EGFR and BRAF fusion as a novel mechanism of resistance to lazertinib, 3rd-generation EGFR-TKI, in EGFR-mutant NSCLC

Seo-Yoon Jeong

Department of Medical Science

The Graduate School, Yonsei University

EGFR and BRAF fusion as a novel mechanism of resistance to lazertinib, 3rd-generation EGFR-TKI, in EGFR-mutant NSCLC

Seo-Yoon Jeong

Department of Medical Science

The Graduate School, Yonsei University

EGFR and BRAF fusion as a novel mechanism of resistance to lazertinib, 3rd-generation EGFR-TKI, in EGFR-mutant NSCLC

Directed by Professor Byoung Chul Cho

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University in partial
fulfillment of the requirements for the degree of
Master of Medical Science

Seo-Yoon Jeong

June 2021

This certifies that
the Master's Thesis of Seo-Yoon Jeong
is approved.

Thesis Supervisor: Byoung Chul Cho

Thesis Committee Member#1: Hyo Sup Shim

Thesis Committee Member#2: Hye Ryun Kim

The Graduate School
Yonsei University

June 2021

ACKNOWLEDGEMENTS

As I graduate my master degree, a beginning of my new journey has come to me as well. I appreciate to whom touched me with their endless encouragement, invaluable critiques and support. The gift of your presence and acknowledgment is truly appreciated.

First of all, Professor Byoung Chul Cho, who always gave me a huge aspiration and energy by his constant passion and effort for study. Thank you for being my best teacher who leads me in right and path breaking ways. Again, it was grateful to have incredible experiments in good condition with astonishing colleagues. Also, I sincerely appreciate to Professor Hyo Sub Shim and Hye Ryun Kim who participated as committee members for this thesis. Without their attention and insightful critiques, such this outstanding research result would not have been possible.

Specially to Doctor Jiyeon Yun who gave me the most assist to my thesis, I deeply appreciate for your patience and endless opportunities to complete my successful thesis. Honestly, I could not have completed my master's degree without you, Dr. Yun. You are my unreplaceable mentor, my words can never tell you how I admire you personally and academically.

Additionally, to Seung Yeon Oh, Seul Lee, Ji won Lee, Su Hwan Lee, Hyeong-seok Joo and Dr. Jae Hwan Kim, Dr. Chae Won Park, Dr. Sang Bin Lim, thank you for being a place where I can lean on. Because of all your

presence, I was able to gain strength and stand up again. I sincerely pray that you all will have a full of luck anytime, anywhere. Especially Seung Yeon Oh, please feel free to contact me anytime if you need a help. Thank you very much. And although it was a short time, Kim Tae-yul, whom I met through a precious relationship, thank you for your unbelievable help. Also, I want to give the greatest thanks to all the members who spent most of the time crying and laughing together. It was great to spend two years to challenge ourselves and work together. I will never forget the CBC Laboratory and miss the time we spend in.

Lastly, I would love to share the joy of this graduation with my loving mom, dad and my siblings, Eun woo, Hye Yoon and Hyun Woo who always give endless support.

Thank you again

2021.06

Seoyoon Jeong

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SUPPLEMENTARY FIGURES

ABSTRACT

EGFR and BRAF fusion as a novel mechanism of resistance to lazertinib, 3rd-generation EGFR-TKI, in EGFR-mutant NSCLC

Seo-Yoon Jeong

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Byoung Chul Cho)

EGFR-TKI is an established first-line therapy for NSCLC with activating EGFR mutations. Lazertinib (YH25448), 3rd-generation EGFR-TKI, has been reported as an outstanding drug that had similar efficacy as osimertinib which was investigated as a first-in-class drug. Apart from its significant clinical benefits, it inevitably triggers an acquired drug resistance. Diverse resistance mechanisms to 3rd-generation EGFR TKI have been reported including loss of EGFR T790M, acquirement of EGFR C797S mutation, Met

amplification, activation of other bypass pathway. However, a large part of the resistance mechanisms remains unknown so far.

To explore the mechanism of resistance to lazertinib, I established lazertinib-resistant cell lines with four NSCLC cells including the patient-derived cell line (PDC), patient-derived tumor xenograft cell line (PDTC) and ATCC cell lines. I found that the EGFR/BRAF fusion mRNA and protein were specifically upregulated in an established lazertinib-resistant cell line. Consistently, I detected the EGFR/BRAF fusion gene expression in patient-derived xenografts obtained from patients who experienced acquired resistance to lazertinib. Most notably, combination treatment of lazertinib and MEK inhibitor obviously overcame lazertinib-acquired resistance with EGFR/BRAF fusion *in vitro* and *in vivo.*

These findings indicate that the combination therapy of EGFR and MEK inhibitors might be a promising therapeutic option for overcoming lazertinib-acquired resistant NSCLC patients with EGFR/BRAF fusion gene in clinic.

Key words: Lazertinib, YH25448, EGFR-TKI, NSCLC, resistant mechanism, EGFR/BRAF fusion, gene alteration, trametinib

EGFR and BRAF fusion as a novel mechanism of resistance to lazertinib, 3rd-generation EGFR-TKI, in EGFR-mutant NSCLC

Seo-Yoon Jeong

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Byoung Chul Cho)

I. INTRODUCTION

In worldwide, lung cancer is one of the major causes of cancer-related mortality.[1] Among non–small cell lung cancer (NSCLC), Epidermal growth factor receptor (EGFR)-mutated lung cancer constitutes a significant portion of major driver oncogenes.[2, 3] According to many reports, EGFR is activated by binding with the ligands including EGF, transforming growth factor-alpha (TGFA), epiregulin (EREG), heparin-binding EGF-like growth factor (HBEGF), amphiregulin (AREG), betacellulin (BTC) and epigen (EPGN).[4] It

induces diverse cellular differentiation, proliferation, migration and survival in cells. EGFR-activating mutations in the kinase domain in NSCLC drive both initiation and maintenance of oncogenic signaling.[5] These mutations constantly activate downstream pathway which are related with cell growth and survival signaling pathways, inducing dependency on the EGFR pathway for growth of tumor.

There have been advances in molecular-targeted therapies in EGFR mutation-positive NSCLC patients; EGFR tyrosine kinase inhibitors (TKIs). EGFR-TKI is an established first-line therapy for NSCLC with EGFR-activating mutations. EGFR-TKIs block the EGFR-induced downstream signaling pathway by binding to the ATP-binding pocket of the EGFR tyrosine kinase domain directly. For the discovery of a treatment for EGFR-mutated NSCLC patients, many scientists have been exploring this and some trials are currently being clinically used.[6]

Approximately 40 to 50% of NSCLC patients harbor EGFR-activating mutations, such as missense mutation in exon 21 (L858R) or in-frame deletions in exon 19 (Ex19del).[7] The frequency is significantly related with ethnicity, gender and smoking history.[8,9] These mutations react to 1st- and 2nd- generation EGFR-TKIs including gefitinib, erlotinib and afatinib.[10] The 1st- generation EGFR-TKIs (gefitinib and erlotinib) and 2nd- generation EGFR-TKI (afatinib) have shown a significant advance of prognosis of EGFR mutation-positive lung cancer patients with EGFR mutations via multiple clinical trials.[11] Even though

with the dramatic initial response, their long-term efficacy is questionable. Despite the introduction of such brilliant treatment strategies, a dismal prognosis has advanced in NSCLC patients continuously, with a 5 yrs survival rate of less than 10%. Most patients inevitably get disease progression after 9-13 months of treatment.[12]

Almost 50 to 60% of patients have the gatekeeper T790M mutation in EGFR kinase as an acquired resistance mechanism to either 1st- or 2nd- generation EGFR-TKIs.[13,14] EGFR T790M mutation is the major resistance mechanism after 1st- and 2nd- generation EGFR-TKI treatment. Then the 3rd-generation EGFR-TKIs including osimertinib, rociletinib, olmutinib and lazertinib are developed for T790M mutant NSCLC. As follows, targeting EGFR T790M mutation with 3rd- generation EGFR-TKI is established as a standard treatment approach. Nevertheless, acquired resistance occurred in clinical by 3rd-generation EGFR-TKIs. EGFR C797S mutation has been reported as the major resistant mechanism of the 3rd- generation EGFR-TKIs.[15]

Osimertinib has been successfully developed for NSCLC with EGFR activating and T790M mutations. Based on the clinical results from AURA3 trial and FLAURA trial, osimertinib meaningfully enhanced progression free survival (PFS) compared to 1st- generation EGFR-TKIs and has become a standard of treatment for T790M-mutant NSCLC or an ideal decision for treatment-naïve EGFR-mutant NSCLC.[16,17]

Lazertinib has also shown promise for the treatment of this patient population, according to a phase I/II dose-escalation study (NCT03046992) published in

the lancet oncology.[18] Phase III study is being conducted to evaluate the efficacy and safety of lazertinib as a first-line treatment in locally advanced or metastatic NSCLC patients with EGFR mutations. The patients with locally advanced or metastatic NSCLC positive for EGFR sensitizing mutations, treatment-naïve and eligible for an initial treatment with an EGFR-TKI were enrolled in this study.

Among the 3rd- generation EGFR-TKIs, we report about lazertinib, which is a potent and irreversible EGFR-TKI. Lazertinib has been developed to target various activating EGFR mutations, including T790M. It is also known as a promising drug which has high efficacy specifically for brain metastasis and high selectivity for T790M mutation. Moreover, the clinical trials are also ongoing to evaluate the efficacy and safety of standing as the first-line therapy for patients who had progression in locally advanced or metastatic NSCLC.

Although lazertinib has shown a great benefit in clinic for EGFR mutant NSCLC patients, the patient will get resistance and undergo the disease progression like other EGFR-TKI agents. Many resistance mechanisms to 3rd- generation EGFR-TKI have been reported. In EGFR-dependent resistance mechanisms, 1) loss of the T790M mutation, 2) mutation in C797, G796, L792, L718, 3) Exon20 mutation and 4) other mutations have been reported. In addition, resistance caused by EGFR-independent mechanisms can be acquired. For example, MET amplification, PI3K pathway activation, RAS–MAPK pathway activation and HER2 amplification have

been reported.[19] These acquired resistance mechanisms activate alternative bypass pathways and make uncontrolled downstream signaling and histologic transformation. Among the reported genetic alterations accompanying with acquired resistance to the 3rd- generation EGFR-TKIs, it is well known that EGFR C797S intrinsic mutation induces acquired resistance to the 3rd-generation EGFR –TKIs. It would be the target of next generation EGFR-TKI (4th- generation EGFR-TKI. However, the remaining resistance mechanisms to 3rd- generation EGFR-TKI including acquired fusions are largely unknown. Especially, there is no previous reporting about acquired resistance mechanism of lazertinib.

In order to investigate the novel mechanism of resistance to lazertinib, we evaluated with the multiple lazertinib-resistant NSCLC models. We established lazertinib-resistant cell lines, using ATCC cell lines, patient-derived cells (PDCs) and patient-derived xenografts (PDXs). Also, we conducted Whole-exome sequencing and RNA-sequencing to find the gene alteration and expression change.

Here, we found a novel EGFR/BRAF fusion gene, as a key driver of the acquired resistant mechanism of lazertinib, in NSCLC cell lines and patient derived xenograft samples. We also present a combination treatment of lazertinib and trametinib that showed a strong antitumor effect in lazertinib-acquired resistant NSCLCs. This combination therapy of EGFR and MEK

inhibitors might be a promising therapeutic option for overcoming lazertinib-acquired resistant NSCLC patients in clinic.

II. MATERIALS AND METHODS

2.1. Cell culture and Reagents

PC9 and PC9GR cells were provided by J.C. Lee (Korea Institute of Radiological and Medical Science, Seoul, Korea). All cell lines were maintained in RPMI-1640 (Sigma-Aldrich) medium supplemented with 10% fetal bovine serum (FBS) and 1% solution of antibiotics. Also these were incubated with 5% CO_2 at 37°C and the media were exchanged every 2–3 days. Lazertinib-resistant cell lines were generated *in vitro* by escalating doses of lazertinib (0.1~1uM) for over six months. After establishment, all the resistant cell lines were maintained in lazertinib 1uM. All reference compounds were purchased from Selleckchem, except lazertinib, which was provided by Yuhan Corporation.

2.2. Patient-derived cells

YU-1150 cell line was derived from malignant effusions of NSCLC patient. YU-1150 was originally cultured on collagen-coated plates in a serum-free defined medium (ACL4) supplemented with 5% FBS. YUX-1024 cell line was derived from its PDX model. The cells sustained the driver oncogenes that were observed in the patients. Cells began to be used *in vitro* study after cells

were enriched in an epithelial cell adhesion molecule (EpCAM)-positive cell population with purity of over 95%. All patient samples were obtained after written informed consent of the patients using an institutional review board-approved protocol.

2.3. Anti-proliferation Assay

NSCLC cell lines expressing EGFR mutation were exposed to the various concentrations of various drugs. Cells were seeded at a density of 2.5×10^3 to 3×10^3 per well onto 96-well plates in 100 μL. After 72 hrs incubated with indicated drugs, cell viability was measured by measuring the entire amount of ATP with the CellTiter-Glo® 2.0 Assay kit (Promega) according to the manufacturer's instruction. Dose-response curves were generated to the data using the GraphPad Prism.

2.4. Colony forming assay

Cells were seeded onto 12 or 48-well culture plates and incubated for 12 days with the indicated concentration of compounds. Cells were washed with phosphate-buffered saline (PBS). Then cells were fixed and stained with 5% crystal violet in 4% paraformaldehyde for 24 hrs. For evalutation clonogenicity, images were captured and then colonies were eluted with 1% sodium dodecyl sulfate (SDS). The optical density value was measured at 470

nm using a SpectraMax 250 microplate reader (Molecular Devices).

2.5. Antibodies and Western blot

Specific primary antibodies for p-EGFR (2234), EGFR (4267), p-BRAF (2696), p-AKT (9271), AKT (9272), p-ERK (4370), ERK (9107), p-S6 (4858), S6 (2217) were purchased from Cell Signaling Technologies. The immunoblots were detected using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Massachusetts, USA). The membrane was re-blotted with a GAPDH (PAB13195) purchased from Abnova (Taipei, Taiwan) as an internal control. Cell lysates were centrifuged at 1300rpm for 15 min at 4°C and the resultant supernatants (cytosolic fraction) were qualified and quantified for getting protein. The same extents of protein were separated by SDS–PAGE and then transferred to nitrocellulose membrane.

2.6. RT-qPCR analysis

The total RNA of the cells was isolated with Trizol reagent (Invitrogen). And RNA of the tissue was isolated with RNeasy Mini kit (QIAZEN). The isolated RNA samples were reverse-transcribed to cDNA. The cDNA was generated by random hexamers directed reverse transcription using 2 ug of RNA in a total volume of 20 μl. Primer annealing at 25℃ for 5 min, cDNA synthesis at 42℃ for 60 min and enzyme deactivation at 70℃ for 15

min. Enzyme inactivation terminated by incubation for 5 min at 94℃. Real-time PCR was performed with the reaction mixture included 2ug of cDNA, PowerSYBR Green PCR master mix (Thermo Fisher) and 100 pmol of each primer pairs of EGFR/BRAF targeted sequences. To provide a quantitative control for reaction efficiency, the relative expression was normalized by 18s.

2.7. T&A Cloning and Transformation

After synthesis of cDNA, target genes were amplified with HotstarTaq plus DNA polymerase(Qiazen), following the manufacturer's instructions. Using two pairs of EGFR/BRAF primers #1, #2 (Table 1), we generate a product for cloning, the following PCR cycles were used: 5 min at 95℃; 50 cycles of 10 sec at 98℃, 30 sec at 59℃, 1 min at 72℃; 10 min at 72℃; and hold at 4℃. The PCR product was analyzed by 0.2 to 2 % of agarose gel electrophoresis with safer-pinky DNA Gel staining solution (genDEPOT, TX, USA). Then DNA purification was conducted with Gel extraction kit (QIAZEN). Using T&A cloning vector kit (Real Biotech Corporation, Taiwan), the target DNA product was ligated with empty vector and transformed into E. coli DH5α cells in keeping with the manufacturer's instructions. Positive clones were cultured in a flask containing Luria Broth with ampicillin (100 ng/l), grown overnight at 37°C in a shaking incubator. Plasmid was purified with Plasmid mini kit (QIAZEN) and confirmed with HindIII restriction enzyme(Takara) at 37℃ overnight. Confirmed clones were PCR-screened

directly for the existence of inserts and then direct sequencing using the M13 universal primer set.

2.8. Small interfering RNA transfection

PC9GR_YH1R cells were seeded at a density of 1.5 x 10^5 cells in a 6-well plate and incubated overnight to attach in the growth medium. For DsiRNA knockdown of EGFR/BRAF fusion gene, PC9GR_YH1R cells were transfected with 20uM of EGFR/BRAF-specific DsiRNA #1, #2 and non-targeted scrambled negative control siRNA(IDT). Transfection was conducted using Lipofectamine RNAiMAX (Thermo Fisher Scientific) without antibiotics. Twenty-four hours after the transfection, cells were collected and analyzed.

2.9. Xenograft study

All mice were controlled in agreement with the Animal Research Committee's Guidelines at Yonsei University College of Medicine and all facilities were approved by AAALAC. PC9GR_YH1R (1x10^7 cells) were injected subcutaneously into the NOG mice, then the tumor was transplanted into nude mice. Tumor growth was measured twice weekly; after a formation of palpable lesions, mice were dispensed for testing. Once tumor volumes reached approximately 180–220 mm^3, mice were randomly separated into appropriate treatment groups. Five mice allocated into the groups which are

vehicle, lazertinib (10mg/kg), trametinib (0.5mg/kg) and combination of lazertinib (10mg/kg) and trametinib (0.5mg/kg) groups . After treatment, tumor size was measured every other day. The average tumor volume in each group was validated in mm^3 and calculated by the following formula:

tumor volume = 0.523 × (large diameter) × (small diameter) ^{2}

All mice were sacrificed in 55 days after treatment.

2.10. Identification and Analysis of Fusion gene

The RNA samples were sequenced using the HiSeq 2000 system. Fusion genes were evaluated using both fusion Arriba and fusioncatcher. The fusion transcript was detected based on STAR aligner by Arriba algorithm (Arriba_2.1.0).[20,21] For more concise results, another somatic fusion calling program, fusioncatcher which includes Bowtie, STAR, and BLAT was used. We could get somatic fusion genes in paired-end RNA-sequencing data with fusioncatcher. To ignore the fusion events which are not fully expressed, the minimum coverage fraction was cut off at 0.15. Reads were aligned to the GRCh37/hg19 reference genome. Fusion transcript partners were shown by the circos plot, which was from RNA-seq data of fusion transcript candidate with the highest coverage. The fusion junction was confirmed by RT-qPCR and Sanger sequencing.

2.11. Statistical analysis

All data were obtained as the mean ± SEM. Data were evaluated by one-way ANOVA, followed by the Student's t-test. Dose-response curves were organized using the GraphPad Prism. A P-value less than 0.05 was considered statistically significant. Survival analysis was accomplished using a Kaplan–Meier survival curve and a log-rank test comparing each group.

III. RESULTS

3.1 Established acquired resistant cells to lazertinib showed the resistance to lazertinib

To explore the potential mechanism of the resistance to lazertinib (YH25448), we established lazertinib-resistant lung cancer cells using a cell line-based model. The following cell lines were examined: 1. Patient-derived tumor xenograft cells, YUX-1024, harboring the exon21 L858R missense mutation, 2. EGFR-TKI naïve PC9-cells harboring the exon19del mutation, 3. Patient-derived tumor cells, YU-1150, harboring the EGFR exon21 L858R and T790M mutations, and 4. PC9GR-cells harboring the EGFR exon19del and T790M mutations. As shown in Supplementary Figure 1, T790M-negative cell lines are sensitive to gefitinib, one of the 1st- generation EGFR-TKIs. However, T790M-positive cell lines are resistant to gefitinib. Notably, all four cell lines were sensitive to lazertinib and osimertinib, both of the promising 3rd-generation EGFR-TKIs, whether they have T790M mutation or not.

Established lazertinib-resistant cells were generated in culture by growing each cell lines based on stepwise dose escalation. For over six mos, acquired resistant cell lines were cultured in increasing concentrations of lazertinib up to 1μM, as previously described methods (Figure 1A). Also, these established resistant cell lines were subsequently maintained in media containing 1μM lazertinib. Finally, we generated four acquired resistant models from NSCLC cell lines with

different EGFR mutations. Lazertinib-resistant cell lines are denoted by YH1R (YH25448 1uM resistant). These are designated YUX-1024_YH1R, PC9_YH1R, YU-1150_YH1R, and PC9GR_YH1R, respectively. The newly created resistant cell lines got completely different characteristics from their previous cell line, exemplified by cell morphology or proliferation rate. (Supplementary Figure 2)

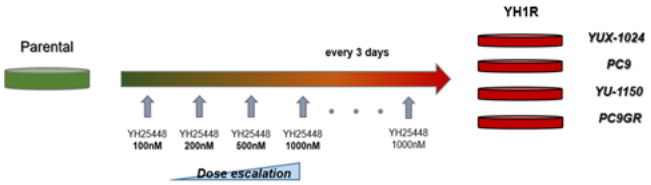
Whole exome sequencing showed that these resistant cells preserved their original activating mutations in EGFR and T790M mutation. Furthermore, the common acquired C797S mutation, which confers resistance to 3rd-generation EGFR-TKIs, was not detected in any of the four resistant cell lines. (Supplementary Figure 3)

Next, we assessed the effect of lazertinib by anti-proliferation assay. We demonstrated that all of the YH1R cells kept growing in the presence of lazertinib, whereas this same drug concentration markedly reduced cell growth of parental cells (Figure 1B). The resistant cell lines exhibited 27- to 84.4- fold resistance to lazertinib compared with each parental cell line *in vitro*. Consistent with these data, treatment with lazertinib resulted in a significant decrease in colony formation by parental cells, but not by YH1R cells (Figure 1C).

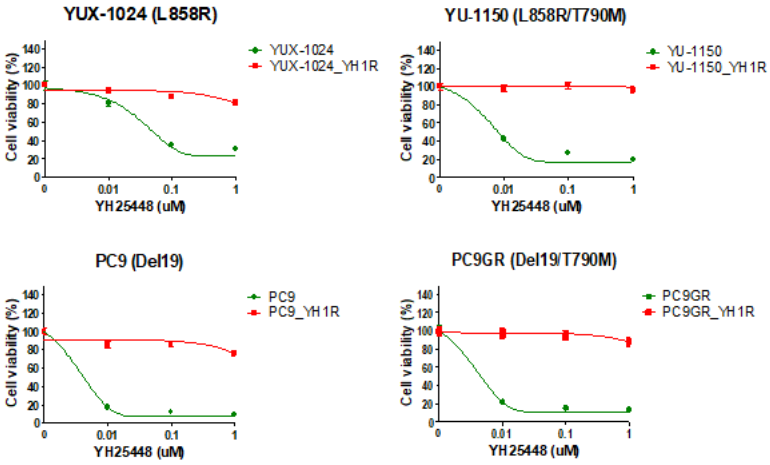
To investigate the underlying mechanism of resistance to the lazertinib, firstly, we tested whether there was any difference in the downstream signaling (Figure 1D). We harvested lysates from both parental and YH1R cells treated

for 6 hrs with 0, 0.1 and 1 μM of lazertinib. In response to treatment, resistant cells commonly demonstrated inactivated EGFR although downstream molecules of EGFR pathway were still activated. As shown in Figure 1D, ERK phosphorylation and S6 phosphorylation were not suppressed by lazertinib in the resistant cell lines. These results suggested that the resistant cells adopt a new mechanism for activating MEK/ERK pathway. In contrast to the persistent ERK phosphorylation, AKT was inactivated in the YU-1150_YH1R and PC9_YH1R cells regardless of lazertinib treatment. This is different from other models, YUX-1024_YH1R and PC9GR_YH1R, which have persistent activation of both AKT and ERK pathways in the presence of lazertinib. It seems that MAP kinase-related pathway is the key factor of the resistance mechanism of lazertinib. The ERK cascade can be activated by various factors.[22] Since aberrant ERK signaling which was caused by downregulation of negative regulator or amplification of MAPK1 (mitogen-activated protein kinase1, ERK2), abnormal MAP/ERK signaling was caused.[23]

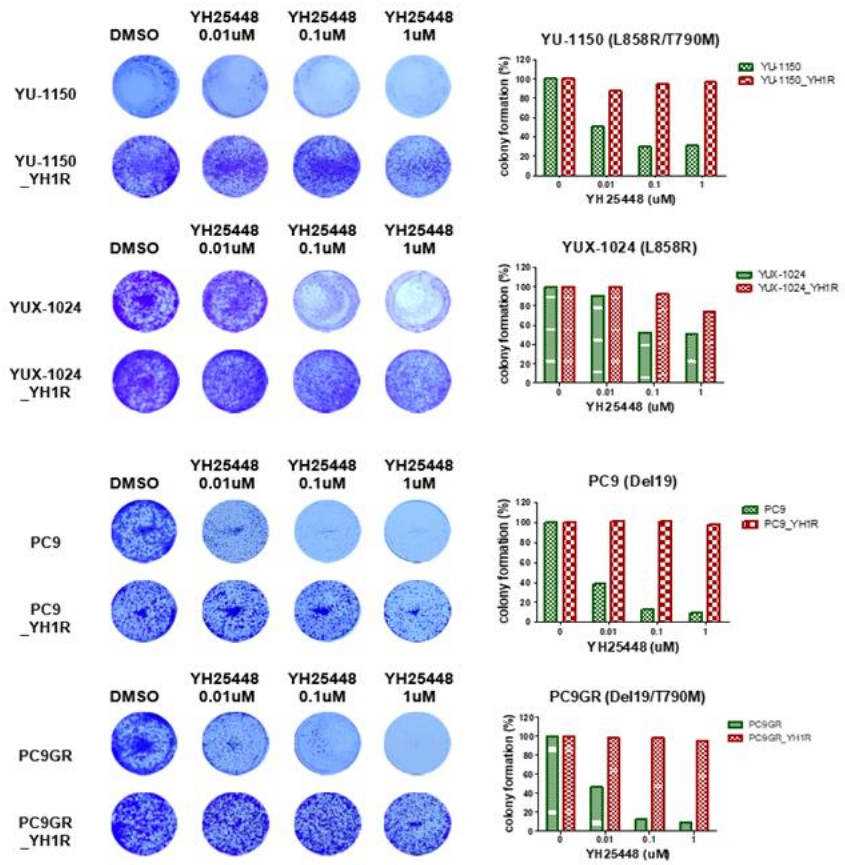
A



B



C



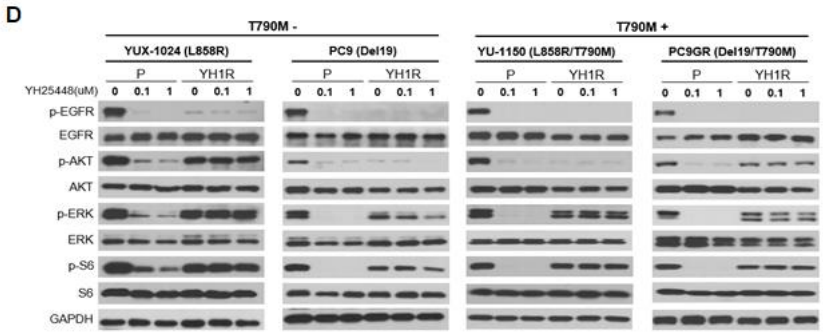
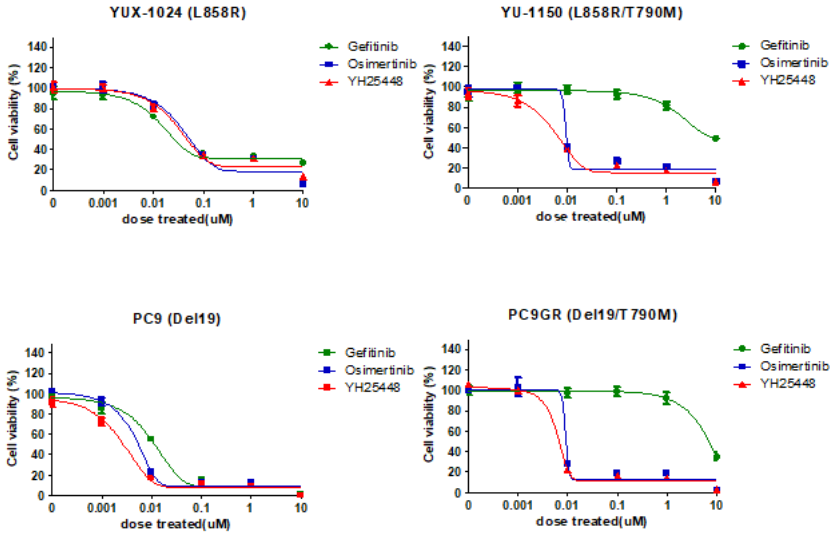
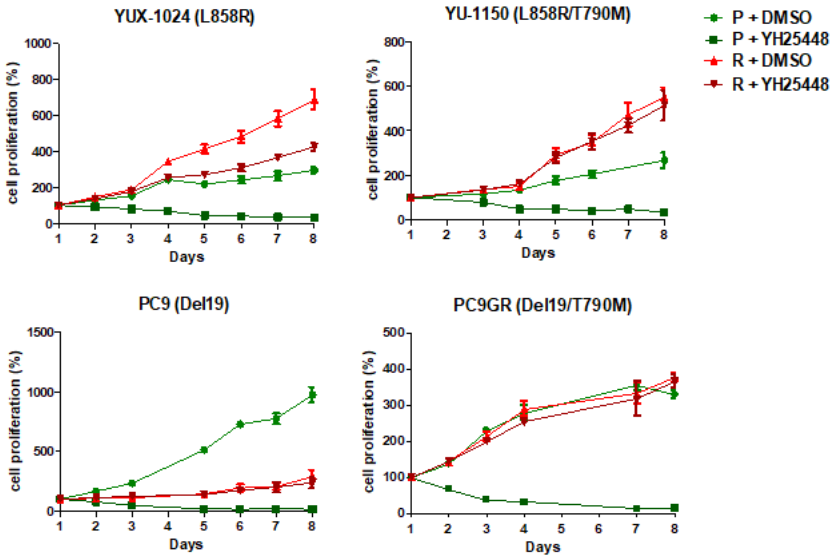


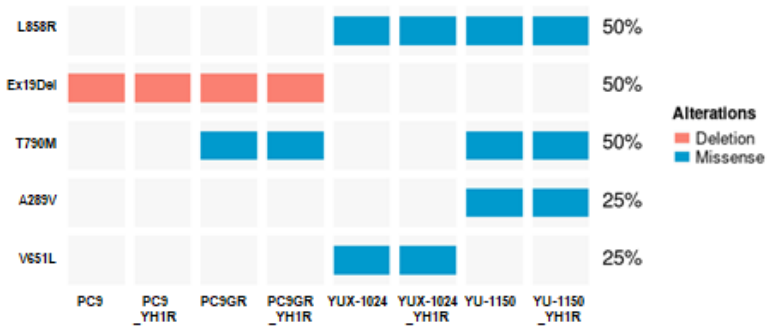
Figure1. Generation and characterization of established lazertinib-resistant cell lines (A) Graphic diagram of the protocol for generation of lazertinib-resistant cell models. The Illustration depicts the lazertinib treatment and time for establishment. (B) Growth-inhibitory effect of lazertinib determined via CellTiter-Glo. Cells were seeded on a 96-well plate and cultured in the indicated concentrations of lazertinib. Following 72 hrs of incubation, the cells were subjected to MTT assay. Data are presented as averages ± SD of triplicate independent experiments. (C) Representative colony formation images of parental and lazertinib resistant cells. Cells were grown in the absence or presence of the lazertinib for 14 days. All cells were fixed and stained with crystal violet, representing quantification. (D) Western blot analysis in parental and lazertinib resistant cells. Cells were plated in a medium containing 0, 0.1 and 1μM of lazertinib for 6 hrs.



Supplementary Figure 1. Drug sensitivity test in each parental cell lines



Supplementary Figure 2. Cell proliferation test of parental and established lazertinib-resistant cell lines



Supplementary Figure 3. The change of EGFR mutation in parental and established lazertinib-resistant cell line

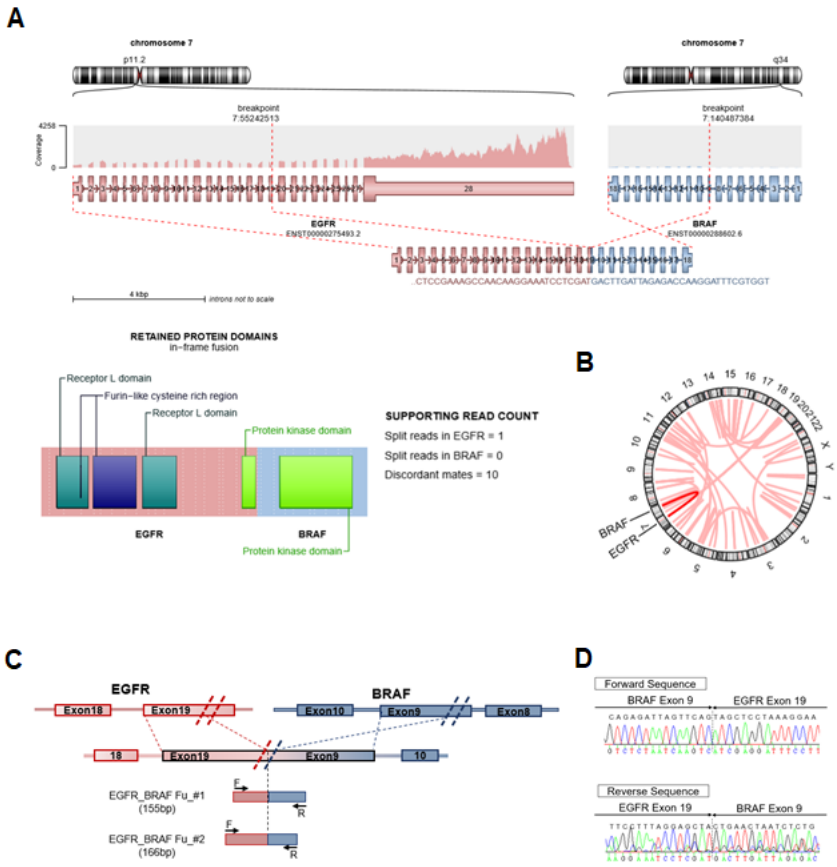
3.2 RNA-seq revealed that EGFR/BRAF fusion gene appeared in PC9GR_YH1R

To investigate the genetic alteration of lazertinib-resistant cells, whole-exome sequencing (WES) was conducted. Also, we aimed to identify whether the resistant cell lines harbor significant changes of gene expression level associated with resistance to EGFR-TKIs by RNA-seq. RNA-sequencing was performed to assess the gene expression profiling of the resistant cells relative to each parental cell. In the beginning, we expected the existence of common acquired genetic alterations in several cell lines. Even though the expression profiles of each resistant cell line were varied, the common targetable alterations were not found among the four resistant cells. Consequently, the mechanism of resistance in each cell line was analyzed independently. Based on the absence of any targetable driver by WES and RNA-seq, we sought to identify acquired gene fusion associated with resistance to EGFR-TKIs. We used two algorithmically different software tools, Arriba and Fusioncatcher, for the detection of gene fusions from RNA-seq data. We performed at a comparable level with two popular tools (Arriba and Fusioncatcher) and identified a novel fusion event with potential therapeutic implications in the established lazertinib resistant cells. Since false positive was found easily in the case of gene fusion, we found the target fusion genes which were involved in both software tools. Arriba and Fustioncatcher tools commonly confirmed an acquired EGFR/BRAF fusion (EGFR exon 1 through

19 fused with BRAF exon 9 through 18; chr7 p11.2:5524513 EGFR_chr.7 q34:140487384 BRAF) in PC9GR_YH1R cell line. (Figure 2A,B) To determine if EGFR/BRAF fusion gene was expressed in PC9GR_YH1R cell, we confirmed the mRNA and protein level of the fusion gene using PCR and Western blot. To identify the fusion gene, two different sets of RT-PCR primers (each transcript sizes are 155 base pairs and 166 base pairs) were designed (Figure 2C, Table 1). As shown in Figure 2D, breakpoint region on gene-level with exact single-base resolution fusion point of PCR product was indicated by Sanger sequencing (Hg19 coordinates). Also, the in-frame fusion of EGFR exon19 and BRAF exon9 was confirmed by fusion-specific transcription.

In Figure 2E-F, general PCR and Quantitative Real-time PCR analyses confirmed that EGFR/BRAF fusion was expressed in PC9GR_YH1R cells and the expression level of EGFR/BRAF fusion mRNA in PC9GR_YH1R cells was significantly higher than PC9GR parental cells. For clarification, we conducted general PCR, RT-qPCR, and western blot in four pairs of cell lines. As we expected, EGFR/BRAF fusion mRNA were expressed in PC9GR_YH1R cells only. Also, using EGFR_BRAF_Fu#1 primers, EGFR/BRAF expression level was relatively higher in PC9GR_YH1R cells than parental cells. (Figure 2G-H). Notably, the fusion protein of EGFR/BRAF was validated by western blot. The p-BRAF (Ser445) antibody detected two bands and the upper band indicated EGFR/BRAF fusion protein. This band appeared only in PC9GR_YH1R cells (Figure 2I). Overall, PC9GR_YH1R cells expressed

mRNA and protein of EGFR/BRAF fusion gene. Consistent with the mRNA level data, the EGFR/BRAF fusion protein was markedly expressed in PC9GR_YH1R, compared with that in PC9GR cells.



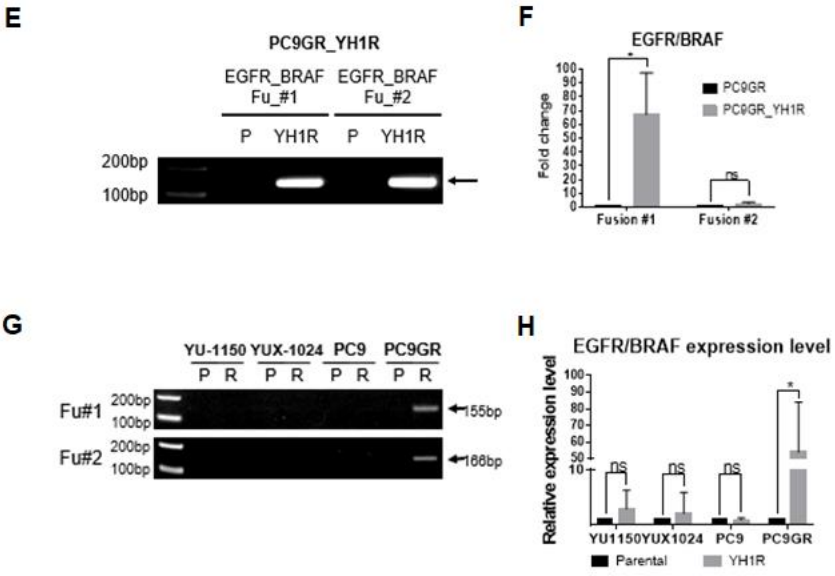


Figure 2. Validation of the novel EGFR/BRAF fusion gene by fusion-specific RNA sequencing in PC9GR_YH1R cell line (A) Chromosomal ideograms showed EGFR/BRAF fusion gene between EGFR (chr.7 p11.2) and BRAF (chr.7 q34). Schematic of EGFR/BRAF fusion gene and transcript. (B) Circos plot of acquired-fusion gene in tracks with chromosomes circularly arranged. Red lines represent fusion genes, and the bold line represents EGFR/BRAF fusion gene. (C) To identify the fusion gene, two different sets of RT-PCR primers (each transcript sizes are 155 base pairs and 166 base pairs) were designed. (D) Sanger sequencing of PCR product confirming a fusion between EGFR (exon19) and BRAF (exon9). (E, F) Confirmation of EGFR/BRAF mRNA expression level in a PC9GR_YH1R cell line by general PCR and quantitative RT-PCR. (G) Each pair of four cell lines was examined for EGFR/BRAF expression by general PCR. (H) Using EGFR_BRAF Fu_#1 PCR primer, EGFR/BRAF mRNA expression was quantified. (I) EGFR/BRAF fusion protein was detected by Western blot analysis. 18S and GAPDH were used as a control. EGFR/BRAF fusion mRNA and protein were expressed only in PC9GR_YH1R cell line among four paired cell lines.

Table 1. The primer sequences of RT-qPCR

Gene	Primer sequence	
	Primer F	Primer R
EGFR_BRAF Fu_#1	ctggatcccagaaggtgaga	gcagacaaacctgtggttga
EGFR_BRAF Fu_#2	gttcggcacggtgtataagg	ctccatcaccacgaaatcct

The real-time PCR (RT-PCR) analysis of EGFR/BRAF was conducted using two sets of primer sequences. The forward and reverse primer sites are located at exon 19 of EGFR and exon 9 of BRAF, respectively. The gene fusions breakpoints were amplified and then gene fusion was validated by Sanger sequencing. The PCR products were separated on a 2% agarose gel and extracted for Sanger sequencing. All sequencing services were provided by Macrogen Inc. (Seoul, Korea)

3.3 EGFR/BRAF fusion gene also existed in patient-derived tumor xenograft samples obtained from patients who experienced acquired resistance to lazertinib

In order to obtain the meaningful clinical evidence, it is important that the EGFR/BRAF fusion gene is found in patient samples. To check whether there is a corresponding fusion gene in the actual patient samples, we harvested RNA from patient-derived tumor xenografts (denoted by PDTX) who had EGFR activating mutation and experienced acquired resistance to lazertinib. The treatment history of two patients who had become resistance to lazertinib were described in Figure 3A. Unfortunately, the pre-treatment samples did not exist. So it was impossible to compare to pre- and post-lazertinib models. We conducted general PCR in YHIM-1045 and YHIM-1035 to amplify EGFR/BRAF fusion transcript with two pairs of primers which were described in Figure 2C. Interestingly, EGFR/BRAF fusion mRNA existed in both of PDTXs consistent with PC9GR_YH1R cell lines. The previous data was consolidated by the observation that two additional PDTXs showed high EGFR/BRAF mRNA expression of which samples were resistant to lazertinib (Figure 3B). Also, Sanger sequencing analysis of EGFR/BRAF fusion gene confirmed that two lazertinib-resistant PDTX models had EGFR/BRAF fusion gene (Figure 3C). In Supplementary Figure 4, YHIM-1045 and YHIM-1035 PDTX samples expressed the same mRNA as EGFR/BRAF fusion gene of PC9GR_YH1R cell.

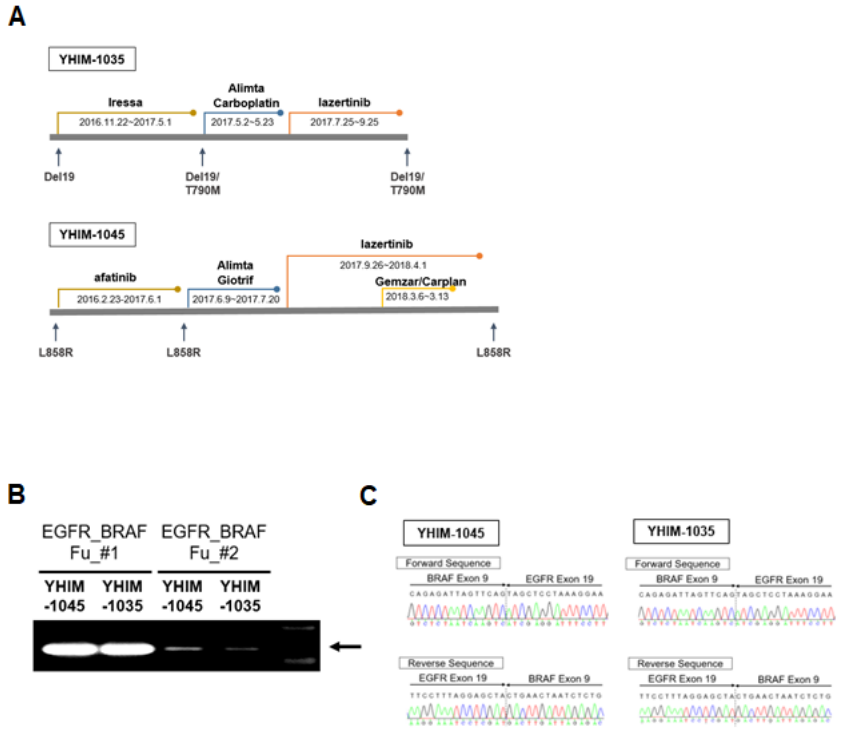


Figure 3. EGFR/BRAF fusion mRNA was also identified in patient-derived tumor xenograft samples (A) Clinical information for patients with resistance to lazertinib. (B) EGFR/BRAF fusion mRNA was expressed in patient-derived tumor xenograft samples. (C) Sanger sequencing data depicting the fusion between of EGFR and BRAF in two cases of patient-derived tumor xenograft.

3.4 EGFR/BRAF fusion knockdown restored sensitivity to lazertinib

To demonstrate that EGFR/BRAF fusion gene directly reduces sensitivity to lazertinib and acquires resistance to lazertinib, EGFR/BRAF fusion gene was specifically knocked-down by Dicer substrate small interfering RNA (DsiRNA) shown in Table 2. As mentioned above, EGFR/BRAF fusion mRNA and protein were upregulated in PC9GR_YH1R cells. Later, we knocked down EGFR/BRAF fusion gene by siRNA transfection. After the silencing of EGFR/BRAF with two of siRNAs targeting EGFR/BRAF fusion gene (denoted by siE/B#1, siE/B#2), mRNA and protein were evaluated through the comparison with siControl transfected cells (denoted by siCon) by RT-qPCR and western blot (Figure 4A, B). As a result, EGFR/BRAF mRNA expression level of siE/B#1- and siE/B#2- transfected cells were downregulated relatively to the transfected cell with siCon. Also, EGFR/BRAF fusion protein expression levels were downregulated.

If the EGFR/BRAF fusion gene is an important factor that influences lazertinib-resistance, EGFR/BRAF knocked-down PC9GR_YH1R cells will acquire sensitivity to lazertinib. As shown in Figure 4C, EGFR/BRAF knocked-down PC9GR_YH1R cells led to the more significant reduction of cell growth compared to the siCon under lazertinib treatment in a dose-dependent manner. All things considered, these data suggest that EGFR/BRAF fusion gene is responsible for the acquisition of acquired resistance to lazertinib in PC9GR_YH1R cells.

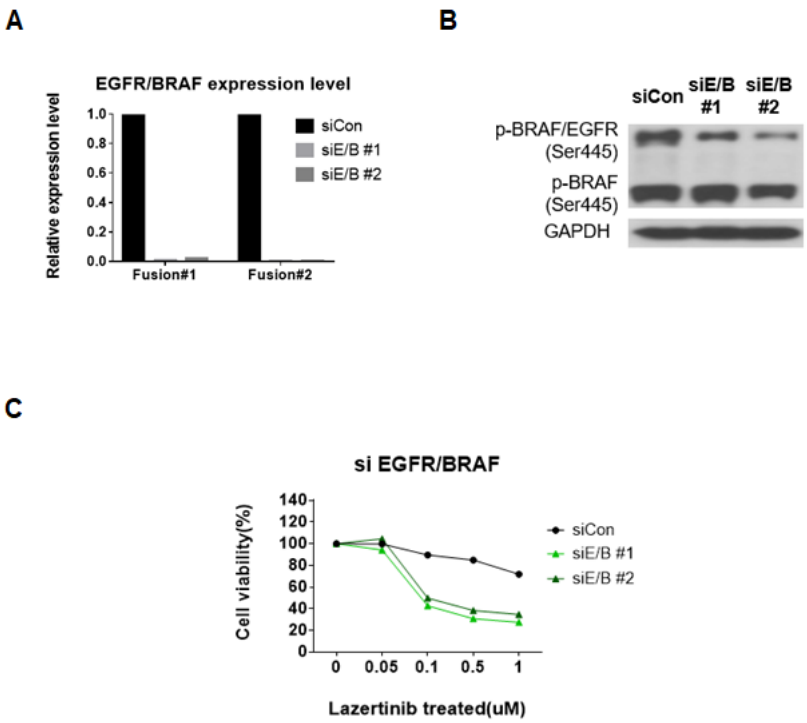


Figure 4. Knockdown of EGFR/BRAF fusion gene restored sensitivity to lazertinib in lazertinib-resistant NSCLC cells *in vitro* (A, B) Downregulation of EGFR/BRAF fusion gene expression in mRNA and protein. PC9GR_YH1R cell was transfected with siE/B #1, siE/B #2 or siCont. After 24 hrs, the mRNA and protein levels of EGFR/BRAF were detected by RT-qPCR(A) and western blot(B) analysis. (C) Twenty-four hours after transfection, the cells were incubated with lazertinib (0.05, 0.1, 0.5, and 1 uM) for 72 hrs and the cell growth was determined. The percentage of growth was shown relative to the growth of lazertinib-untreated control cells.

Table 2. The DsiRNA sequences for knockdown the EGFR/BRAF fusion gene

	Sequence
siEGFR/BRAF #1	5'-AAGGAAAUCCUCGAUGACUUGAUTA-3'
siEGFR/BRAF #2	5'-CUCGAUGACUUGAUUAGAGACCAAG-3'

3.5 Combination treatment of MEK inhibitor and lazertinib showed potential killing effect *in vitro* and *in vivo*

To overcome EGFR/BRAF fusion-induced resistance, we investigated whether inhibition of BRAF or RAS/MAPK pathway using MEK inhibitor could reverse or overcome the intrinsic lazertinib-resistance in PC9GR_YH1R cell line. As shown in Figure 1D, AKT and ERK signals were still activated under lazertinib treated condition in PC9GR_YH1R in an EGFR-independent manner. In cases of EGFR-TKI resistance owing to genetic fusion, the combination treatment of EGFR-TKI and inhibitor-related with fusion gene was effective in overcoming this resistance.[24,25]

Therefore, we tested the effects of BRAF or MEK inhibitors and lazertinib combination treatment in PC9GR_YH1R cells. As expected, the sensitivity to lazertinib was synergistically restored when cells were treated with lazertinib and BRAF inhibitor, dabrafenib, or MEK inhibitor, trametinib and selumetinib. (Figure 5A, B) Consistent with the cell viability test, the colony forming assay showed the combined effect of each drug. Especially, the treatment of trametinib with lazertinib showed the most effective combination efficacy in EGFR/BRAF fusion-positive cell line, PC9GR_YH1R. Treatments with either lazertinib or trametinib alone did not have significant killing effects in PC9GR_YH1R cells. On the other hand, the co-treatment of lazertinib and tramentinb had a strong killing effect in PC9GR_YH1R cells. These results might have been caused by colony heterogeneity. In a further study, single

clonal assay should be conducted to evaluate the cause of the combination effect.

To confirm the combination efficacy of trametinib and lazertinib, we conducted *in vivo* experiment using PC9GR_YH1R xenografts. When tumor volumes reached around 180–220 mm^3, the treatment began. Mice were randomly allocated into groups of five to receive either vehicle, lazertinib, trametinib, or lazertinib and trametinib combination. The nude mice were killed 55 days after injections. Studies with PC9GR_YH1R xenograft tumors revealed a 30% reduction in tumor volume with the combination treatment of lazertinib and trametinib compared with the lazertinib-single treatment group. (Figure 5C, D, and Supplementary Figure 5) The data showed excellent inhibitory effects compared to lazertinib monotherapy or trametinib monotherapy *in vivo*. Moreover, there was no difference in body weight. Our model suggests that co-targeting EGFR and MEK may be a promising approach for overcoming EGFR/BRAF fusion gene-induced intrinsic resistance to lazertinib in EGFR-mutated NSCLC patients.

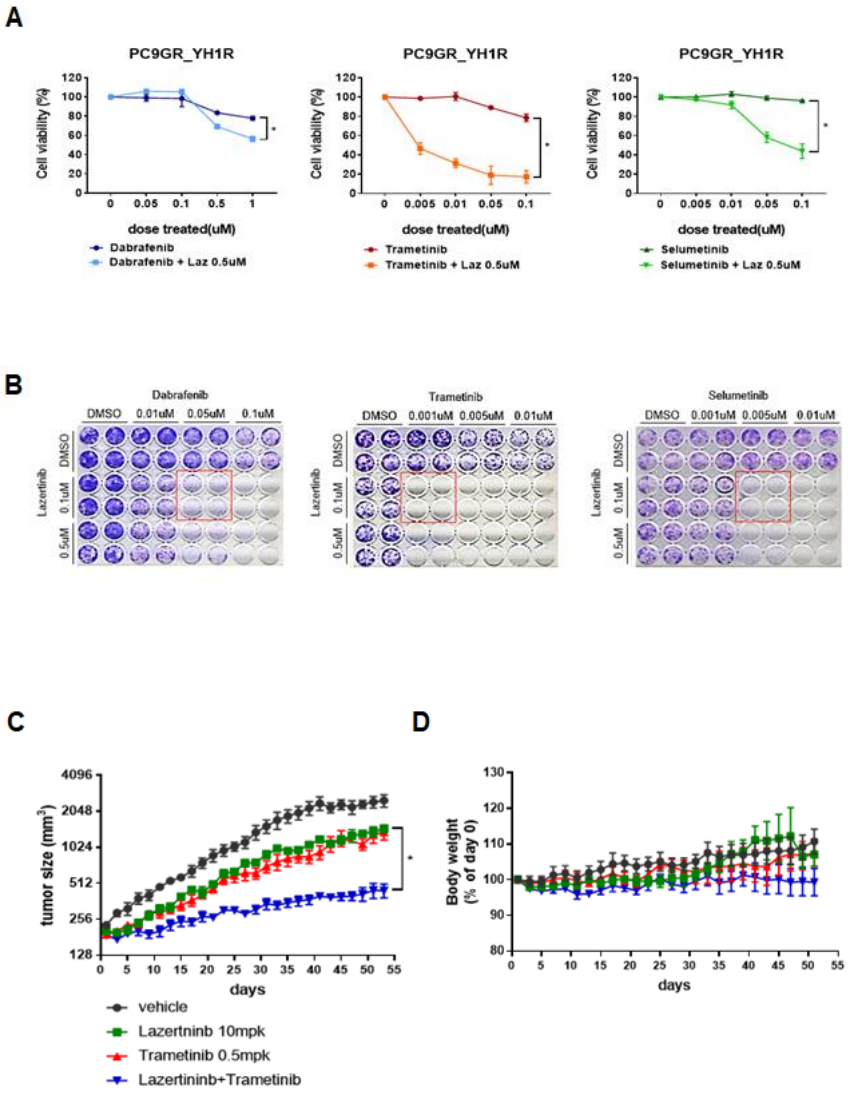
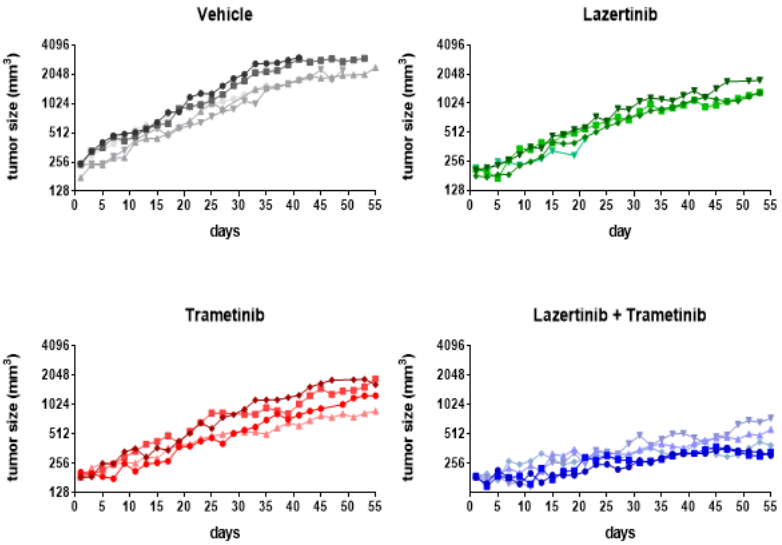


Figure 5. Combined inhibition of EGFR and MEK was effective in reducing growth of cells with lazertinib resistance *in vitro* and also combination treatment led potential killing effect in vivo (A) PC9GR_YH1R cells were treated with BRAF inhibitor (dabrafenib) or MEK inhibitor (trametinib, selumetinib) and lazertinib for 72 hrs. Then cell growth was determined by CellTiter-glo. (B) Colony formation assay was conducted in PC9GR_YH1R cell line treated with BRAF inhibitor (dabrafenib) or MEK inhibitor (trametinib, selumetinib) and lazertinib. Each drug was treated with indicated concentrations. Then the cells were cultured for 2 wks to grow clones. Trametinib and lazertinib had the most effective combination efficacy in PC9GR_YH1R cell *in vitro*. (C, D) Inhibition of PC9GR_YH1R xenograft growth by lazertinib, trametinib, and their combination in nude mice. PC9GR_YH1R tumor-bearing mice were treated with vehicle control, 10 mg/kg lazertinib, 0.5 mg/kg trametinib, and combination of 10 mg/kg lazertinib and 0.5 mg/kg trametinib Q.D. Combination therapy of lazertinib and trametinib also showed synergistic antitumor efficacy in the lazertinib-resistant model *in vivo*.



Supplementary Figure 4. Tumor volume of individual mice

IV. DISCUSSION

The results of the study comprehensively demonstrated the mechanism of resistance to lazertinib. We identified the novel lazertinib-resistance mechanism, namely, the fusion of EGFR and BRAF as an acquired genetic alteration in PC9GR_YH1R cells. Also, we found the same acquired mutation in patient-derived tumor xenografts from the patients who had experienced lazertinib resistance. These findings indicate that EGFR/BRAF fusion gene is the key driver inducing resistance to lazertinib. We demonstrated that with knockdown functional study. Also, we provided the overcoming strategy with lazertinib and trametinib combination treatment in lazertinib-resistance NSCLC model.

Diverse resistance mechanisms of 3rd- generation EGFR-TKIs have been reported. Loss of T790M, acquisition of EGFR C797X mutation, cMET amplification, and other tract activation are included. However, it is necessary to develop the other unknown mechanisms. Until now, the resistance mechanisms induced by lazertinib have not been reported.

In our study, one of the established lazertinib-resistant cell lines, PC9GR_YH1R cells, obtained the acquired EGFR/BRAF fusion gene. It was reported that approximately 2 % of EGFR-mutant NSCLC had BRAF fusions

as acquired mutation.[26] It has been shown to be minor, but RTK fusions with concurrent EGFR activating mutation are actionable and in a largescale survey.[27]

Among the resistant mechanisms related to fusion by 1st- generation EGFR-TKI, RET fusion was the most common one, and in particular, KIF5B–RET fusion and CCDC6-RET fusion were well reported. In addition, among RTK fusions occurring after 2nd- and 3rd- generation EGFR-TKIs, RET and ALK fusion accounted for the largest ratio and these were reported as the major mechanisms. Recently, gene fusion has emerged as an oncogenic mutation that induces resistance to 3rd- generation EGFR-TKI. Although BRAF fusion was not known as a major resistant mechanism such as RET, and ALK fusion, it has been being studied as a potential oncogenic mechanism consistently and the following research will be needed.

BRAF is a molecule that composes the RAS/MAP kinase pathway. It is a serine/threonine-specific protein kinase that acts on cell proliferation, differentiation, migration, and signal transduction. If oncogenic BRAF is generated through uncontrolled binding with Ras protein, BRAF cannot be autoinhibited and forms a dimer with CRAF. Then downstream signaling is continuously activated. According to reports, BRAF rearrangement occurs due to BRAF fusion, BRAF kinase domain duplication and N-terminal deletion. Especially, in the case of BRAF fusion, the 3' kinase domain and the RKTR motif (Arg-Lys-Thr-Arg dimerization domain) which is related to dimer

formation are maintained and 5' N-terminal Ras binding domain (RBD) disappeared. Then Raf protein continues to activate MAP kinase signaling, resulting in oncogenic properties regardless of RAS activation. Mostly, exon 8-11 of BRAF including kinase domain is cleaved. Of these, about 52% of BRAF fusions were found to be fused within exon 9 of BRAF. Until now, a few partners of BRAF fusion were already reported, for example, AGK, STAT3, TRIM24, AGAP3, and so on.[28-31]

To elucidate the resistance mechanism of lazertinib, fusion analysis was performed with RNA sequencing data. It showed the expression of EGFR and BRAF fusion gene. As a result, EGFR and BRAF were discovered as innovative fusion partners. Although only one case of established-resistant cell lines showed EGFR/BRAF fusion, it functioned as an inducer of resistance to lazertinib in a setting of treatment. The BRAF gene of PC9GR_YH1R model is also cleaved at exon 9 to form EGFR/BRAF fusion as usual. Also, two cases of patient-derived tumor xenografts, which were from patients who exhibited cancer progression after the lazertinib treatment, had the EGFR/BRAF fusion transcript. This showed the possibility that lazertinib resistant patients expressed EGFR/BRAF fusion gene. However, it entailed the limitation owing to a lack of fusion gene occurring cases and there are no pre-treatment samples to compare with post-treatment samples.

Many reports and studies showed the possibility of MEK inhibitors to

directly overcome BRAF fusion-positive cancers.[32] The underlying mechanism behind the expression of EGFR/BRAF fusion gene was related with MEK/ERK pathway leading to constant ERK activation. In several lazertinib-resistant cancer models, EGFR was inactivated, but the downstream molecules such as ERK, AKT, S6 were still activated under lazertinib treatment condition. (Figure 1D).

There are many ongoing clinical trials to evaluate the combined effects of other inhibitors and trametinib, a promising partner of lazertinib we confirmed. The partners of trametinib which are ongoing are multi-RTK inhibitor (pazopanib), BRAF inhibitor (dabrafenib, palbociclib), PI3K inhibitor (buparlisib), mTOR inhibitor (everolimus), and AKT inhibitor (afuresertib, uprosertib).[35] Also, there are some reports that the co-treatment of EGFR-TKI and MEK inhibitor affects the response to EGFR-TKI.[33,34] Therefore, a clinical trials using the co-treatment of lazertinib and trametinib might be expected.

There are several studies about toxicities arising under co-treatment of trametinib and osimertinib, another 3rd- generation EGFR-TKI.[36] However, we confirmed that there was no toxicity in our study, even though lazertinib-resistant NSCLC model with acquired EGFR/BRAF fusion gene was able to successfully overcome the resistance with the combination therapy of lazertinib and trametinib. (Figure 5D)

In conclusion, we uncover a novel EGFR/BRAF fusion as a mechanism of resistance to lazertinib. NSCLC cells harboring EGFR/BRAF

fusion significantly responded to co-treatment of lazertinib and trametinib *in vitro* and *in vivo*. Our finding suggests that the combination treatment of lazertinib and trametinib is a promising therapy for patients harboring EGFR/BRAF fusion mutation.

However, there is a need to further confirm clinical relevance for EGFR/BRAF fusion in lazertinib failed NSCLC patients.

V. CONCLUSION

In conclusion, we found a novel EGFR/BRAF fusion gene, as a key driver of acquired resistant mechanism of lazertinib, in NSCLC cell lines and patient-derived xenografts with acquired resistance to lazertinib. Also, combination treatment of lazertinib and trametinib showed a strong antitumor effect and no toxicity in lazertinib-acquired resistant NSCLCs *in vitro* and *in vivo*. These findings indicate that the combination therapy of lazertinib and trametinib might be a promising clinical option for the NSCLC patients who had acquired resistance to lazertinib.

REFERENCE

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69 –90.

2. Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer 2007;7:169–81.

3. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res 2004;64:8919–23.

4. Singh B, Carpenter G, Coffey RJ. EGF receptor ligands: recent advances. F1000Res 2016;5:F1000 Faculty Rev-2270.

5. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.

6. Melosky B. Review of EGFR TKIs in metastatic NSCLC, including ongoing trials. Front Oncol 2014;4,244.

7. Zhang YL, Yuan JQ, Wang KF, Fu XH, Han XR, Threapleton D, et al. The prevalence of EGFR mutation in patients with non-small cell lung cancer: a systematic review and meta-analysis. Oncotarget 2016;7:78985-78993. doi:10.18632/oncotarget.12587.

8. Shu Y, Wu X, Tong X, Wang X, Chang Z, Mao Y, et al. Circulating tumor DNA mutation profiling by targeted next generation sequencing provides guidance for personalized treatments in multiple cancer types. Sci Rep 2017;7:583.

9. Zhang Z, Stiegler AL, Boggon TJ, Kobayashi S, Halmos B. EGFR-mutated lung cancer: a paradigm of molecular oncology. Oncotarget 2010;1:497–514.

10. Taniguchi H, Yamada T, Wang R, Tanimura K, Adachi Y, Nishiyama A, et al. AXL confers intrinsic resistance to osimertinib and advances the emergence of tolerant cells. Nat Commun 2019;10: 259

11. Sequist LV, Yang JC, Yamamoto N, O'Byrne K, Hirsh V, Mok T, et al. Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations. J Clin Oncol 2013;31:3327–34.

12. Xia B, Zhang S, Ma S. Management of non-small cell lung cancer with mutation: the role of radiotherapy in the era of tyrosine kinase inhibitor therapy-opportunities and challenges. J Thorac Dis. 2017;9:3385–3393.

13. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med

2005;2:e73.

14. Lin Y, Wang X, Jin H. EGFR-TKI resistance in NSCLC patients: mechanisms and strategies. Am J Cancer Res 2014;4:411-35.

15. Thress KS, Paweletz CP, Felip E, Cho BC, Stetson D, Dougherty B, et al. Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring EGFR T790M. Nat Med 2015;21:560–2.

16. Mok TS, Wu YL, Ahn MJ, Garassino MC, Kim HR, Ramalingam SS, et al. Osimertinib or Platinum-Pemetrexed in EGFR T790M-Positive Lung Cancer. N Engl J Med. 2017;376:629–640.

17. Soria JC, Ohe Y, Vansteenkiste J, Reungwetwattana T, Chewaskulyong B, Lee KH et al. Osimertinib in Untreated EGFR-Mutated Advanced Non-Small-Cell Lung Cancer. N Engl J Med. 2018;378:113–125.

18. Ahn MJ, Han JY, Lee KH, Kim SW, Kim DW, Lee YG, et al. Lazertinib in patients with EGFR mutation-positive advanced non-small-cell lung cancer: results from the dose escalation and dose expansion parts of a first-in-human, open-label, multicentre, phase 1-2 study. Lancet Oncol 2019;20:1681-1690.

19. Leonetti, A., Sharma, S., Minari, R., Paola Perego P., Elisa Giovannetti E., Marcello Tiseo M. Resistance mechanisms to osimertinib in -mutated non-small cell lung cancer. *Br J Cancer* 121, 725–737 (2019)

20. Uhrig S, Ellermann J, Walther T, Burkhardt P, Frohlich M, Hutter B, et al. Accurate and efficient detection of gene fusions from RNA sequencing data. Genome Res 2021;31:448-460.

21. Hass BJ, Dobin A, Li B, Stransky N, Pochet N, Regev A, et al. Accuracy assessment of fusion transcript detection via read-mapping and de novo fusion transcript assembly-based methods. Genome Biol. 2019;20:213.

22. Guo YJ, Pan WW, Liu SB, Shen ZF, Xu Y, Hu LL. ERK/MAPK signalling pathway and tumorigenesis. Exp Ther Med. 2020;19:1997–2007.

23. Ercan D, Xu C, Yanagita M, Monast CS, Pratilas CA, Montero J, et al. Reactivation of ERK Signaling Causes Resistance to EGFR Kinase InhibitorsCancer Discov 2012;2:934-947.

24. Miyanaga A, Shimizu K, Noro R, Seike M, Kitamura K, Kosaihira S, et al. Activity of EGFR-tyrosine kinase and ALK inhibitors for EML4–ALK-rearranged non–small–cell lung cancer harbored coexisting mutation. BMC Cancer 2013;13:262.

25. Xu H, Shen J, Xiang J, Li H, Li B, Zhang T, et al. Characterization of acquired receptor tyrosine-kinase fusions as mechanisms of resistance to EGFR tyrosine-kinase inhibitors. Cancer Manag Res 2019;11:6343-6351.

26. Vojnic M, Kubota D, Kurzatkowski C, Offin M, Suzawa K, Benayed R, et al: Acquired BRAF rearrangements induce secondary resistance to EGFR therapy in EGFR-mutated lung cancers. J Thorac Oncol 2019;14:802-815,

27. Cho BC, Cheng Y, Zhou C, Ohe Y, Imamura F, Lin MC, et al. Mechanisms of acquired resistance to first-line osimertinib: preliminary data from the phase III FLAURA study. Ann Oncol. 2018; 29 (viii740, LBA50)

28. Latysheva NS, Babu MM. Discovering and understanding oncogenic gene fusions through data intensive computational approaches. Nucleic Acids Res 2016;44:4487-503.

29. Leonetti A, Sharma S, Minari R, Perego P, Giovannetti E, Tiseo M.. Resistance mechanisms to osimertinib in -mutated non-small cell lung cancer. Br J Cancer 2019;121**:**725–737.

30. Sheikine Y, Pavlick D, Klempner SJ, Trabucco SE, Chung JH, Rosenzweig M, et al. BRAF in Lung Cancers: Analysis of Patient Cases Reveals Recurrent BRAF Mutations, Fusions, Kinase Duplications, and Concurrent AlterationsJCO Precis Oncol 2018:2: PO.17.00172.

31. Nguyen-Ngoc T, Bouchaab H, Adjei AA, Peters S. BRAF Alterations as Therapeutic Targets in Non–RAF Alterations as Ther J Thoracic Oncol 2015 Oct;10:1396-403.

32. Zhu YC, Wang WX, Xu CW, Zhuang W, Du KQ, Chen G, et al. A patient with lung adenocarcinoma with BRAF gene fusion and response to vemurafenib. Clin Lung Cancer 2019 May;20:e224-e228.

33. Huang MH, Lee JH, Chang YJ, Tsai HH, Lin YL, Lin AM, et al. MEK inhibitors reverse resistance in epidermal growth factor receptor mutation

lung cancer cells with acquired resistance to gefitinib, Mol Oncol 2013;7:112-20.

34. Tricker EM, Xu C, Uddin S, Capelletti M., Ercan D., Ogino A., et al: Combined EGFR/MEK inhibition prevents the emergence of resistance in EGFR-mutant lung cancer. Cancer Discov 5:960-971, 2015

35. Tolcher AW, Peng W, Calvo E. Rational Approaches for Combination Therapy Strategies Targeting the MAP Kinase Pathway in Solid Tumors. Mol Cancer Ther 2018 Jan;17:3-16.

36. Ribeiro, M.F.S.A., Knebel, F.H., Bettoni F., Saddi R., Sacardo KP., Canedo F.S.N.A., et al. Impressive response to dabrafenib, trametinib, and osimertinib in a metastatic *EGFR-mutant*/ *BRAF* V600E lung adenocarcinoma patient. *npj Precis. Onc.* 5, 5 (2021).

ABSTRACT (IN KOREAN)

EGFR 돌연변이를 갖는 NSCLC 환자에서 3 세대 EGFR TKI 인 렉라자 (레이저티닙)에 대한 새로운 내성 기전, 'EGFR/BRAF fusion' 에 대한 연구

<지도교수 조병철>

연세대학교 대학원 의과학과

정서윤

전 세계적으로 폐암은 암 관련 사망의 주요 원인 중 하나입니다. 비소 세포 폐암 (NSCLC) 중에서 표피 성장 인자 수용체 (EGFR) 돌연변이는 폐암 원인의 상당 부분을 차지합니다. 이에 따라 EGFR 돌연변이를 갖는 NSCLC 환자에서 molecular-targeted therapy 인 EGFR tyrosine kinase inhibitors (TKIs) 에 대한 연구가 활발해졌습니다. EGFR TKI 는 EGFR 돌연변이를 활성화하는 NSCLC 에 대해 확립된 first-line therapy 입니다. NSCLC 에서 EGFR 돌연변이의 약 85 %를 차지하는 Exon 19

deletion 과 Exon 21 missense mutation (L858R) 은 1 세대 및 2 세대 EGFR TKI 에 반응합니다. 초기의 좋은 치료효과에도 불구하고 대부분의 환자는 치료 9-13 개월 후 불가피하게 disease progression 이 진행되게 됩니다. 환자의 약 50-60 %는 1 세대 및 2 세대 EGFR TKI 에 대한 후천적 내성 기전으로써 T790M 돌연변이를 갖게 되며, 이에 따라 3 세대 EGFR TKI 가 T790M 돌연변이 NSCLC 을 타겟 하여 개발되었습니다.

Lazertinib 은 3 세대 EGFR TKI 중 하나이며 T790M 내성 돌연변이로 활성화된 EGFR 을 선택적으로 차단합니다. 또한 Lazertinib 은 임상 I / II 상 dose-escalation 연구에서 훌륭한 치료효과를 보였고, 현재 EGFR 돌연변이를 갖는 locally advanced 혹은 metastatic NSCLC 환자에서 first-line treatment 로써의 lazertinib 의 효능과 안전성을 평가하기 위해 진행중인 임상 3 상 연구가 진행 중입니다. Lazertinib 은 임상에서 뛰어난 효과를 보였지만 필연적으로 다른 EGFR TKI와 마찬가지로 내성을 갖게 될 것입니다.

Lazertinib 에 대한 내성 기전을 조사하기 위해, 6 개월 이상의 기간 동안 ATCC, PDC, PDX cell line 에서 lazertinib 에 대한 내성 세포주를 만들었습니다. 기존의 세포주와 비교하여 만들어진 내성세포주(YH1R)에서 새로이 생기는 genetic level, molecular

level, drug screening 의 세가지 측면에서 연구를 진행하였습니다. 그 결과 RNA-seq 분석을 통해 genetic alteration, gene expression level 을 확인하는 과정에서 타겟을 발굴하였습니다. PC9GR_YH1R 세포주에서 새로운 EGFR / BRAF fusion transcript 를 발견하였고, EGFR / BRAF fusion mRNA 및 protein 이 lazertinib 내성 세포주에서 특이적으로 발현되었습니다. 흥미롭게도 EGFR / BRAF fusion gene 이 lazertinib 에 대한 내성을 갖는 환자로부터 얻은 환자 유래 샘플(PDTX)에서 또한 발견되었습니다. 마지막으로 lazertinib 과 MEK inhibitor 의 병용 치료가 EGFR/BRAF fusion 을 갖는 lazertinib resistant model 에서 resistant 를 극복할 수 있다는 것을 in vitro 및 in vivo study 를 통해 증명하였습니다.

본 연구를 통해 lazertinib 에 대한 후천적 내성을 가진 NSCLC 세포에서 lazertinib 의 내성기전으로서 새로운 EGFR / BRAF fusion gene 을 발견했습니다. 또한 lazertinib 으로 획득한 내성 NSCLC model 에서 강력한 항 종양 효과를 보여준 lazertinib 과 trametinib 의 병용 치료를 제시합니다. 이러한 발견은 EGFR 과 MEK inhibitor 의 병용 요법이 임상에서 lazertinib 에 의한 내성을 갖는 NSCLC 환자를 위한 유망한 치료 옵션 일 수 있음을 나타냅니다.

핵심되는 말: Lazertinib, YH25448, EGFR-TKI, NSCLC, resistant mechanism, EGFR/BRAF fusion, gene alteration, trametinib