dovitinib for an

additional 4 hr prior to lysis.

3. Growth inhibition assay

For MTT assays, cultured cells were seeded into 96-well plates (3,000 cells per well). Twenty-four hours after seeding, serial dilutions of appropriate inhibitors were added to the culture. After 72 hr, 50 µl of MTT (5 mg/ml stock solution) was added and the plates were incubated for an additional 4 hr. The medium was discarded and the formazan blue, which was formed in the cells, was dissolved with 100 µl DMSO. The optical density was measured at 540 nm. Relative survival in the presence of drugs was normalized to DMSO control cells after background subtraction. For colony formation assays, cells were seeded into 6-well plates (2.5 x 10^4 cells per well) and treated with the agents indicated. RMPI-1640 containing 10% FBS for 14 days. Compound treatments were replaced at least every 3 days during the assay. After 14 days, cells were fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature and stained with 0.5% crystal violet in 20% methanol for 20 min. Images were captured using flatbed scanner, and the cells were dissolved with 20% acetic acids in 20% methanol for 30 min. Assays were performed independently at least three times.

4. Western blot analysis

Preparation of whole cell protein lysates and Western blot analysis were described previously. Briefly, cells were then chilled on ice, washed twice with ice-cold PBS, and lysed in a buffer (Cell Signaling Technology, Beverly, MA, USA) containing 1

mM PMSF and 1X protease inhibitors (Sigma Aldrich). Protein concentrations were determined using a Bradford assay kit (Bio-Rad Laboratories). Equal amounts of protein in cell lysates were separated by SDS-PAGE, transferred to membranes, immunoblotted with specific primary and secondary antibodies, and the blot was detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific), according to the manufacturer's instructions.

5. Small interfering RNA transfection

The small interfering RNAs (siRNAs) targeting RET or Src were purchased from Invitirogen. Cells were plated at a density of 0.5×10^5 per 60 mm dish 1 day before transfection. Cells were transfected with 10 nmol/L of siRNAs for 72 hr using Lipofectamine (Invitrogen), according to the manufacturer's instructions. Transfected cells were cultured at 37 ℃ for 48 hr and then treated with or without appropriate inhibitors for an additional 24hr prior to lysis. The following sequence:

5'-GGAUUGAAAACAAACUCUAtt-3' for RET siRNA;

5'-GCCUCUCAGUGUCUGACUUCGACAA-3' for Src siRNA;

5'-CGUUAAUCGCGUAUAAUACGCGUAT-3' for scramble siRNA.

Cellular expression of RET and SRC was examined by Western blot analysis.

6. Human Phospho-Kinase Array

The Human Phospho-Kinase Array Kit (R&D Systems) was used to detect the

activation of 49 different protein kinases mediating various aspects of cellular proliferation following the manufacturer's instructions. Briefly, LC-2/ad cells and LC-2/ad DR cells were plated in 10-cm dishes and cultured at 37 °C for 28 hr. After cell lysis, 500 μg of lysate was applied to a membrane-anchored phosphor kinase array and incubated at 4 °C for 24hr. Membranes were exposed to chemiluminescent reagents and detected by X-ray film (AGFA).

7. Xenograft studies

All experiments were in accordance with the Yonsei University Medical College guidelines and regulations approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Six-week-old Nude mice were injected subcutaneously with 8.0 x 10^6 LC-2/ad cells or LCD-2/ad DR cells. Mice were randomized to groups of 7 animals to receive either vehicle, vandetanib or dovitinib (oral, qd) for the indicated duration of treatment. The length, width, and depth of the tumor mass were measured every 2 days using calipers, and tumor volume was calculated as: tumor volume = 0.5236 x length x width x depth (mm^3). The rate of change in body weight (BW) was calculated using the following formula: BW= W/W_0 x 100, where W and W_0 are the body weight on a specific experimental day and on the first day of treatment, respectively.

8. Microarray analysis

Total RNA was extracted using RNeasy columns (Qiagen, Valencia, USA) according to the manufacturers' protocol. RNA purity and integrity were evaluated by ND-1000 Spectrophotometer (NanoDrop), Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was amplified and purified using TargetAmp-Nano Labeling Kit forIlluminaExpression BeadChip (EPICENTRE) to yield biotinylatedcRNA according to the manufacturer's instructions. Briefly, 200~500ng of total RNA was reverse-transcribed to cDNA using a T7 oligo (dT) primer. Second-strand cDNA was synthesized, *in vitro* transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer. 750 ng of labeled cRNA samples were hybridized to each HumanHT-12 v4.0 Expression Beadchipfor 16-18 h at 58°C, according to the manufacturer's instructions (Illumina, Inc.). Detection of array signal was carried out using Amershamfluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences) following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner according to the manufacturer's instructions.

9. Statistical analysis

Data are presented as means ± S.D. of results from three independent experiments with similar patterns. Statistical significant values were compared using ANOVA and Dunnett's post-hoc test.

III. RESULTS

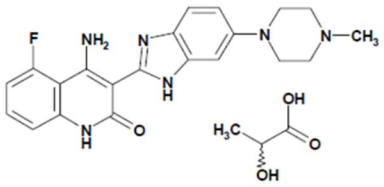
1. Dovitinib inhibits RET kinase activity

Dovitinib, of which chemical structure was shown in **Figure 1A**, targets type Ⅲ,Ⅳ, and Ⅴ RTKs including PDGFRβ, CSF-1R, KIT, FLT3, VEGFR, TrkA, *RET* and FGFRs. To explore *RET* fusion as an additional target indication of dovitinib, we first performed *in silico* modeling of *RET* with dovitinib and two other *RET*-TKIs, sunitinib and vandetanib (**Fig. 1B**). Docking score of *RET* kinase complex with dovitinib, sunitinib and vandetanib were calculated using Glide (**Fig. 1B**). Docking simulation showed almost identical docking structure for dovitinib and two other *RET*-TKIs. Dovitinib-*RET* complex exhibited high docking score (-9.16), which was comparable to those of sunitinib and vandetanib. The structure modeling suggested that dovitinib formed hydrogen bonds with A907 and Q905 sites, which are located within active site of the *RET* kinase. According to Novartis investigator's brochure, dovitinib inhibited *RET* in nanomolar range (7nM) *in vitro* kinase assay. Together, these results strongly suggest that dovitinib has potential to inhibit *RET* kinase activity.

To determine whether dovitinib could inhibit kinase activity of the two most common type of fusions, *CCDC6-RET* and *KIF5B-RET*, we first transfected HEK293 cells with plasmids encoding each fusion variants. Cells were lysed after dovitinib treatment and exogenous expression of *RET* kinase tyrosine 905 (Y905)

was determined by immunoblot analysis (**Fig. 1C**). Dovitinib suppressed the phosphorylation of Y905 in both *CCDC6-RET* and *KIF5B-RET* fusion kinases, resulting in significant reduction of ERK1/2 phosphorylation in *CCDC6-RET* or *KIF5B-RET* plasmid-transfected HEK293 cells. However, ERK1/2 phosphorylation was unaffected by dovitinib in HEK293 cells transfected with mutant $KRAS^{G12V}$ plasmids. Next, we examined whether dovitinib selectively inhibited *RET* activity, leading to decreased ERK1/2 phosphorylation, by transfecting either $KRAS^{G12V}$ or *KIF5B-RET* plasmids into HEK293 cells (**Fig. 1D**). The ERK1/2 phosphorylation induced by $KRAS^{G12V}$ transfection could not be suppressed by treatment with dovitinib, a *RET*-TKI (vandetanib) or a selective EGFR-TKI (gefitinib). On the other hand, dovitinib and vandetanib, but not gefitinib, potently inhibited phosphorylation of *RET* and ERK1/2 in *KIF5B-RET* transfected HEK293 cells. Taken together, these results indicate that dovitinib has ability to specifically inhibits *RET* kinase activity and ERK1/2 signaling mediated by *RET*.

A



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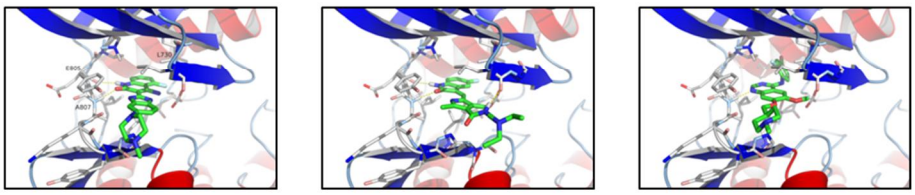
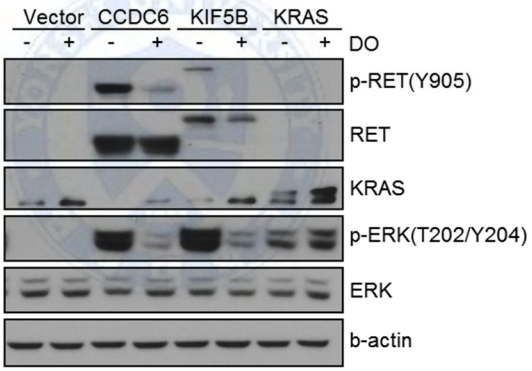


Dovitinib - RET Sunitinib - RET Vandetanib - RET

C



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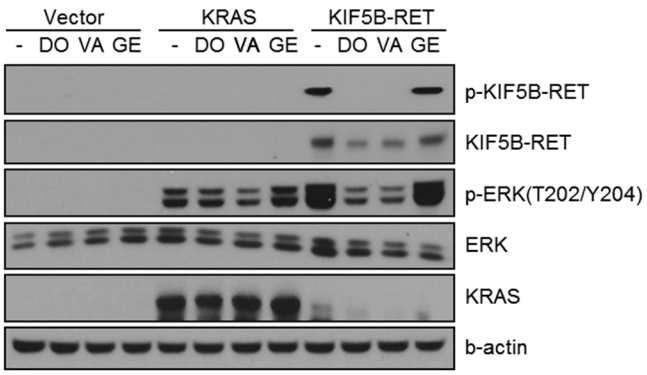


Figure 1. Inhibition of RET-fusion kinase activity by dovitinib. (A) Chemical structure of dovitinib. (B) X-ray structure of dovitinib with RET, sunitinib with RET, and vandetanib with RET. (C) Effects of dovitinib on the phosphorylation levels of RET in HEK293 cells expressing CCDC6-RET, KIF5B-RET, or KRAS. Each transfected HEK293 cells were treated with dmso (-) or dovitinib (DO) for 4hr. The expression levels of indicated protein levels were detected by western blot analysis using the corresponding antibodies. (D) Western blot analysis showing the effect of dovitinib, vandetanib, and gefitnib on expression of indicated proteins. Each transfected cells were treated with indicated inhibitor for 4 hr.

2. Dovitinib induces cell cycle arrest and apoptosis of RET rearranged LADC via selective inhibition of RET kinase

LC-2/ad cells were recently reported to harbor the *CCDC6-RET* rearrangement. we evaluated the effect of dovitinib on the growth of the LC-2/ad cells. Compared with H1299 cells without *RET* rearrangement, dovitinib potently suppressed the growth of LC-2/ad (IC_{50} ≒ 0.2μM), further confirming selective anti-tumor effects of dovitinib against *RET* kinase activity (**Fig. 2A**). Consistent with the results of MTT assays, treatment of dovitinib suppressed the phosphorylation of *RET* and ERK1/2 in a dose-dependent manner (**Fig. 2B**). Moreover, dovitinib inhibited the expression of cyclin D1 and induced p21 and p27 (**Fig. 2B)**, which was in parallel with the fact that dovitinib significantly induced cell cycle arrest at G0/G1 phase (**Fig. 2C**). Furthermore, treatment of dovitinib significantly increased a subpopulation of the annexin V-positive apoptotic cells (**Fig. 2D**).

As dovitinib is known to target multiple tyrosine kinases, the growth suppression effect of dovitinib in LC-2/ad may not be due to selective inhibition of *RET* kinase. To rule out this possibility, introduction of si*RET* in LC-2/ad significantly suppressed the proliferation, whereas siFGFR3, siPDGFR, siFLT3 and siKIT had no significant effect on cell viability (**Fig. 2E and 2F**).

Taken together, these results suggest that dovitinib induces cell cycle arrest and cellular apoptosis via selective inhibition of *RET* kinase and its downstream signaling in lung cancer cells harboring *RET* rearrangement.

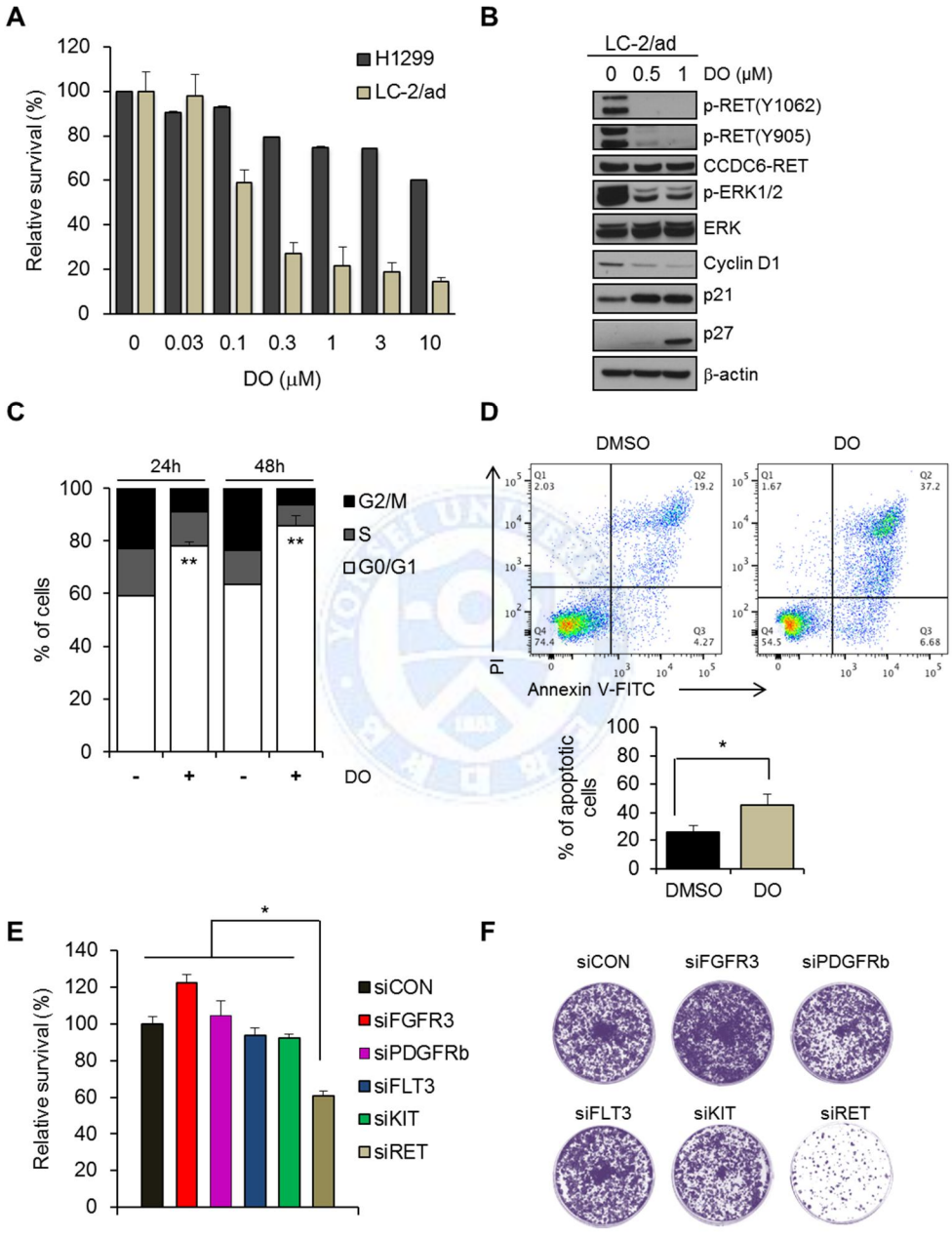
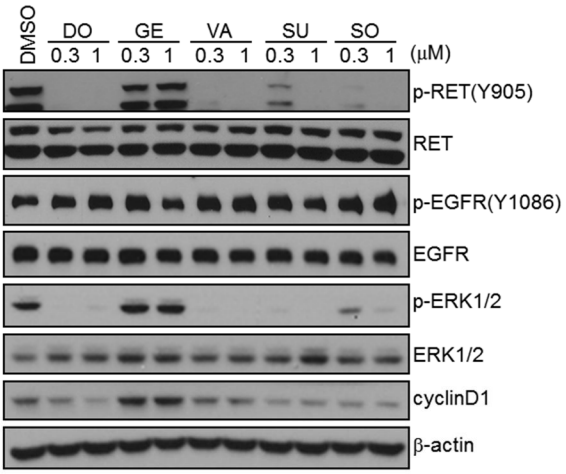

A
Relative survival (%)
H1299
LC-2/ad
DO (μM)
B
LC-2/ad
0 0.5 1 DO (μM)
p-RET(Y1062)
p-RET(Y905)
CCDC6-RET
p-ERK1/2
ERK
Cyclin D1
p21
p27
β-actin
C
24h
48h
% of cells
G2/M
S
G0/G1
DO
D
DMSO
DO
PI
Annexin V-FITC
% of apoptotic cells
E
Relative survival (%)
siCON
siFGFR3
siPDGFRb
siFLT3
siKIT
siRET
F
siCON
siFGFR3
siPDGFRb
siFLT3
siKIT
siRET

Figure 2. Inhibition of RET-fusion driven tumors by dovitinib. (A) Relative survival of H1299 and LC-/2ad against doviitinib after 72 hr treated with dovitinib. (B) Western blot analysis of CCDC6-RET, ERK1/2, and cyclinD1, p21, p27 and β-actin in LC-2/ad cells after treated with dovitinib for 4h. (C) LC-2/ad cells were treated with dovitinib for 24 or 48 hr. Cell cycle distribution was measured by propidium iodide staining and subsequent FACS analysis. Error bars indicate mean ± SEM (n = 3). **p<0.01, vehicle (DMSO) vs. DO (dovitinib) in both 24 hr and 48 hr. (D) FACS analysis via Annexin- V/PI double-staining assay was used to observe the induction of apoptosis. After treating with dmso or dovitinib for 72 hr, cells were stained with annexin V-FITC/PI double staining. Error bars indicate mean ± SEM (n=3). *p<0.05, dmso vs. dovitinib. (E) MTT assay of siCON, siFGFR3, siPDGFRb, siFLT3, siKIT, or siRET-transfected LC-2/ad cells. Indicated siRNA-transfected LC-2/ad cells were incubated for 72 hr before treating with MTT solution**.** **(**F) Colony formation assay of siCON, siFGFR3, siPDGFRb, siFLT3, siKIT, or siRET-transfected LC-2/ad cells. . Indicated siRNA-transfected LC-2/ad cells were incubated for 14 days before staining with crystal violet.

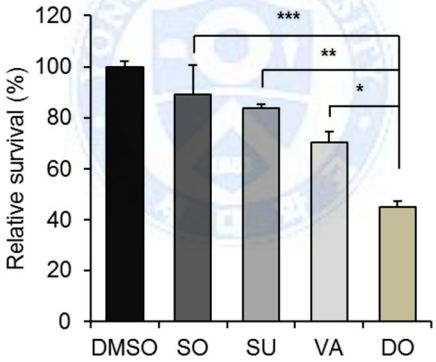
3. Dovitnib inhibits growth of RET rearranged LADC more effectively than other RET-TKIs

We next compared antitumor effects of dovitinib with other known *RET*-TKIs, such as vandetanib, sunitinib and sorafenib, using gefitinib, a selective EGFR-TKI, as a negative control. Contrary to gefitinib, dovitinib suppressed the phosphorylation of *RET* and ERK1/2 and the expression of cyclin D1 as potently as other known *RET*-TKIs (**Fig. 3A**). Notably, compared with other *RET*-TKIs, dovitinib exerted the most potent inhibitory effects on the proliferation of LC-2/ad cells in MTT assays (**Fig. 3B**). The superior antitumor activity of dovitinib to vandetanib, sunitinib and sorafenib in LC-2/ad cells became more evident through long-term (14 days) colony formation assays (**Fig. 3C**). Taken together, our data suggest that dovitinib inhibits growth of *RET* rearranged LADC at least as effectively as other known *RET*-TKIs.

A


DMSO
DO
GE
VA
SU
SO
0.3 1 0.3 1 0.3 1 0.3 1 0.3 1 (μM)
p-RET(Y905)
RET
p-EGFR(Y1086)
EGFR
p-ERK1/2
ERK1/2
cyclinD1
β-actin

B


Relative survival (%)
120
100
80
60
40
20
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DMSO
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C

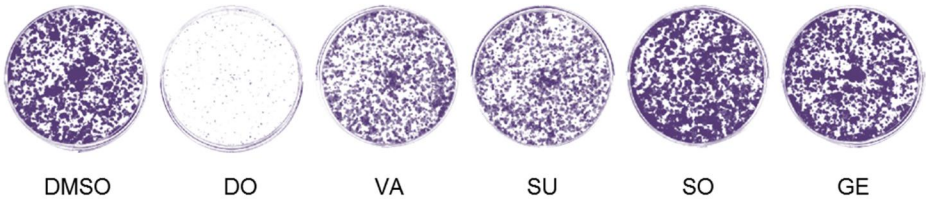
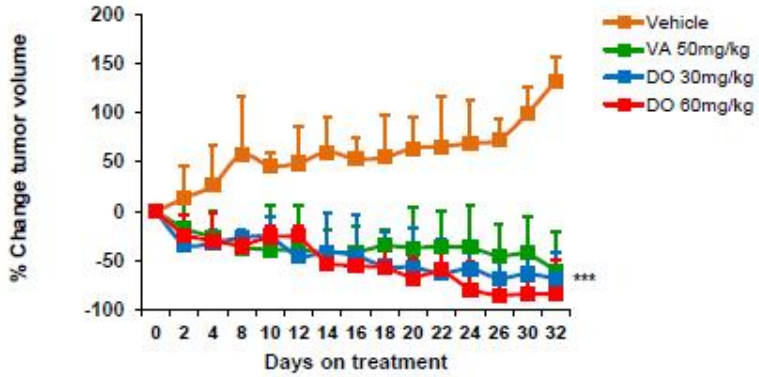

DMSO
DO
VA
SU
SO
GE

Figure 3. Differences in the cell growth inhibition efficacy of RET-fusion driven NSCLC between dovitinib and other RET inhibitors. (A) Western blot analysis of indicated markers in LC-2/ad cells after treated with dovitinib, gefitinib, vandetanib, sunitinib, or sorafenib for 4hr. (B) MTT assays of LC-2/ad. Cells were treated with 1M of sorafenib, sunitinib, vandetanib, or dovitinib for 72 hr, and cell viability was determined using MTT (5ug/ml) solution by measuring the absorbance at 540nm in a microplate reader. Means are drived from five replicate (n=3). Error bars indicate mean ± SE of three replicates. *P < 0.05, vandetanib versus dovitinib; **P < 0.01, ***P < 0.001, dovitinib versus sunitinib or sorafenib in LC-2/ad cells, respectively. (C) Colony formation assay of LC-2/ad cells. Cells were grown in the absence or presence of the indicated inhibitor for 14 days. All cells were fixed and stained with crystal violet (0.005%), represent quantification.

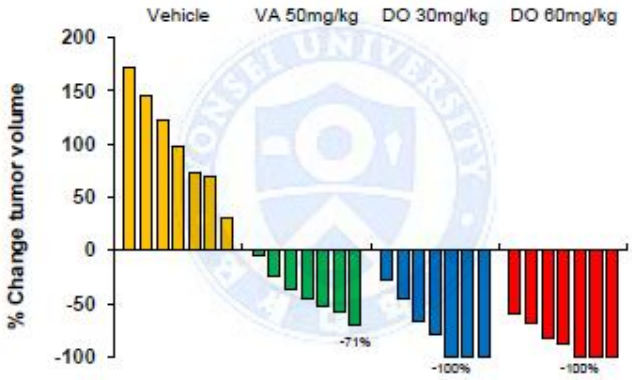
4. Anti-tumor activity of dovitinib in LC-2/ad xenograft models

To evaluate anti-tumor activity of dovitinib *in vivo*, we tested the effects of dovitinib and vandetanib on the growth of LC-2/ad xenograft tumors established in nude mice. Mice treated with dovitinib showed remarkable tumor regression at all doses without significant weight loss at 30 mg/kg and 60 mg/kg doses of dovitinib (**Fig. 4A**). In waterfall plots, the percent volume changes of the individual tumors were greater in dovitinib treatment group compared with those in vandetanib group, showing complete tumor regression (-100%) in 3 out of 7 mice treated with two different doses of dovitinib (**Fig. 4B**). Dovitinib suppressed phosphorylation of *RET* and ERK1/2 in a dose dependent manner relative to vehicle treatment, as assessed by immunoblots and immunohistochemistry of tumor tissues from xenograft (**Fig. 4C and 4D**). Taken together, our *in vitro* and *in vivo* findings confirm that dovitinib is effective against *RET* fusion-positive LADC.

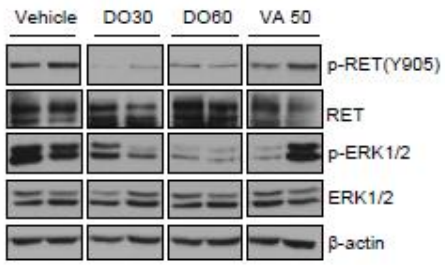
A


% Change tumor volume
200
150
100
50
0
-50
-100
0 2 4 8 10 12 14 16 18 20 22 24 26 30 32
Days on treatment
Vehicle
VA 50mg/kg
DO 30mg/kg
DO 60mg/kg
***

B


Vehicle
VA 50mg/kg
DO 30mg/kg
DO 60mg/kg
% Change tumor volume
200
150
100
50
0
-50
-100
-71%
-100%
-100%

C


Vehicle
DO30
DO60
VA 50
p-RET(Y905)
RET
p-ERK1/2
ERK1/2
β-actin

D

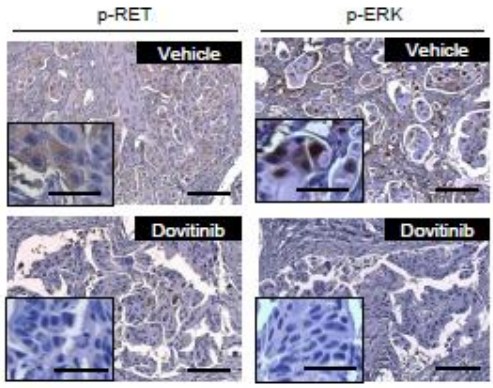

p-RET
p-ERK
Vehicle
Vehicle
Dovitinib
Dovitinib

Figure 4. Anti-tumor activity of dovitinib against RET-fusion driven tumors. (A) Mice bearing LC-2/ad cells were treated with vehicle only (Vehicle), dovitinib (DO 30 mg/kg), dovitinib (DO 60 mg/kg), or vandetanib (VA 50 mg/kg) for 32days. Average percent change in tumor volume relative to initial tumor volume is shown. Error bars represent SEM. ***P < 0.0001 for vehicle vs. dovitinib 30mg/kg treatment group. (B) Waterfall plots showing the percent change in volume (relative to initial tumor volume) for the individual tumors in each treatment group. Maximum regression tumors for each group are indicated. (C) Mice bearing LC-2/ad cells expressing CCDC6-RET were orally administered a dose of 0(vehicle), 30 mg/kg, 60 mg/kg dovitinib (DO), or 50 mg/kg vandetanib (VA) and the tumors were collected and lysed at 6hours postdosing. The expression levels of phospho-RET, RET, phospho-ERK, ERK, and β-actin were detected by immunoblot analysis using the appropriate antibodies. (D) Immunohistochemical (IHC) analysis of LC-2/ad xenograft tumor samples. Mice were orally administered a dose of 0(vehicle), or 60 (dovitinib 60 mg/kg) for 6 hr and sacrificed, and tumors were stained with indicated antibodies. Photos shown are representative fields in each group in low and high magnification. Scale bars in the main images and in the insets indicate 100 and 50 μM, respectively.

5. Src activation in acquired dovitinib-resistant cells

In most targeted agents, acquired resistance eventually develops after an initial response.[20] To identify potential mechanisms of acquired resistance to dovitinib, we established LC-2/ad DR cells with acquired resistance to dovitinib by exposing LC-2/ad cells to increasing doses of dovitinib. LC-2/ad DR cells showed strong resistance to dovitinib (IC_{50}> 3 μmol/L; **Fig. 5A**). Next, LC-2/ad and LC-2/ad DR cells were subjected to genome-wide gene expression profiling using cDNA microarray. Gene-set enrichment analysis against Kyoto Encyclopedia of Genes and Genomes (KEGG) database showed that focal adhesion pathway was significantly enriched, with Src contributing significantly to the core enrichment, in LC-2/ad DR cells, as compared with LC-2/ad cells (**Fig. 5B**). LC-2/ad DR cells displayed higher phosphorylation level of Src and focal adhesion kinase (FAK) in phospho-kinase array than LC-2/ad cells, which was confirmed by immunoblots (**Fig. 5C and 5D**). Increased Src activation in LC-2/ad DR cells led to constitutive ERK1/2 phosphorylation unresponsive to dovitinib treatment (**Fig. 5D**). Interestingly, contrary to LC-2/ad cells, LC-2/ad DR cells did not activate RET kinase any more, suggesting the loss of addiction to *RET* fusion in these cells (**Fig. 5D**).

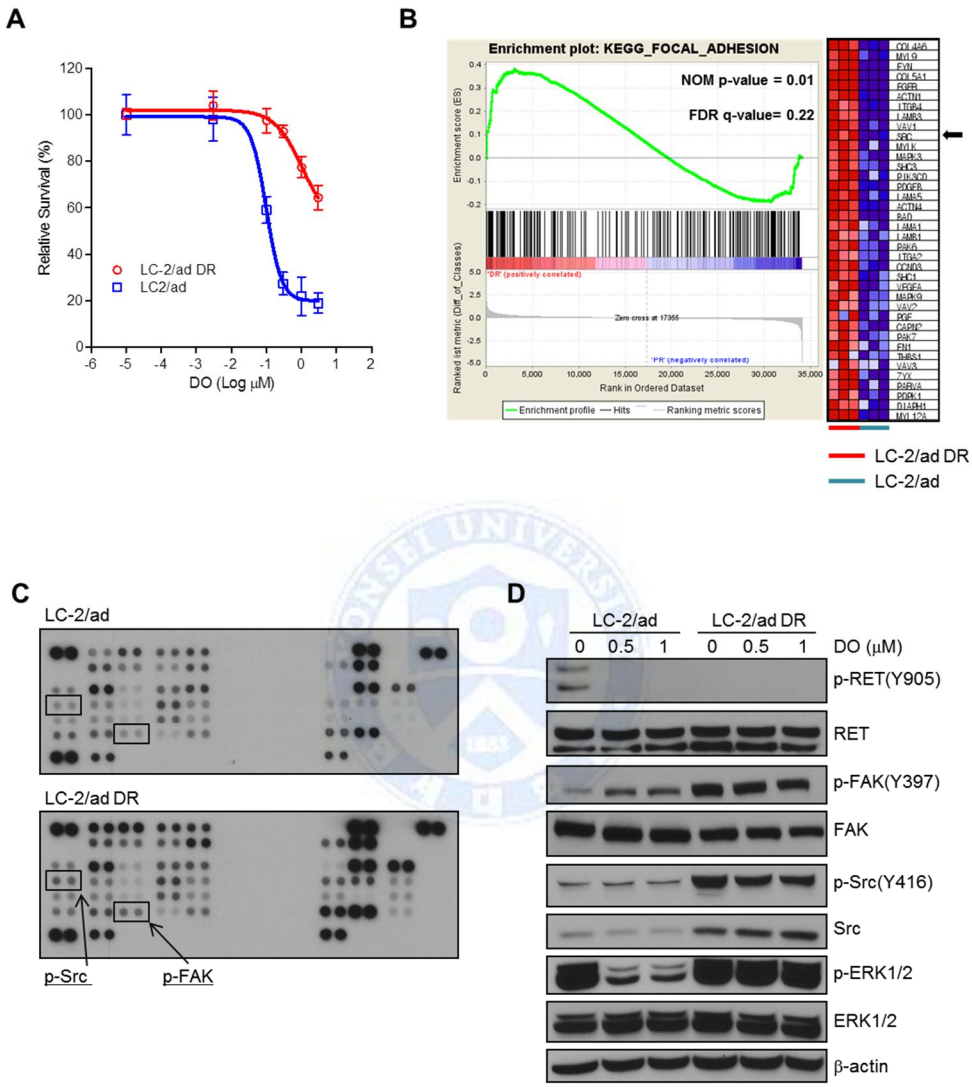

A
Relative Survival (%)
DO (Log μM)
LC-2/ad DR
LC2/ad
B
Enrichment plot: KEGG_FOCAL_ADHESION
NOM p-value = 0.01
FDR q-value= 0.22
Enrichment score (ES)
Ranked list metric (Diff_of_Classes)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
LC-2/ad DR
LC-2/ad
C
LC-2/ad
LC-2/ad DR
p-Src
p-FAK
D
LC-2/ad
LC-2/ad DR
DO (μM)
p-RET(Y905)
RET
p-FAK(Y397)
FAK
p-Src(Y416)
Src
p-ERK1/2
ERK1/2
β-actin

Figure 5. Src hyperactivation in acquired dovitinib-resistant cells. (A) MTT assay comparing cell proliferation of indicated parental LC-2/ad cells and their corresponding acquired dovitinib-resistant LC-2/ad DR cells upon treatment with dovitnib for 72 hr. (B) The GSEA demonstrating a significant enrichment of the set of focal adhesion pathway genes among significantly differentially expressed genes between LC-2/ad and LC-2/ad DR cells. (C) The phosphorylation state was detected by R&D Systems Proteome Profiler Phospho-Kinase Array in LC-2/ad or LC-2/ad DR. Key activated kinase are indicated (FAK and Src). (D) Lysates from LC-2/ad and LC-2/ad DR cells were evaluated by western blotting to determine total and phosphorylated protein levels from the arrays in (C).

6. Src inhibition overcomes dovitinib resistance *in vitro*

Previous studies have demonstrated that Src sits on the signaling node mediating resistance to multiple anticancer agents.[21, 22] The increased activation of Src led us to hypothesize that Src activation directly mediated acquired resistance to dovitinib in LC-2/ad DR cells. To test this hypothesis, we inhibited Src activity using either saracatinib, a selective Src inhibitor, or siRNA targeting Src (siSrc) in LC-2/ad DR cells. Although dovitinib and other known *RET*-TKIs could not inhibit tumor growth in colony formation assays, saracatinib significantly exerted growth inhibitory effects on LC-2/ad DR cells (**Fig. 6A**). Furthermore, siSrc could efficiently inhibit tumor growth only in LC-2/ad DR cells (**Fig. 6B**). On the other hand, si*RET* could not inhibit tumor growth in LC-2/ad DR cells, suggesting the minimal role of *RET* signaling in survival of these cells (**Fig. 6B**). Consistent with cell viability data, inhibition of *RET* kinase by dovitinib or si*RET* abrogated ERK1/2 activity only in *RET*-dependent LC-2/ad cells, and inhibition of Src by saracatinib or siSrc abrogated ERK1/2 activity only in Src-dependent LC-2/ad DR cells (**Fig. 6C and 6D**). Finally, concurrent inhibition of *RET* and Src signaling did not show synergistic antitumor effect in LC-2/ad DR cells, further supporting complete loss of dependence on *RET* signaling for survival upon acquisition of acquired resistance to dovitinib (**Fig. 6E**).

Given the activation of FAK and mRNA upregulation of integrin, we

hypothesized that FAK-integrin axis was involved in the Src activation. To rule out this hypothesis, we used PF-562,271, ATP-competitive reversible inhibitor of FAK. Unexpectedly, PF-562,271 had neither anti-proliferation effect nor inhibition of downstream ERK1/2 activation in LC-2/ad DR cells (data not shown). Expressions of integrins in LC-2/ad DR cells were similar with LC-2/ad cells (data not shown).

Taken together, these data indicate an essential role of Src in mediating the acquired resistance to dovitinib in *RET* fusion-positive LADC.

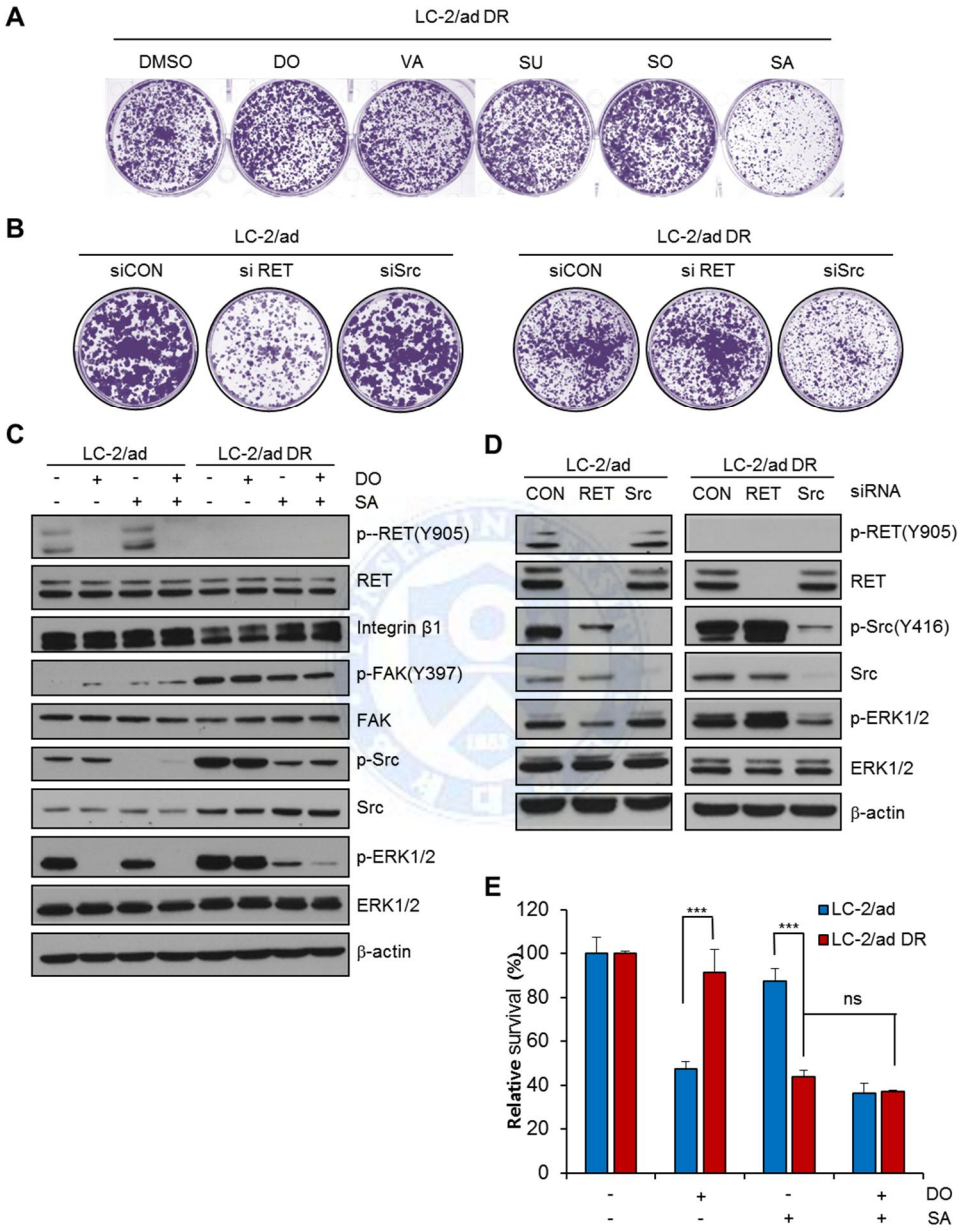

A
LC-2/ad DR
DMSO
DO
VA
SU
SO
SA
B
LC-2/ad
LC-2/ad DR
siCON
si RET
siSrc
siCON
si RET
siSrc
C
LC-2/ad
LC-2/ad DR
DO
SA
p--RET(Y905)
RET
Integrin β1
p-FAK(Y397)
FAK
p-Src
Src
p-ERK1/2
ERK1/2
β-actin
D
LC-2/ad
LC-2/ad DR
CON RET Src
CON RET Src
siRNA
p-RET(Y905)
RET
p-Src(Y416)
Src
p-ERK1/2
ERK1/2
β-actin
E
Relative survival (%)
120
100
80
60
40
20
0
LC-2/ad
LC-2/ad DR
***
***
ns
DO
SA

Figure 6. Loss of dependency on RET-fusion and gain of dependency on Src in dovitinib-resistant cells. (A) Colony formation assay of LC-2/ad DR cells. Cells were grown in the absence or presence of the indicated inhibitor for 14 days. All cells were fixed, stained with crystal violet (0.005%). (B) Colony formation assay of LC-2/ad and LC-2/ad DR cells. Cells were transfected with indicated siRNA and grown for 14 days. All cells were fixed, stained with crystal violet (0.005%). (C) Western blot analysis assessing the effect of dovitinib, saracatinib, or combination treatment on LC-2/ad and LC-2/ad DR cells. Cells were treated indicated inhibitor(s) for 4 hr. Lysates were evaluated by western blotting with the indicated antibodies. (D) Western blot analysis of assessing the dependency, whether cell survival is mediated by *CCDC6-RET* or Src. Cells were transfected with siCON, si*RET*, or siSrc for 72 hr. Lysates were evaluated by western blotting with the indicated antibodies. (E) MTT assay of single or combination treatment of dovitinib and saracatinib in LC-2/ad and LC-2/ad DR cells. LC-2/ad and LC-2/ad DR cells were incubated for 72h with indicated inhibitors before treating with MTT solution. Error bars indicate mean ± SE of three replicates. ***P < 0.001, dovitinib or saracatinib treated LC-2/ad versus LC-2/ad DR; ns, saracatinib treated LC-2/ad DR versus combination treated LC-2/ad DR.

IV. DISCUSSION

In our study, we evaluated and compared antitumor activity of dovitinib in *RET* fusion-driven LADC models. Dovitinib efficiently suppressed growth of *RET*-rearranged LADC *in vitro* and *in vivo* by inhibiting autophosphorylation of *RET* and was shown to be at least as efficacious as other known *RET*-TKIs, such as sorafenib, sunitinib and vandetanib. Our data provided the preclinical evidence supporting a rationale for the clinical development of dovitinib in patients with *RET* fusion-driven LADC. Furthermore, we found that increased activation of Src mediated acquired resistance to dovitinib by phosphorylating *RET* downstream effector ERK1/2 in *RET* fusion-positive LADC.

Given that *RET* rearrangement is a druggable target in LADC, a number of clinical trials have been designed to investigate the therapeutic effects of multikinase inhibitors targeting *RET*, such as vandetanib (trial registration ID: NCT01823068), sunitinib (NCT01829217), cabozantinib (NCT01639508) and lenvatinib (NCT01877083), in *RET* fusion-positive LADC. In a recent report of preliminary efficacy in patients with *RET* fusion-positive LADC, cabozantinib, an inhibitor of MET, vascular endothelial growth factor receptor (VEGFR) and *RET*, achieved partial responses in 2 patients and durable stable disease in 1 patient.[12] Vandetanib, an inhibitor of VEGFR, EGFR and *RET*, showed a dramatic response in a patients with advanced *RET* fusion-positive LADC.[16] Moreover, alectinib (CH5424802), which has been known to be a selective inhibitor of ALK, showed

antitumor activity against *RET* fusion-driven LADC models by inhibiting oncogenic *RET* fusion signaling.[23] Our data support the inclusion of dovitinib in the therapeutic armamentarium of targeted agents currently being tested for *RET* fusion-positive LADC.

Targeted agents that exploit genetic vulnerabilities in human cancers have now been clinically validated as effective cancer therapies. However, clinical efficacy of these agents is limited by the rapid emergence of drug resistance, which remains a substantial challenge to the clinical management of advanced cancers.[20, 24-27]To our knowledge, this is the first report on the mechanism of acquired resistance to the *RET* inhibitor describing a link between Src-dependent survival signal activation and drug resistance in *RET* fusion-positive LADC.

Src regulates many fundamental cellular processes, including cell growth, differentiation, migration and drug resistance.[21, 22, 28, 29] In our study, acquisition of acquired resistance to dovitinib is attributable to the activation of a Src-driven bypass signaling pathway. We observed marked activation and expression of Src in LC-2/ad DR cells, as compared with the LC-2/ad cells. The blockade of Src signaling by saracatinib or siSrc resulted in the marked suppression of ERK1/2 phosphorylation, suggesting a dominant role of Src on ERK1/2 activation in LC-2/ad DR cells. Consistent with these results, treatment with saracatinib and siSrc dramatically suppressed cell growth in LC-2/ad DR cells. The disappointing lack of synergy in the combined treatment of dovitinib and saracatinib may be due to the

fact that survival of LC-2/ad DR cells no longer depend on the activity of *RET* fusion kinase (**Fig. 5D and 6E**). Similar to our findings, loss of addiction to mutant EGFR resulted in gain of addiction to both HER2/HER3 and PI3K/Akt signaling to acquire EGFR-TKI resistance.[30]

Integrins are a family of adhesion receptors for extracellular matrix (ECM) proteins, integrins can transduce biochemical signals into the cell to regulate a variety of cellular functions, including proliferation, migration, survival and drug resistance.[31-34] Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase which mediates integrin signaling pathways. Src family of cytosolic protein tyrosine kinases function intimately with FAK in integrin signaling pathways.[35, 36] In our study, focal adhesion kinase (FAK) activity was increased in LC-2/ad DR cells (**Fig. 5D**). These data also raise the question as to whether FAK could play an important role in the enhanced Src activation, resulting in the acquisition of dovitinib resistance in LC-2/ad DR cells. However, the inhibition of FAK activity using PF-562,271 could not suppress the downstream ERK1/2 activation and LC-2/ad DR cell growth (data not shown). Protein levels of integrins in LC-2/ad DR cells were similar with LC-2/ad cells and treatment of intergrin β1 could not inhibit the cell growth in LC-2/ad DR cells (data not shown). These data suggest that integrin-mediated FAK activation may not be critically involved in acquired resistance to dovitinib in LC-2/ad DR cell. Instead, Src, as a common node downstream of multiple drug resistance pathways, might be activated by various signal inputs from

specific RTKs, nonreceptor tyrosine kinases or loss of protein tyrosine phosphatase activity.[21, 29] Therefore, it remains to be further studied what mediates the enhanced activity of Src.

V. CONCLUSION

In conclusion, these findings warrant further evaluation of clinical development of dovitinib in RET rearrangement patients and targeting Src signaling is effective in RET rearranged LADC with acquired resistance to dovitinib.

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ABSTRACT (IN KOREAN)

Dovitinib (TKI258)의 RET fusion 비소세포폐암의 항 종양 활성과 획득 내성기전

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연세대학교 대학원 의과학과

강 찬 우

RET fusion은 폐선암에서 새롭게 발견된 driver gene이다. Dovitinib은 FGFR, VEGFR, 그리고 PDGFR에 대한 키나아제 저해제로 RET fusion 폐선암에 대한 항 종양 활성에 대해서는 아직 밝혀진 바가 없다. 본 연구의 목적은 RET fusion 폐선암에 대한 dovitinib의 항 종양 활성과 획득 내성기전을 밝히는데 있다. 인실리코 분석결과 dovitinib은 RET kinase domain 부분에 강하게 결합하는 것을 확인하였고, CCDC6-RET fusion을 갖는 폐선암 세포주인 LC-2/ad의 RET과 ERK의 인산화를

억제하는 동시에 세포주기의 G1기에 억류시켰다. 이러한 dovitinib의 치료적 효과는 RET을 siRNA를 이용한 저해효과와 같은 결과를 보였다. 또한 CCDC6-RET이나 KIF5B-RET을 발현하는 HEK293 세포주에 dovitinib을 처리했을 때 선택적으로 RET 키나아제의 활성을 선택적으로 억제하는 것을 확인할 수 있었다. 종양 이종이식 (tumor xenograft)을 통해서 dovitinib이 RET fusion을 발현하는 종양의 성장을 억제하였고 이것은 인비트로에서 확인한 결과를 뒷받침하는 것을 알 수 있었다. 더 나아가 이 연구에서는 RET fusion 폐선암에서 dovitinib의 내성기전을 밝히기 위해 dovitinib 획득 저항 세포주 (LC-2/ad DR)을 구축하였고, 이 세포주는 RET siRNA에 의해서 ERK의 인산화와 세포성장이 억제되지 않았다. Micro array 분석을 통해 Src의 인산화와 발현이 dovitinib 저항 세포주에서 높게 확인되었고 Src 키나아제 저해제인 saracatinib을 처리하였을 때 ERK의 인산화와 세포성장이 감소하는 것을 확인하였다. 따라서 이번 연구는 dovitinib이 RET fusion 폐선암에 대한 치료방법이 될

수 있다는 가능성과 dovitinib에 대한 획득 내성은 Src을 표적하여 극복할 수 있음을 시사한다.

핵심되는 말: 폐 선암, 비소세포폐암, RET rearrangement, dovitinib (TKI258), Src, 획득 저항성