이학석사 학위논문

비소세포성폐암 세포에서 ALK 억제제의 내성기전연구

Combined cetuximab and pemetrexed therapy enhances cytotoxicity against crizotinib resistant non-small cell lung cancer cells by downregulating thymidylate synthase

2016년 8월

서울대학교 융합과학기술대학원

분자의학 및 바이오제약학과

김 혜 진

ABSTRACT

Combined cetuximab and pemetrexed therapy enhances cytotoxicity against crizotinib resistant non-small cell lung cancer cells by downregulating thymidylate synthase

Hye-Jin Kim

Department of Molecular Medicine and Biopharmaceutical Sciences

World Class University Graduate School of Convergence Science and Technology

Seoul National University

Although non-small cell lung cancer (NSCLC) cells with anaplastic lymphoma kinase (ALK)-rearranged initially show a dramatic response to crizotinib, these cells eventually develop resistance to

crizotinib. This resistance is caused by secondary mutations and copy number gain in the ALK gene. However, other resistance mechanisms through activation of the bypass tracts have yet to be clearly elucidated. To investigate the mechanisms of acquired resistance to ALK fusion-directed treatment in NSCLC, I generated crizotinib-resistant NSCLC cell lines in vitro through chronic exposure of an ALK fusion NSCLC line (SNU2292) to crizotinib. Interestingly, the resultant SNU2292-CR cells maintained activation of epidermal growth factor receptor (EGFR) and expressed increased levels of transforming growth factor alpha (TGF-α), an EGFR ligand. Additionally, I found thymidylate synthase (TS) was overexpressed in SNU2292-CR cells compared with the parental cells. These data showed that reduction of TS enhanced synergistic inhibition of cell proliferation when cetuximab, an EGFR inhibitor, was combined with pemetrexed, a TS inhibitor. As a result, combined therapy was found to exhibit a synergistic growth inhibitory effect against crizotinib-resistant cells by downregulating TS expression. Taken together, these results support a potential role of activation of the bypass tracts in acquired resistance to ALK-directed treatment in ALK-rearranged NSCLC,

and provide insights into strategies for preventing and/or overcoming this resistance in patients.

Keywords : EML4-ALK, TGF-α, TS, Crizotinib, Pemetrexed

Student Number : 2012-24143

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Non-small cell lung cancer (NSCLC) is increasingly recognized as a heterogeneous disease, and these differences in protein expression can drive therapeutic decision-making (1-3). The most prevalent mutated or rearranged oncogenes identified in NSCLCs are KRAS, epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), and ROS1, among others (2,4). Crizotinib is the first food and drug administration-approved treatment for patients with ALK-positive NSCLC (5). To date, only the final data from one phase III randomized trial has been published, which evaluated the use of crizotinib as a second-line therapy (6). Multiple phase III randomized trials are in progress to assess the efficacy of crizotinib as first-line chemotherapy (7). Eventually, most patients on treatment with crizotinib develop resistance to this

drug within 1 year of treatment. Most clinical trials in progress on ALK-positive patient populations involve new generation ALK inhibitors, or crizotinib in combination with novel drugs to bypass known resistance mechanisms (8,9). However, a detailed understanding of the most prevalent mechanism(s) of resistance and strategies to overcome the most common forms of acquired resistance to ALK TKIs is still lacking.

Thymidylate synthase (TS) is involved in DNA synthesis during the production of thymine nucleotides and is a target of cancer chemotherapy. DNA and RNA synthesis is inhibited through TS inhibition, for instance, by 5-fluorouracil, capecitabine and pemetrexed. As a result, they show anti-cancer effects on cancer cells (10-12). Pemetrexed is an anti-cancer agent that inhibits TS in addition to many other folate-dependent enzymes such as DHFR, glycinamide ribonucleotide transformylase (GART) and

aminoimidazole carboxamide ribonucleotide transformylase , resulting in anti-proliferative effects thorough DNA and RNA synthesis and repair, eventually inducing cell cycle arrest and DNA damage (13,14). Especially, pemetrexed is effective in ALK-positive NSCLC and acts as a second-line agent for advanced NSCLC. Some cancer types including NSCLC and colorectal cancer (CRC) are associated with elevated expression of TS with poor clinical data after chemo-based therapy, supporting evidence of high levels of TS expression as a mechanism of resistance to antifolate drugs such as pemetrexed (15-17). In addition, TS expression is regulated by the EGFR signaling pathway (18,19). EGFR has been detected in the nuclei of cancer cells and in primary tumor specimens of various origins, as well as in those of other highly proliferative tissues. When localized to the nucleus, EGFR may operate as a transcriptional regulator (20,21). EGFR

downregulates a variety of nucleotide synthesis-related genes, including TS. Nuclear EGFR activates TS gene transcription, and TS-bound EGFR promoter activities are inhibited by EGFR blockage. Combination of an EGFR inhibitor with a TS inhibitor is known to effectively inhibit the proliferation of cancer cells with the highest levels of EGFR (19).

In this study, I established crizotinib-resistant cell lines from EML4-ALK-positive, crizotinib-sensitive, SNU2292 human NSCLC cells. I demonstrate that TGF-α-induced EGFR activation increases TS levels, representing a mechanism of resistance to crizotinib. Dual inhibition of EGFR by cetuximab and TS by pemetrexed inhibited the downstream effectors, restoring crizotinib sensitivity to these cells. These data support the development of novel, therapeutic approaches for treating EML4-ALK-positive NSCLC. Furthermore, these data support a potential role of EGFR

activation in acquired resistance to ALK-directed treatment in EML4-ALK-positive NSCLC, providing unique insights into potential strategies for preventing and overcoming disease resistance

MATERIALS AND METHODS

Reagents

Crizotinib(PF-02341066) and pemetrexed were purchased from Selleck (Houston, TX, USA). Cetuximab was kindly provided by Merck, respectively. Stock solutions were prepared in dimethyl sulfoxide(DMSO), stored at -20℃. Crizotinib and pemetrexed were diluted in fresh medium before each experiment, and the final concentration of DMSO was <0.1%.

Cell lines and Cell culture

The NSCLC cell lines SNU2292 (variant 3 E13:A20) purchased from the Korean Cell line Bank (22) . And other EML4-ALK fusion NSCLC cell line NCI-H3122 (variant 1 E13:A20) provided by ATCC (American Type Culture Collection). All cell lines were maintained in RIMI-1640 culture media supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 10ul/mL gentamicin in a humidified atmosphere containing 5% CO2 at 37℃ condition.

Generation of SNU2292 CR cells

Crizotinib Resistance cell line SNU2292-CR cells generated during 6 month. Generation of SNU2292-CR cell line by exposed the crizotinib for sensitive parental cell line SNU2292. Cell were seeded 70% of dish and maintenance when cells density over 70% of total dish concentration. Gradually increase the amount of drug from 1 to 10 μM and cells were expanded in RPMI-1640 culture media supplemented with 10% fetal bovine serum (FBS) and 10ul/mL gentamicin in a humidified atmosphere containing 5% CO2 at 37℃ condition.

Western blot analysis

Cultured cells were washed cold PBS and Cell harvest with RIPA buffer [50mM Tris-Cl(pH7.4), 150mM NaCl, 1% NP40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sufate(SDS), 50mM sodium fluoride, 1mM sodium pyrophosphate, 2mM phenylmethylsulfonyl fluoride, 1mg/mL pepstatin A, 0.2mM leupeptin, 10μg/mL aprotinin, 1mM sodium vanadate, 1mM nitrophenylphosphate, and 5mM benzamidine] containing protease

& phosphatase inhibitors. And centrifuge 15000rpm 20min. After quantitative protein supernatant containing equal concentration and a 20μl of total protein were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Followed by resolved protein transfer to nitrocellulose membrane about 1-2 hours. After blocking this membrane by 1% BSA and skim milk powder mixture for 1hr at room temperature and overnight incubation at 4℃ with primary antibody. Antibodies against p-EGFR, EGFR, p-MAPK, MAPK, p-AKT(s473), AKT, TS(Thymidylate Synthase), caspase3, Bad and Bcl-2 purchased from Cell signaling Technology (Beverley, MA, USA). ACTIN purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) was used as a control. And TGF-α purchased from R&D systems and PARP purchased from BD Biosciences (San Jose, CA, USA).

Cell growth inhibition assay

The viability of cells was assessed using MTT assays (Sigma-Aldrich, St Louis, MO, USA). A total of 3 × 103 cells were seeded in 96-well plates, incubated for 24 h, and treated for 72 h with indicated drugs at 37 ℃. Following treatment, MTT solution was added to each well and incubated for 4 h at 37 ℃. The medium was then removed, and dimethyl sulfoxide was added and mixed thoroughly for 30 min at room temperature. Cell viability was determined by measuring absorbance at 540 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cell cycle Analysis

Cells were washed twice in phosphate buffered solution (PBS), fixed in 70% ethanol, and stored at −20℃ until required for analysis. Before analysis, the cells were washed in 1% BSA and incubated with 1% BSA 300μl and 10μg/mL RnasA (Sigma Aldrich, St Louis, MO, USA) at 37℃ 20 minutes. Next, the cells were stained with 14μg/mL PI (Propidium Iodide, Sigma Aldrich) and 37℃ 10 minutes incubation. The cell cycle was confirmed using a FACS Calibur flow

cytometer (BD Biosciences; San Jose, CA, USA) equipped with a ModFit LT Program (Verify Software House, Inc.).

Human RTK arrays

Phospho-RTK antibody arrays kit (R&D Systems, Minneapolis, MN, USA) were used according to the manufacturer's protocol. Briefly, Nitrocellulose membrane contained duplicate spots for 42 anti-receptor tyrosine kinase of 42 incubated with Control and Crizotinib 1μM/ml treatment 500μg/ml protein lysates overnight at 4℃ respectively. Bound phosphor-RTKs were detected by using chemiluminescence.

Human TGF-α Immunoassay

For the quantitative determination of human Transforming Growth Factor alpha (TGF-α) concentration in cell culture supernatants using Human TGF-α Immunoassay kit (R&D Systems) were used according to the manufacturer's protocol. Briefly, add 100μl of assay diluent RD1W each well and prepared reagents, standard and

samples adds 50μl each well incubate for 2hr at room temperature. Next, four times washes by wash buffer each well and aspirated wash buffer. Adding TGF-α conjugate 200μl and incubate for 2hr at room temperature. After Repeat the aspiration and washes four time each well. Lastly adds each well 200μl of substrate solution and incubate for 30min at room temperature. Add 50μl of stop solution to each well within 30min at the same condition. When color changed from blue to yellow measures using microplate reader. Absorbance measured by ELISA 540nm wavelength and pre automix 5min. The data linearized by plotting the log of the TGF-α concentrations versus of the O.D. on a linear scale on offer.

Colony forming assay

For each cell line, 3000 cells were plated in 6-well plates for 48h, prior to drug- treatment. After addition of various indicated concentrations of crizotinib, cetuximab, and pemetrexed, cells were grown for 14days in a humidified atmosphere containing 5% CO2 at 37℃, during which period the medium was not changed. Cells were then fixed with 4% formaldehyde and colonies were stained with

coommassie blue. The colonies were counted under a Bio-Rad Gel DOC and counting number average from three independent experiments in same condition.

Sub-cellular fractionation

Cells were fractionated using Qproteome Cell Compartment Kit (Qiagen) according to manufacturers instruction. Briefly, cells incubated in a lysis buffer were centrifuged at 1000×g for 10 min to produce a supernatant containing cytosolic proteins. The pellet was resuspended in a extraction buffer and re-centrifuged at 6000×g for 10 min. The supernatant served as the membrane proteins. Using cell compartment kit for extraction of cytosol and membrane protein.

Reverse transcription and Real-time PCR

Total RNA extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) following manufacturer's instructions. cDNA was synthesized 1μg of total RNA with ImProm-Ⅱ revers

transcriptase (Promega, a Corporation, Madison, WI, USA). And amplified by RT-PCR using AmpliTaq Gold DNA polymerase and Quantitative real-time PCR, cDNA was amplified using Premix Ex Taq (TaKaRa, Shiga, Janpan) with SYBR Green I (Molecular Probes, Eugene, OR, USA) by Step One Plus system (Applied Biosystems). Primer were synthesized by Macrogen Inc. and Bionics Inc. (Seoul, Korea). Actin expression was used as control. The primers used for RT-PCR are as follows Table1.

Table 1. Primer sequences

No.	Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
1	EGF	TGCCAACTGGGGGTGCACAG	CTGCCCGTGGCCAGCGTGGC
2	TGF-α	CCTGCTGCCCGCCCGCCCGT	GCTGGCAGCCACCACGGCCA
3	Amphiregulin	CCATTATGCAGCTGCTTTGGAGC	TGTTTTTCTTGGGCTTAATCACCT
4	HB-EGF	CTCCCACTGGATCCACAAAC	GGCATGGGTCTCTCTTCTTC
5	Epiregulin	CACCGAGAAAGAAGGATGGA	GGGATCGTCTTCCATCTGAA
6	Betacellulin	CCCCAAGCAGTACAAGCATT	TGAACACCACCATGACCACT
7	EGFR	GGACGACGTGGTGGATGCCG	GGCGCCTGTGGGGTCTGAGC
8	Actin	AGAGCTACGAGCTGCCTGAC	GGATGCCACAGGACTCCA

Mutational analysis of the ALK gene

Genomic DNA was extracted from SNU2292-CR cells, using QIAamp DNA mini kit(Qiagen) and ALK (Exon 20-Exon28) was then sequenced. The primers used in the sanger-sequencing were as follow Table 2.3.

Table 2. Primer sequences for ALK Secondary mutation sites analysis

No.	Exon	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
1	Exon 20	ATGGAGATCCAGGGAGGCTT	ATTCAGCCCCTACACTGCAC
2	Exon 21	ACCCCCAAGCTGCCTCATTAT	CAGCCTACAGAGTCCGCAAG
3	Exon 22	ACTCTGTAGGCTGCAGTTCTC	ACATGCTAGGGACAACACGAT
4	Exon 23	AGATTTGCCCAGACTCAGCTC	CTCCTGTCCTTGGCACAACA
5	Exon 24	TGTAGCTGCATGTTCACGGT	AAGCACACAGATCAGCGACA
6	Exon 25	ATCCTAGTGATGGCCGTTGT	CCCATTCTTGAGGGGCTGAG
7	Exon 26	CTGAACCGCCAAGGACTCAT	CCCAGGAGCACCACCTTATG
8	Exon 27	AATGTGGGTGGGTGTGTCTA	TTTTTGAAAAGAAAAACTGCTTAG
9	Exon 28	GTTCACACCCTCAACGTATT	TGTTGACCAAAGGGAGAAA

Table 3. Primer sequences for EML4-ALK

No.	Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
1	EML4-ALK	CCCACCAAAAGCATAAAACG	TGCCAGCAAAGCAGTAGTTG

Annexin V assay of apoptosis

Cells were treated with a indicated drug 72 h, at which point cells were collected and stained with Annexin V- fluorescein isothiocyanate and propidium iodide (Pharmingen, BD Sciences, San Jose,CA,USA). Apoptotic cell death was measured by counting the number of cells that stained positive for Annexin V- fluorescein isothiocyanate as assessed by fluorescence-activated cell sorting analysis.

Statistics

The statistical significance of the results was calculated by unpaired Student's test, and P values of < 0.05 were considered to be statistically significant.

RESULT

Establishment of Crizotinib- Resistant Cell Line

I investigated the resistance mechanisms of crizotinib in NSCLC. The NSCLC cell line SNU2292 has EML4-ALK variant 3, and H3122 cells have variant 1 (Supplementary figure S1). Each cell line is highly sensitive to treatment with crizotinib. I established a SNU2292-CR cell line by chronically exposing SNU2292 cells to increasing concentrations of crizotinib for 6 months in vitro. Single cell clones were isolated from a pool of resistant cells to generate the resultant cell lines. The half-maximal inhibitory concentration value in SNU2292-CR cells was higher than that of parental cells, after which cell proliferation inhibition was analyzed by an MTT assay (Figure 1A). The IC50 values of H3122 and SNHU2292 cell lines were 0.3 μM and 0.8 μM respectively, while the resistant cell line IC50 value was higher than 10 μM. The phosphorylation status of ALK and EGFR kinases, and AKT and ERK, downstream targets of both genes, were determined for NCI-H3122, SNU2292 and SNU2292-CR cell lines. Treatment of H3122 cells and parental

SNU2292 cells with crizotinib markedly inhibited tyrosine phosphorylation of EGFR and ALK in addition to AKT and ERK. In contrast, phosphorylation of these proteins persisted at higher levels in crizotinib-resistant SNU2292-CR cells following treatment (Figure 1B). Then, annexin V staining was performed to determine apoptotic cell death that was influenced by crizotinib in SNU2292 and SNU2292-CR cells. Treatment with crizotinib resulted in induction of annexin-positive cells in SNU2292 cells, but not in SNU2292-CR cells. This data suggested that cell death was induced in SNU2292 but not in SNU2292-CR. In addition, cleaved and activated caspase-3 was increased as a result of treatment with crizotinib, which is consistent with the results of the apoptosis assay (Figure 1C). Taken together, these data suggest that SNU2292-CR cells have acquired resistance to crizotinib compared with the parental cells.

FIGURE1. Establishment Crizotinib Resistance Cell Line.

(A) Crizotinib-resistant sublines of the EML4-ALK fusion SNU2292 NSCLC cell line were generated by culturing in increasing concentrations of crizotinib over a 6-month period. SNU2292 and resistant (SNU2292-CR) cell lines were treated with crizotinib at the indicated concentrations and cell viability was measured after 72 h. The percentage of viable cells is shown relative to untreated controls. (B) H3122, SNU2292 and SNU2292-CR cells were exposed to increasing concentrations of crizotinib (0.1 and 1 μM) for 24 h. Whole-cell extracts were analyzed by western blotting with antibodies recognizing phosphorylated and total EGFR, ALK, AKT, ERK and TS. Actin was used as a loading control. (C) SNU2292 and SNU2292-CR cells were treated with increasing concentrations of crizotinib (0.1 and 1 μM) for 72 h. Cells were fixed with 70% ethanol, stained with propidium iodide and analyzed by flow cytometry. Proportions of cells in G1, S and G2-M phase were quantified using the ModFit LT program (Verity Software House Inc) (left figure). SNU2292 and SNU2292-CR cells were treated with crizotinib (1 μM) for 72 h. Whole-cell extracts were

analyzed by western blotting with antibodies recognizing cleaved and total caspase-3 (right figure).

Fig. 1A

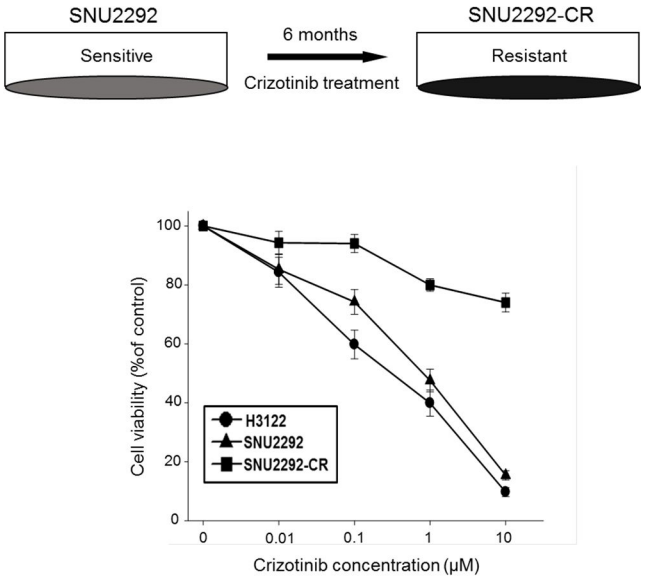

SNU2292
Sensitive
6 months
Crizotinib treatment
SNU2292-CR
Resistant
Cell viability (%of control)
100
80
60
40
20
0
H3122
SNU2292
SNU2292-CR
0
0.01
0.1
1
10
Crizotinib concentration (μM)

Fig. 1B

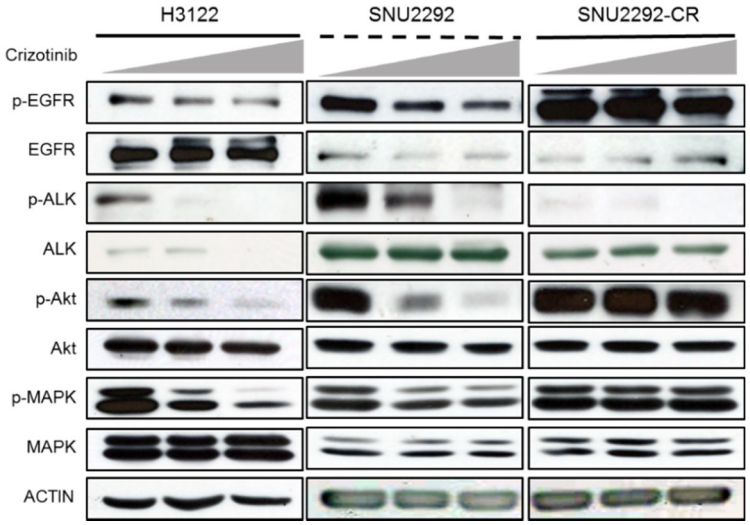
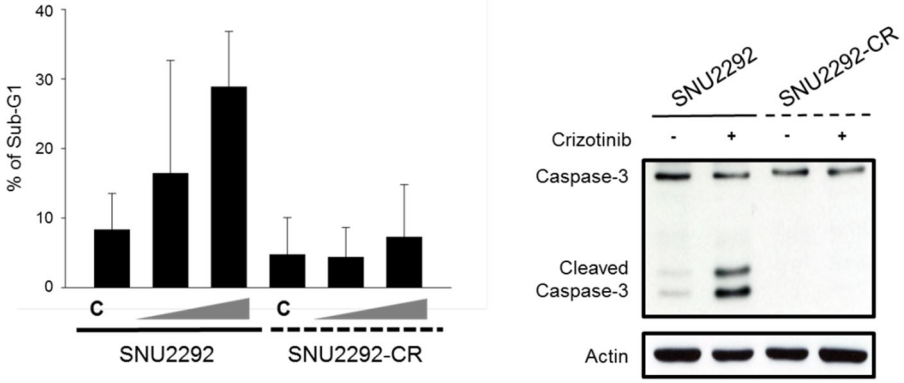

H3122
SNU2292
SNU2292-CR
Crizotinib
p-EGFR
EGFR
p-ALK
ALK
p-Akt
Akt
p-MAPK
MAPK
ACTIN

Fig. 1C


40
30
20
10
0
% of Sub-G1
C
C
SNU2292
SNU2292-CR
SNU2292
SNU2292-CR
Crizotinib
-
+
-
+
Caspase-3
Cleaved
Caspase-3
Actin

Activation of EGFR induced by TGF-α confer acquired resistancne to crizotinib-sensitive SNU2292 cells

I next determined whether aberrant activation of other receptors may have a role in mediating crizotinib resistance. I performed a phospho-receptor tyrosine kinase (phospho-receptor tyrosine kinase) array to compare the effects of crizotinib on the phosphorylation of 42 receptor tyrosine kinases in SNU2292 and SNU2292-CR cells. In SNU2293-CR cells, EGFR, platelet-derived growth factor receptor (PDGFR), insulin receptor (IR), and AXL were all phosphorylated, compared with the parental cells (Figure 2A). Among them, phosphorylation of EGFR and AXL were reconfirmed by western blot (Figure2B). Expression of total protein levels was not changed. There, I focused on increased phosphorylation of EGFR or AXL. First, we examined the levels of EGFR ligands, such as TGF-α, EGF, amphiregulin (AREG), HB-EGF (HBEGF), betacellulin (BTC), and epiregulin (EREG). I assessed the mRNA levels of each EGFR ligand and found TGF-α was significantly increased by 12-fold in SNU2292-CR cells. EGF,

AREG, and BTC were increased approximately two-fold (Figure 3A). Then, the DNA levels of the ligands for copy number gain were assessed by quantitative RT-PCR, and TGF-α was only increased by approximately 2-fold (Figure3B), however, the other ligands remained unchanged (data not shown). Consistent with these results, ELISA showed that secretion of TGF-α was increased and protein expression of TGF-α was also increased in a similar pattern in SNU2292-CR (Figure 3C). In addition, expression of TGF-α was detected by western blotting after subcellular fractionation. Crizotinib inhibited AKT and ERK phosphorylation in normal media (NM). However, inhibition was less effective in conditioned media (CM) from SNU2292-CR cells (Supplementary Figure S3). Furthermore, to investigate whether TGF-α secretion plays a role in crizotinib resistance, I examined the effect of exogenous TGF-α on crizotinib sensitivity in SNU2292 and H3122 cells. I found that exposure to TGF-α induces resistance to crizotinib in these cells (Figure 3D). Crizotinib inhibited AKT and ERK phosphorylation in SNU2292 and H3122 cells in the absence of EGF, whereas it failed to inhibit downstream signaling, including the phosphorylation of AKT and ERK, in the

presence of EGF. Combination of crizotinib and cetuximab re-inhibited phosphorylation of AKT and ERK. Cells were treated with cetuximab for TGF-α blockage. These data suggested that TGF-α-induced activation of EGFR might underlie resistance to crizotinib in SNU2292-CR cells. In experiments comparing the colony formation inhibition of crizotinib-treated cells and cells treated with a combination of crizotinib and cetuximab, I observed that the colony formation inhibition effects on SNU2293-CR cells were further inhibited in a dose-dependent manner compared with parental cells (Figure 3E). In addition, combination with crizotinib and cetuximab effectively reduced the activity of AKT and ERK more than single agent treatment. These results reveal that TGF-α-induced EGFR activation conferred resistance to crizotinib, and cetuximab inhibition of EGFR activity downregulated the downstream molecules in SNU2292-CR cells.

Phosphorylation of AXL was higher than in SNU2292 cells (Figure 2A, 2B). Therefore, I examined whether combination with crizotinib and AXL inhibitor (XL-184) is effective. I observed that it was not effective in restoring crizotinib sensitivity (data not shown). Previous studies have shown that acquired resistance to crizotinib

is associated with a secondary mutation in EML4–ALK fusion–expressing NSCLC. Furthermore, genomic DNA sequence analysis of the ALK kinase domain in SNU2292–CR cell lines revealed no mutations (Supplementary Figure S2).

Figure2. Increased expression and activation of EGFR in the Crizotinib Resistant NSCLC cells.

(A) SNU2292 and SNU2292-CR cells were treated with 1 μM crizotinib for 24 h and cell lysates were hybridized to a phospho-receptor tyrosine kinase (RTK) array. In the array, each RTK was spotted in duplicate. Hybridization signals at the corners served as controls. (B) SNU2292 and SNU2292-CR cell extracts were analyzed by western blotting with antibodies recognizing phosphorylated and total EGFR, AXL, PDFGR, and IR. Actin was used as a loading control. Data are representative of three independent experiments.

Fig. 2A

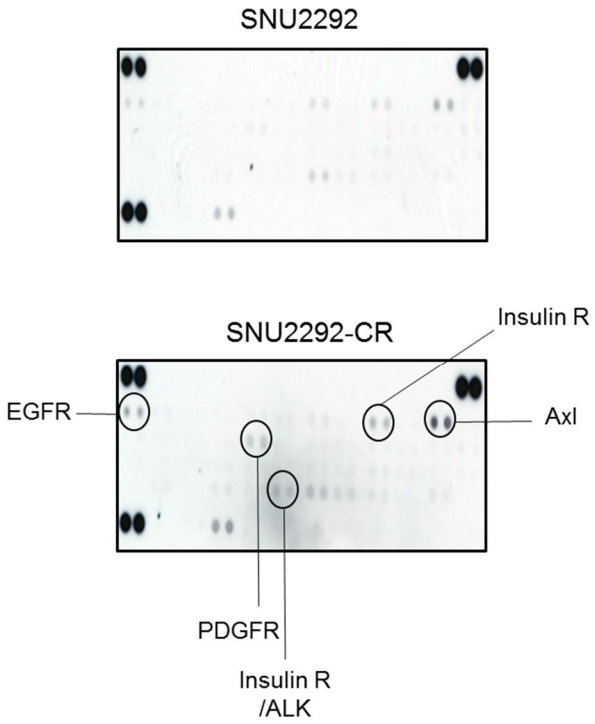


Fig. 2B

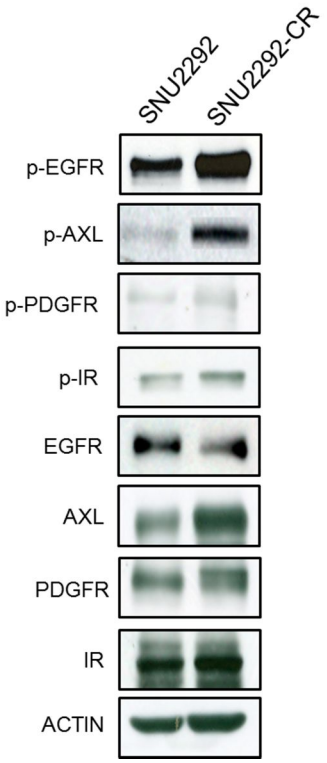

SNU2292
SNU2292-CR
p-EGFR
p-AXL
p-PDGFR
p-IR
EGFR
AXL
PDGFR
IR
ACTIN

Figure3. Activation of EGFR axis induced by TGF-α and Inhibition of EGFR restores Crizotinib sensitivity in NSCLC cells.

(A) Levels of TGF-α, EGF, amphiregulin, HB-EGF, betacellulin, and epiregulin mRNA were determined via quantitative RT-PCR. Differences in parental SNU2292 versus SNU2292-CR cells are shown. Data are expressed as the mean±s.d., *P<0.05. **(B)** gDNA level of TGF-α and EGF was quantified by real-time quantitative-PCR. Data are expressed as the mean±s.d., *P<0.05. **(C)** Cells were cultured overnight in medium containing 10% FBS and then incubated for 24 h in serum-free medium, after which the culture supernatants were collected and assayed for TGF-α with an ELISA. Data are expressed relative to the corresponding value for H2292 (left). Cells were subjected to biochemical fractionation to separate the nuclei from the cytosolic material. Western blots are shown for TGF-α. The loading controls were Integrin V (membrane marker) and α-tubulin (cytosol marker). **(D)** Prior to harvesting, SNU2292 and H3122 cells were serum-starved for 24 h, then grown for 3 h in the presence of crizotinib (0.1 μM), and cetuximab (100 μg/ml), followed by 30 min of TGF-α (100 ng/mL) stimulation. Whole-cell extracts were analyzed by western blotting

with antibodies recognizing phosphorylated and total EGFR, AKT, ERK and TS. Actin was used as a loading control. **(E)** Cells were treated with cetuximab and/or crizotinib at the indicated concentrations for 14 days in normal growth media. The surviving colonies were stained as described in the materials and methods. Representative plates and data for the number of colonies formed are shown. Data are representative of three independent experiments. **(F)** Cells were treated with cetuximab and/or crizotinib at the indicated concentrations for 14 days in normal growth media. The surviving colonies were stained as described in the materials and methods. Representative plates and data for the number of colonies formed are shown. Data are representative of three independent experiments.

Fig. 3A

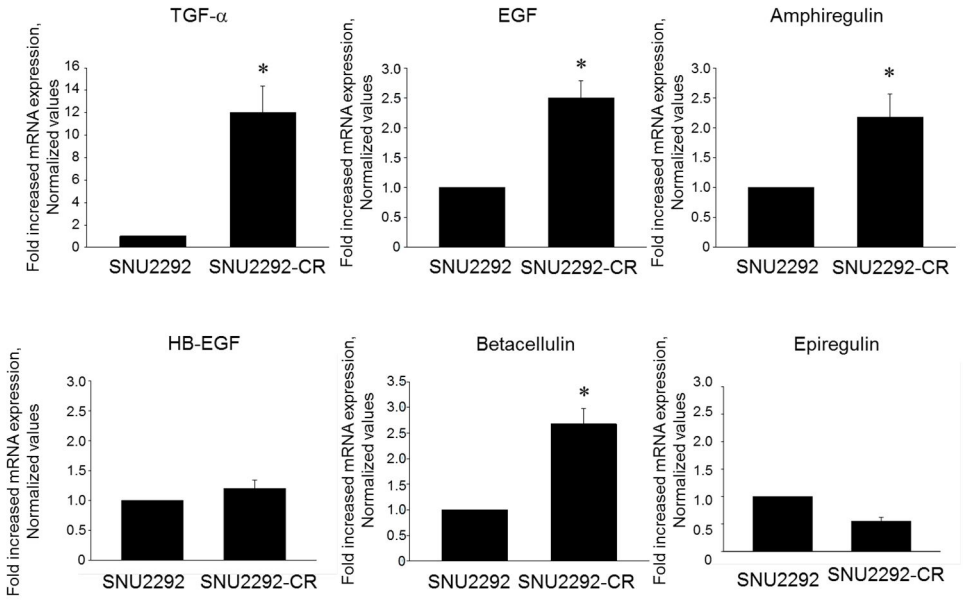

TGF-α
EGF
Amphiregulin
HB-EGF
Betacellulin
Epiregulin
Fold increased mRNA expression, Normalized values
SNU2292
SNU2292-CR
*

Fig. 3B

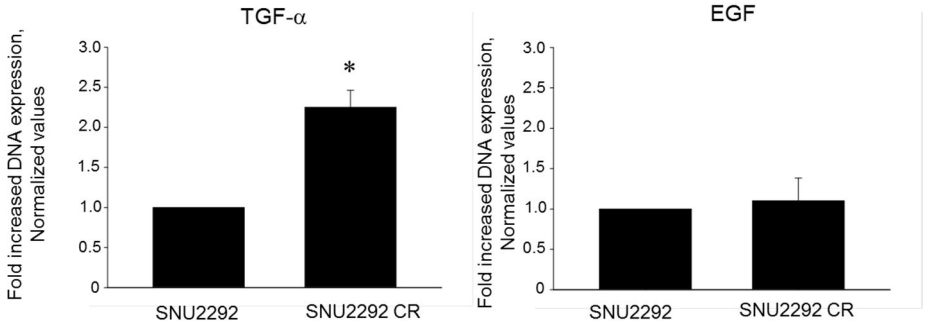

TGF-α
Fold increased DNA expression, Normalized values
3.0
2.5
2.0
1.5
1.0
0.5
0
*
SNU2292
SNU2292 CR
EGF
Fold increased DNA expression, Normalized values
3.0
2.5
2.0
1.5
1.0
0.5
0
SNU2292
SNU2292 CR

Fig. 3C

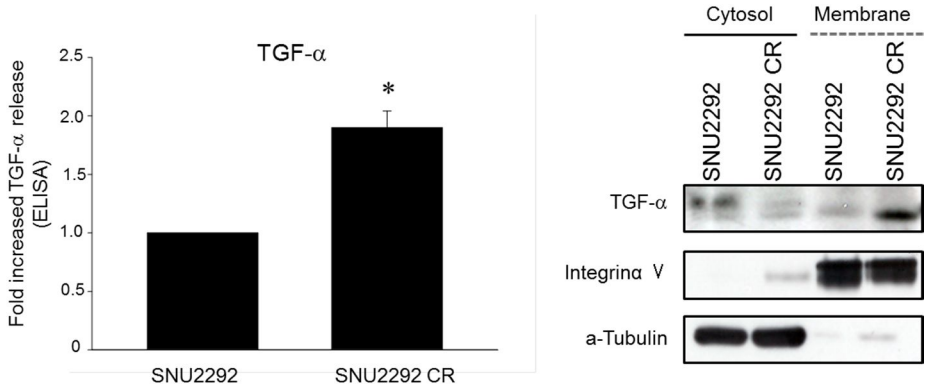

TGF-α
Fold increased TGF-α release (ELISA)
2.5
2.0
1.5
1.0
0.5
0
*
SNU2292
SNU2292 CR
Cytosol
Membrane
SNU2292
SNU2292 CR
SNU2292
SNU2292 CR
TGF-α
Integrinα V
a-Tubulin

Fig. 3D

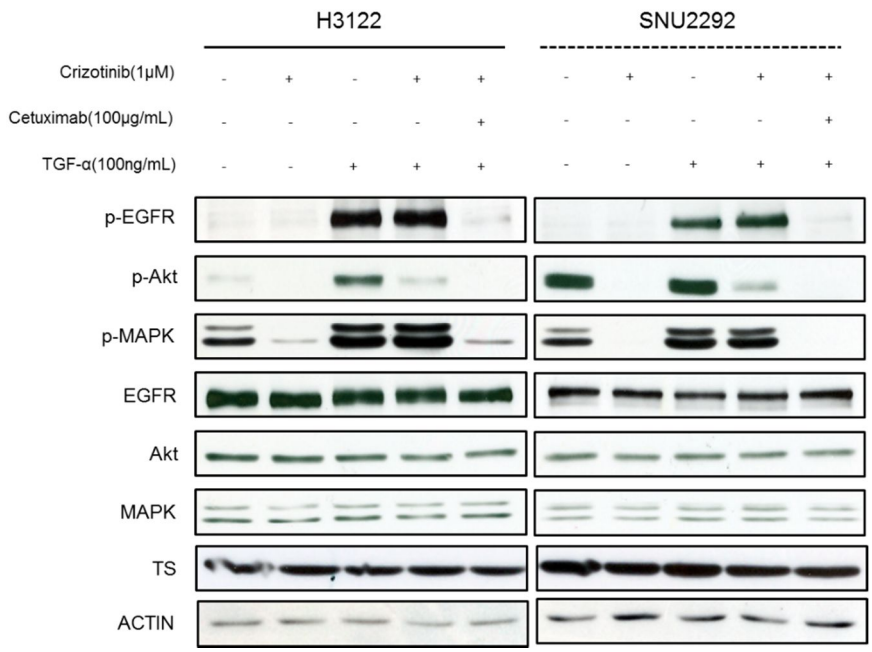
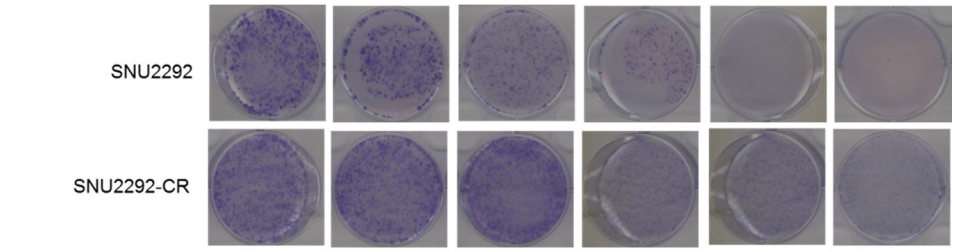
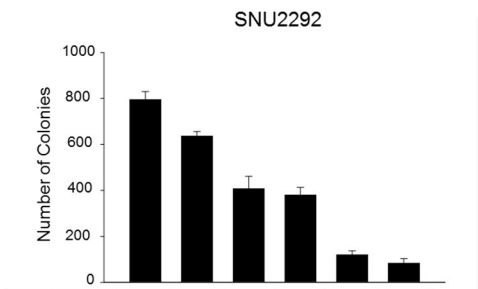

H3122
SNU2292
Crizotinib(1μM)
Cetuximab(100μg/mL)
TGF-α(100ng/mL)
p-EGFR
p-Akt
p-MAPK
EGFR
Akt
MAPK
TS
ACTIN

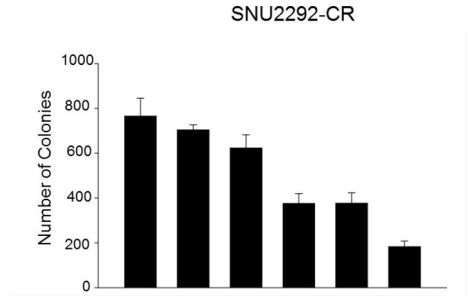
Fig. 3E



Cetuximab (mg/ml)	–	100	–	100	–	100
Crizotinib (μM)	–	–	0.1	0.1	1	1

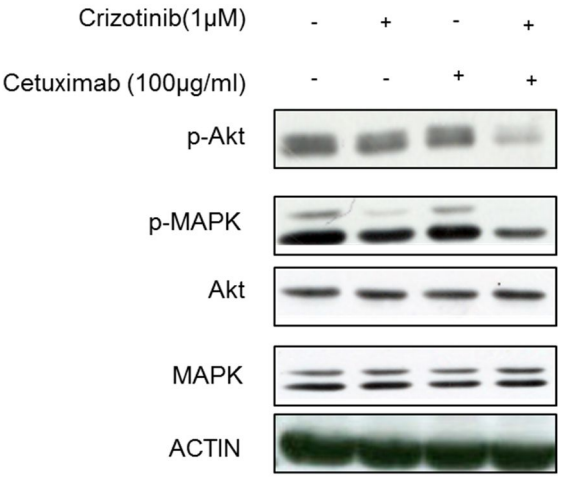


Cet (mg/ml)	–	100	–	100	–	100
Crizo (μM)	–	–	0.1	0.1	1	1



Cet (mg/ml)	–	100	–	100	–	100
Crizo (μM)	–	–	0.1	0.1	1	1

Fig. 3F


Crizotinib(1μM) - + - +
Cetuximab (100μg/ml) - - + +
p-Akt
p-MAPK
Akt
MAPK
ACTIN

Combination with cetuximab and pemetrexed enhances cytotoxicity against crizotinib-resistant cells

It was previously reported that thymidylate synthase (TS) is closely related with EGFR expression (9), and overexpression of TS results in resistance to pemetrexed, a TS inhibitor (ref). Therefore, I evaluated TS expression with crizotinib resistance because SNU2292-CR cells have highly phosphorylated EGFR expression (Figure 1B). TS expression was increased in SNU2292-CR cells, and SNU2292-CR cells were found to be more resistant to pemetrexed than SNU2292 (Figure 4A). Previously, I showed that TGF-α-induced EGFR activation confers resistance to crizotinib, and targeting TGF-α in combination with cetuximab-sensitized crizotinib-resistant cell lines. In addition, TS is highly overexpressed in crizotinib-resistant cells. These results showed the critical role of TS in determining pemetrexed sensitivity, as reported in a previous study (29). On this basis, we reasoned that the inhibition of TGF-α induced EGFR activation may restore pemetrexed sensitivity caused by induction of TS. To test this

hypothesis, I investigated the effect of combined treatment with cetuximab and pemetrexed on the proliferation of SNU2292-CR cells. As shown in Figure 4A, treatment of cells with this drug combination had a greater colony formation inhibitory effect compared with treatment with single-agent cetuximab in SNU2292-CR cells. Cetuximab enhanced the inhibitory effect of pemetrexed on cell proliferation in a dose-responsive manner. In addition, SNU2292-CR cells were simultaneously exposed to cetuximab with pemetrexed at a fixed ratio. Combined treatment with cetuximab and pemetrexed exerted synergistic effects (CI<1; Figure 4B). To further confirm the previous data, I performed western blotting to determine the phosphorylation and total protein levels of EGFR, AKT, ERK, and TS (Figure 4C). Cetuximab treatment slightly decreased phosphorylation of EGFR, AKT, ERK and TS. However, combined treatment with cetuximab and pemetrexed led to a significant decrease in phosphorylated AKT and ERK. TS was also decreased after treatment with combination therapy. Annexin V-propidium iodide (PI) staining showed that treatment with a combination of cetuximab and pemetrexed resulted in a greater induction of apoptosis than treatment alone (Figure 4D).

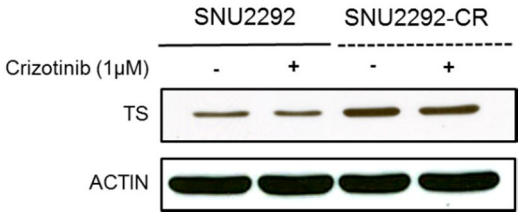
Treatment with 100 mg/ml cetuximab and 0.01 μM pemetrexed resulted in 27.6% of cells stained with FITC+/PI- (apoptotic) compared with cetuximab alone (4.2%) or pemetrexed alone (22.0%). In addition, cleaved caspase-3 and PARP were more increased after combination treatment. Taken together, these data show that combined treatment with cetuximab and pemetrexed significantly inhibits the proliferation of crizotinib-resistant SNU2292-CR cells.

Figure4. Combination with cetuximab and pemetrexed has a synergistic effect on crizotinib-resistant cells.

(A) SNU2292-CR cells were treated with crizotinib (1 μM) for 24 h. Whole-cell extracts were analyzed by western blotting with antibodies recognizing TS. Actin was used as a loading control (left). SNU2292 and SNU2292-CR cells were treated with pemetrexed at the indicated concentrations and cell viability was measured after 72 h. The percentage of viable cells is shown relative to untreated controls (right). **(B)** Cells were treated with cetuximab and/or pemetrexed at the indicated concentrations for 14 days in normal growth media. The surviving colonies were stained as described in the materials and methods. Representative plates and data for the number of colonies formed are shown. **(C)** Combination index values were calculated using the CalcuSyn software. SNU2292-CR cells were treated with varying concentrations of cetuximab and pemetrexed. Addictive effect (CI=1), synergism (CI<1) and antagonism (CI>1) were calculated. **(D)** SNU2292-CR cells were treated with cetuximab and/or pemetrexed at the indicated concentrations for 24 h. Whole-cell extracts were analyzed by western blotting with antibodies

recognizing phosphorylated and total EGFR, AKT, ERK, and TS. Actin was used as a loading control. **(E)** Cells were treated with cetuximab (100 mg/ml) and/or pemetrexed (0.01 μM) for 48 h. Cells were stained with Annexin V–FITC and PI (left). Whole−cell extracts were analyzed by western blotting with antibodies recognizing caspase−3 and PARP. Actin was used as a loading control (right). Data are representative of three independent experiments.

Fig. 4A


SNU2292
SNU2292-CR
Crizotinib (1μM)
-
+
-
+
TS
ACTIN

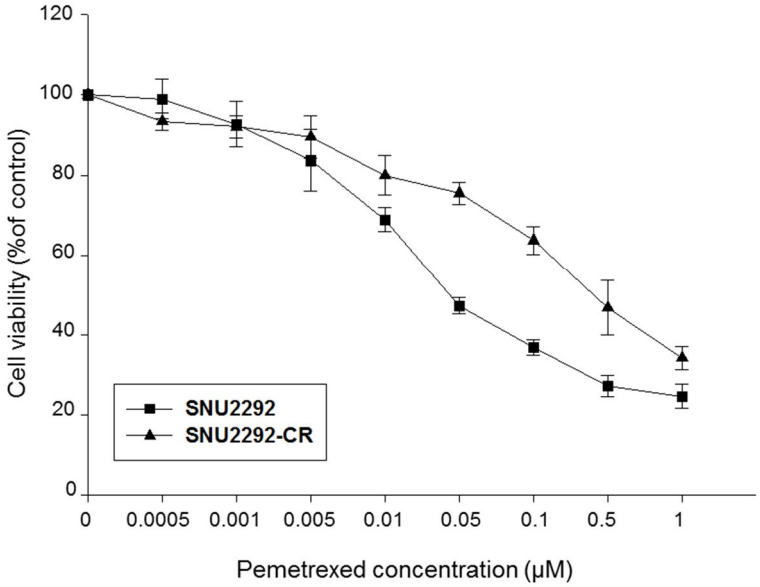
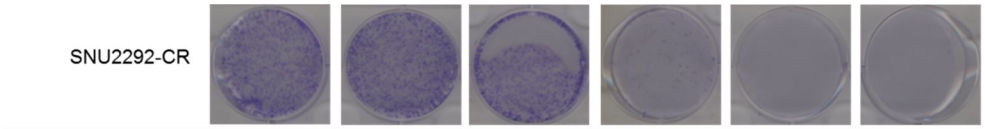

120
100
80
60
40
20
0
Cell viability (%of control)
SNU2292
SNU2292-CR
0
0.0005
0.001
0.005
0.01
0.05
0.1
0.5
1
Pemetrexed concentration (μM)

Fig. 4B



Cetuximab (mg/ml)	–	100	–	100	–	100
Pemetrexed (μM)	–	–	0.01	0.01	0.1	0.1

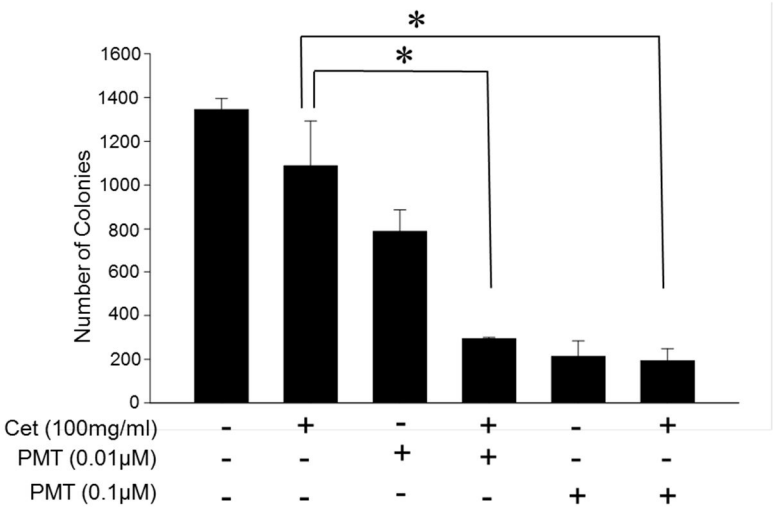


Fig. 4C

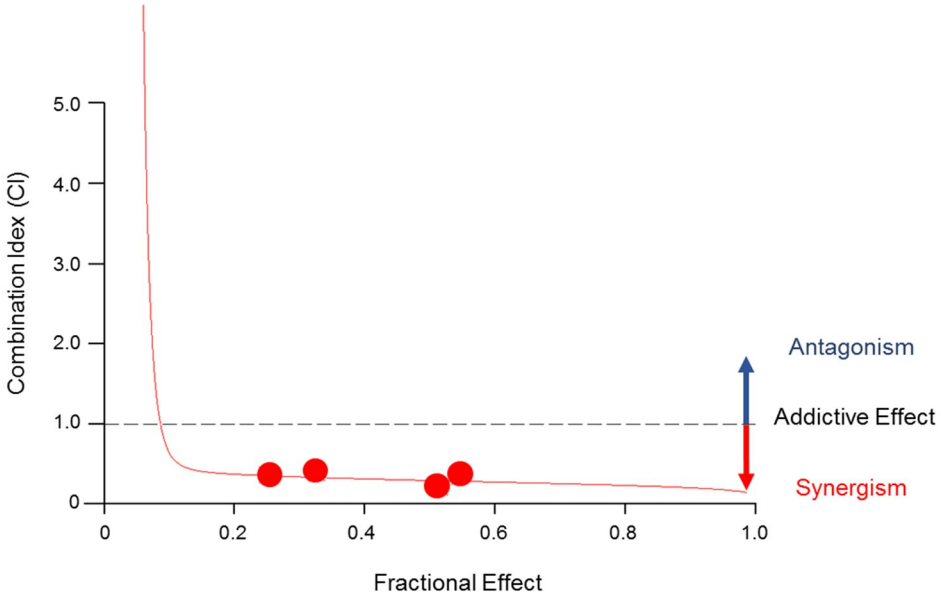

5.0
4.0
3.0
2.0
1.0
0
Combination Idex (CI)
0
0.2
0.4
0.6
0.8
1.0
Fractional Effect
Antagonism
Addictive Effect
Synergism

Fig. 4D

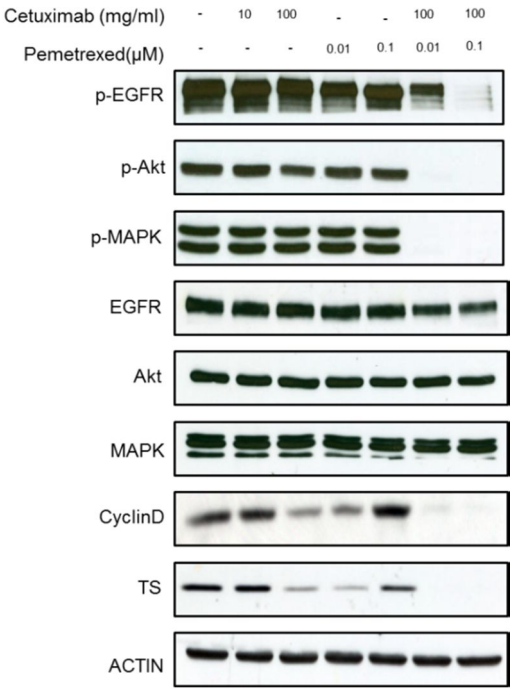
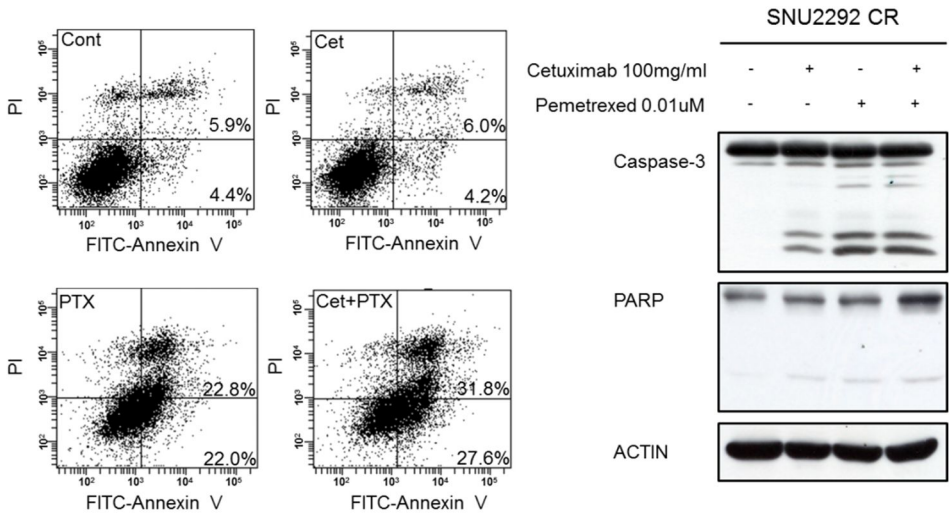
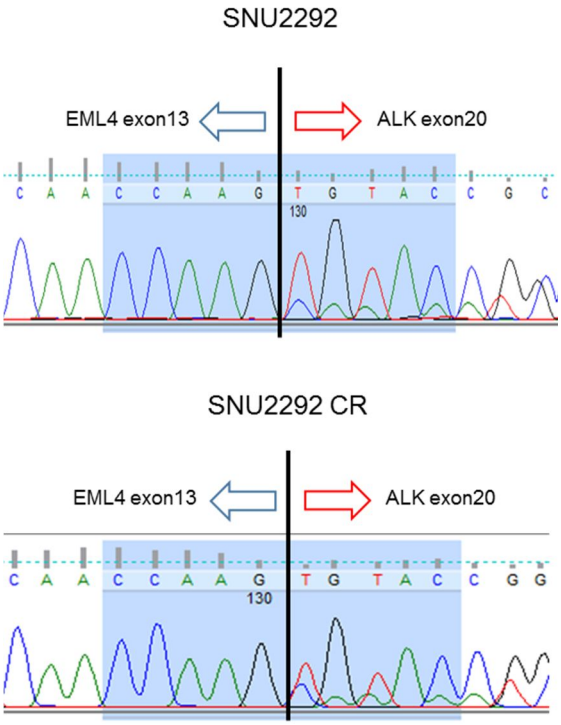

Cetuximab (mg/ml) - 10 100 - - 100 100
Pemetrexed(μM) - - - 0.01 0.1 0.01 0.1
p-EGFR
p-Akt
p-MAPK
EGFR
Akt
MAPK
CyclinD
TS
ACTIN

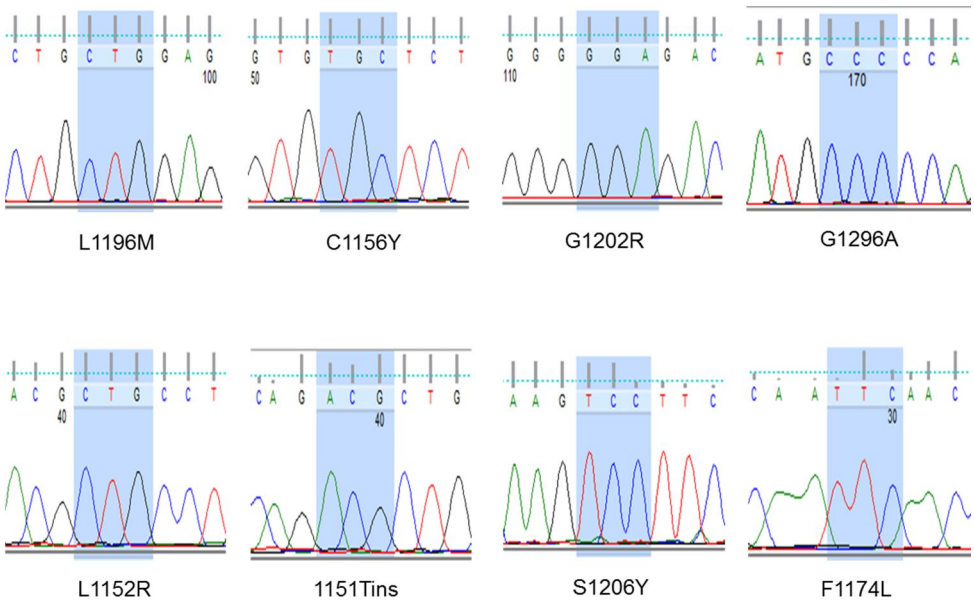
Fig. 4E


Cont
5.9%
4.4%
Cet
6.0%
4.2%
PTX
22.8%
22.0%
Cet+PTX
31.8%
27.6%
PI
FITC-Annexin V
SNU2292 CR
Cetuximab 100mg/ml - + - +
Pemetrexed 0.01uM - - + +
Caspase-3
PARP
ACTIN

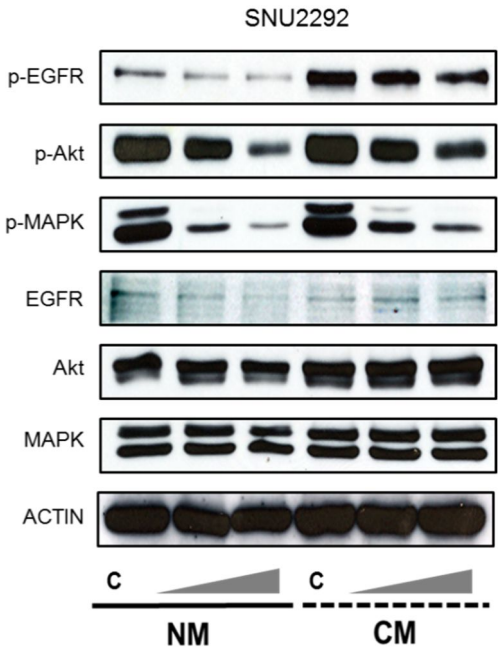
Supplementary figure S1



Supplementary figure S2



Supplementary figure S3



DISCUSSION

In this study, I identified a novel, molecular mechanism of acquired resistance to crizotinib in EML4–ALK–positive NSCLC cells. I established crizotinib–resistant cells from SNU2292 EML4–ALK–positive NSCLC cells in vitro. These data provide strong evidence that TGF–α–mediated EGFR activation conferred acquired resistance to crizotinib in this model system. In addition to displaying crizotinib resistance, EGFR activation induced TS overexpression, resulting in resistance to pemetrexed. Thus, treatment with cetuximab and pemetrexed is effective in crizotinib–resistant cells. This strategy and mechanism of combination treatment have not been previously identified in an EML4–ALK–positive NSCLC model, although overcoming crizotinib resistance through combination treatment with EGFR–targeted agents and crizotinib have been reported in an in vitro crizotinib–resistant cancer model.

Patients with ALK–positive NSCLC treated with crizotinib develop acquired resistance to the drug within the first year of therapy (23). Recent studies have elucidated several mechanisms underlying

acquired resistance to crizotinib, which conventionally belong to two classifications. One is an ALK-dependent mechanism, which occurs through mutations in the kinase domain of ALK, including a gatekeeper mutation or ALK fusion gene amplification, the latter alone or in combination with resistance mutations (8, 24-17). Most ongoing trials in ALK-positive NSCLC patients involve new generation ALK inhibitors, which include agents with activity against the L1196M gatekeeper mutation or ROS1 mutations (28,29). The other is an ALK-independent mechanism, as they determine the activation of other pathways, such as EGFR or PI3K/Akt/mTOR (9,30,31). Thus, approaches to circumventing crizotinib resistance are of immense importance. Combination of crizotinib with a small molecule inhibitor of EGFR tyrosine kinase was tested and showed significant growth-inhibitory effects against drug-resistant tumor cells only containing enhanced activation of the EGFR axis without a secondary ALK mutation or ALK gene amplification. However, this combined treatment failed to completely reverse crizotinib resistance, suggesting that other mechanisms might exist that are possibly involved in cellular resistance to this drug. To better understand how cancer cells alter

sensitivity to crizotinib, we generated an in vitro model of crizotinib-acquired resistance and investigated a mechanism of resistance to crizotinib. In this study, I demonstrated that TGF-α-induced EGFR activation results in resistance to crizotinib without secondary ALK mutations or amplification in SNU2292-CR cells. These results support that activation of the EGFR axis is a potential mechanism of bypassing the inhibition of ALK. Therefore, I expected to block activation of an alternative pathway if EGFR activation was inhibited by cetuximab. However, downstream AKT and ERK signaling was still maintained (Figure3F). We previously reported that EGFR inhibition downregulated TS via inhibiting nuclear translocation of EGFR, thus sensitizing cancer cells to fluoropyrimidine. Pemetrexed inhibits purine and pyrimidine synthesis enzymes such as TS. I detected induction of TS in EGFR-activated SNU2292-CR cells and demonstrated treatment with cetuximab and pemetrexed enhanced cytotoxicity in resistant cells. On the basis of our findings, I propose that inhibition of TGF-α-induced EGFR activation may enhance response to crizotinib treatment in appropriately selected EML4-ALK-positive NSCLC patients.

In this study, the measurement of basal gene expression levels allowed us to identify TGF-α-mediated EGFR activation as a mechanism of acquired resistance to crizotinib in EML4-ALK NSCLC cells. EGFR blocking partially restored sensitivity to crizotinib but co-treatment with cetuximab and pemetrexed blocked the downstream signaling in resistant cells, implying that other signaling pathways may also be responsible for drug resistance. However, owing to the limited number of cell lines used in our experiments, it is possible that other important biological factors exist that contribute to drug resistance in EML4-ALK NSCLC.

In conclusion, these work demonstrates that acquired resistance to crizotinib is associated with TGF-α-induced EGFR activation. Although these molecular changes may serve as molecular markers of resistance, they may also be associated with an increased invasive capacity of cancer cells in vitro. I found a further beneficial drug effect in that the combination of EGFR and TS inhibitors increased cytotoxicity in crizotinib-resistant cells by downregulating TS expression. These study may assist in understanding the mechanisms associated with acquired resistance to crizotinib, and serve to identify resistant tumors in clinical trials.

SNU2292-CR cells also represent a potentially useful tool for gaining insights into the mode of action of crizotinib in tumors, in addition to investigating and developing methods to prevent resistance to this drug or other targeted therapies. These findings provide a strong rationale for the combined use of EGFR and TS inhibitors to treat crizotinib-resistant NSCLC in a clinical setting.

REFERENCE

1. Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K, Sougnez C, Greulich H, Muzny DM, Morgan MB, Fulton L, Fulton RS, Zhang Q, Wendl MC, Lawrence MS, Larson DE, et al. **Somatic mutations affect key pathways in lung adenocarcinoma**. Nature. 2008; 455(7216):1069-1075.
2. Govindan R, Ding L, Griffith M, Subramanian J, Dees ND, Kanchi KL, Maher CA, Fulton R, Fulton L, Wallis J, Chen K, Walker J, McDonald S, Bose R, Ornitz D, Xiong D, et al. **Genomic landscape of non-small cell lung cancer in smokers and never-smokers**. Cell. 2012; 150(6):1121-1134.
3. Chen Z, Fillmore CM, Hammerman PS, Kim CF and Wong KK. **Non-small-cell lung cancers: a heterogeneous set of diseases. Nature reviews Cancer.** 2014; 14(8):535-546.
4. Pao W and Girard N. **New driver mutations in non-small-cell lung cancer. The Lancet Oncology.** 2011; 12(2):175-180.
5. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara S, Watanabe H, Kurashina K, Hatanaka H, Bando M, Ohno S, Ishikawa Y, Aburatani H, Niki T, Sohara Y, et al. **Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature.** 2007; 448(7153):561-566.
6. Shaw AT, Kim DW, Nakagawa K, Seto T, Crino L, Ahn MJ, De Pas T, Besse B, Solomon BJ, Blackhall F, Wu YL, Thomas M, O'Byrne KJ, Moro-Sibilot D, Camidge DR, Mok T, et al. **Crizotinib versus**

chemotherapy in advanced ALK-positive lung cancer. The New England journal of medicine. 2013; 368(25):2385-2394.

7. Solomon BJ, Mok T, Kim DW, Wu YL, Nakagawa K, Mekhail T, Felip E, Cappuzzo F, Paolini J, Usari T, Iyer S, Reisman A, Wilner KD, Tursi J, Blackhall F and Investigators P. **First-line crizotinib versus chemotherapy in ALK-positive lung cancer.** The New England journal of medicine. 2014; 371(23):2167-2177.
8. Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T, Yatabe Y, Takeuchi K, Hamada T, Haruta H, Ishikawa Y, Kimura H, Mitsudomi T, Tanio Y, Mano H and Group ALKLCS. **EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors.** The New England journal of medicine. 2010; 363(18):1734-1739.
9. Sasaki T, Koivunen J, Ogino A, Yanagita M, Nikiforow S, Zheng W, Lathan C, Marcoux JP, Du J, Okuda K, Capelletti M, Shimamura T, Ercan D, Stumpfova M, Xiao Y, Weremowicz S, et al. **A novel ALK secondary mutation and EGFR signaling cause resistance to ALK kinase inhibitors.** Cancer research. 2011; 71(18):6051-6060.
10. Longley DB, Harkin DP and Johnston PG. **5-fluorouracil: mechanisms of action and clinical strategies. Nature reviews Cancer.** 2003; 3(5):330-338.
11. Weekes CD, Nallapareddy S, Rudek MA, Norris-Kirby A, Laheru D, Jimeno A, Donehower RC, Murphy KM, Hidalgo M, Baker SD and Messersmith WA. **Thymidylate synthase (TYMS) enhancer region genotype-directed phase II trial of oral capecitabine for 2nd line treatment of advanced pancreatic cancer. Investigational new drugs.** 2011; 29(5):1057-1065.
12. Takezawa K, Okamoto I, Okamoto W, Takeda M, Sakai K, Tsukioka S,

Kuwata K, Yamaguchi H, Nishio K and Nakagawa K. **Thymidylate synthase as a determinant of pemetrexed sensitivity in non-small cell lung cancer.** British journal of cancer. 2011; 104(10):1594-1601.

13. Goldman ID and Zhao R. **Molecular, biochemical, and cellular pharmacology of pemetrexed.** Seminars in oncology. 2002; 29(6 Suppl 18):3-17.
14. Chattopadhyay S, Moran RG and Goldman ID. **Pemetrexed: biochemical and cellular pharmacology, mechanisms, and clinical applications.** Molecular cancer therapeutics. 2007; 6(2):404-417.
15. Nicolson MC, Fennell DA, Ferry D, O'Byrne K, Shah R, Potter V, Skailes G, Upadhyay S, Taylor P, Andre V, Nguyen TS, Myrand SP, Visseren-Grul C, Das M and Kerr KM. **Thymidylate synthase expression and outcome of patients receiving pemetrexed for advanced nonsquamous non-small-cell lung cancer in a prospective blinded assessment phase II clinical trial. Journal of thoracic oncology** : official publication of the International Association for the Study of Lung Cancer. 2013; 8(7):930-939.
16. Johnston PG, Lenz HJ, Leichman CG, Danenberg KD, Allegra CJ, Danenberg PV and Leichman L. **Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors.** Cancer research. 1995; 55(7):1407-1412.
17. Wilson PM, Danenberg PV, Johnston PG, Lenz HJ and Ladner RD. **Standing the test of time: targeting thymidylate biosynthesis in cancer therapy. Nature reviews Clinical oncology.** 2014; 11(5):282-298.
18. Skvortsov S, Sarg B, Lindner H, Lukas P, Hilbe W, Zwierzina H and Skvortsova I. **Cetuximab inhibits thymidylate synthase in colorectal**

cells expressing epidermal growth factor receptor. Proteomics Clinical applications. 2008; 2(6):908-914.

19. Kim HP, Yoon YK, Kim JW, Han SW, Hur HS, Park J, Lee JH, Oh DY, Im SA, Bang YJ and Kim TY. **Lapatinib, a dual EGFR and HER2 tyrosine kinase inhibitor, downregulates thymidylate synthase by inhibiting the nuclear translocation of EGFR and HER2.** PloS one. 2009; 4(6):e5933.

20. Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY, Bourguignon L and Hung MC. **Nuclear localization of EGF receptor and its potential new role as a transcription factor.** Nature cell biology. 2001; 3(9):802-808.

21. Lo HW, Hsu SC, Ali-Seyed M, Gunduz M, Xia W, Wei Y, Bartholomeusz G, Shih JY and Hung MC. **Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway.** Cancer cell. 2005; 7(6):575-589.

22. Ku JL, Kim KH, Choi JS, Jeon YK, Kim SH, Shin YK, Kim TY, Bang YJ, Kim WH and Park JG. **Establishment and characterization of six human lung cancer cell lines: EGFR, p53 gene mutations and expressions of drug sensitivity genes.** Cellular oncology. 2011; 34(1):45-54.

23. Camidge DR, Bang YJ, Kwak EL, Iafrate AJ, Varella-Garcia M, Fox SB, Riely GJ, Solomon B, Ou SH, Kim DW, Salgia R, Fidias P, Engelman JA, Gandhi L, Janne PA, Costa DB, et al. **Activity and safety of crizotinib in patients with ALK-positive non-small-cell lung cancer: updated results from a phase 1 study.** The Lancet Oncology. 2012; 13(10):1011-1019.

24. Huang D, Kim DW, Kotsakis A, Deng S, Lira P, Ho SN, Lee NV,

Vizcarra P, Cao JQ, Christensen JG, Kim TM, Sun JM, Ahn JS, Ahn MJ, Park K and Mao M. **Multiplexed deep sequencing analysis of ALK kinase domain identifies resistance mutations in relapsed patients following crizotinib treatment.** Genomics. 2013; 102(3):157-162.

25. Kim S, Kim TM, Kim DW, Go H, Keam B, Lee SH, Ku JL, Chung DH and Heo DS. **Heterogeneity of genetic changes associated with acquired crizotinib resistance in ALK-rearranged lung cancer. Journal of thoracic oncology** : official publication of the International Association for the Study of Lung Cancer. 2013; 8(4):415-422.

26. Zhang S, Wang F, Keats J, Zhu X, Ning Y, Wardwell SD, Moran L, Mohemmad QK, Anjum R, Wang Y, Narasimhan NI, Dalgarno D, Shakespeare WC, Miret JJ, Clackson T and Rivera VM. **Crizotinib-resistant mutants of EML4-ALK identified through an accelerated mutagenesis screen.** Chemical biology & drug design. 2011; 78(6):999-1005.

27. Lovly CM and Pao W. **Escaping ALK inhibition: mechanisms of and strategies to overcome resistance.** Science translational medicine. 2012; 4(120):120ps122.

28. Shaw AT and Engelman JA. **Ceritinib in ALK-rearranged non-small-cell lung cancer.** The New England journal of medicine. 2014; 370(26):2537-2539.

29. Seto T, Kiura K, Nishio M, Nakagawa K, Maemondo M, Inoue A, Hida T, Yamamoto N, Yoshioka H, Harada M, Ohe Y, Nogami N, Takeuchi K, Shimada T, Tanaka T and Tamura T. **CH5424802 (RO5424802) for patients with ALK-rearranged advanced non-small-cell lung cancer (AF-001JP study): a single-arm, open-label, phase 1-2 study.** The Lancet Oncology. 2013; 14(7):590-598.

30. Tanizaki J, Okamoto I, Okabe T, Sakai K, Tanaka K, Hayashi H, Kaneda H, Takezawa K, Kuwata K, Yamaguchi H, Hatashita E, Nishio K and Nakagawa K. **Activation of HER family signaling as a mechanism of acquired resistance to ALK inhibitors in EML4-ALK-positive non-small cell lung cancer.** Clinical cancer research : an official journal of the American Association for Cancer Research. 2012; 18(22):6219-6226.

31. Ji C, Zhang L, Cheng Y, Patel R, Wu H, Zhang Y, Wang M, Ji S, Belani CP, Yang JM and Ren X. **Induction of autophagy contributes to crizotinib resistance in ALK-positive lung cancer.** Cancer biology & therapy. 2014; 15(5):570-577.

국 문 초 록

폐암은 우리나라에서 상위 5대 암에 포함되어 높은 발병률을 가질 뿐 아니라 발병 후 전이가 빠르게 진행되어 치료가 쉽지 않아 사망률이 높은 암으로 여겨져 왔다. 그 중에서도 비소세포성 폐암(Non-small cell lung cancer; NSCLC)을 치료하기 위하여 2011년에 개발된 크리조티닙(crizotinib)은 ALK 유전자 변이 표현형(EML4-ALK)을 보이는 말기 비소세포성폐암 환자들에게 매우 높은 효과를 나타내었으나 빠른 시간 내에 약제내성을 보이며 이에 대한 새로운 치료방안이 연구의 중요한 실마리로 여겨지고 있다. 본 연구에서는 비소세포성 폐암 세포주인 SNU2292를 이용하여 crizotinib에 저항성을 가지는 세포주(SNU2292-CR)를 제작 후 이를 유발하는 기전을 밝히고 이를 표적으로 하는 적절한 항암요법에 관한 연구를 진행하였다. Crizotinib 저항성 세포주에서는 표피상피세포성장인자수용체(Epidermal growth factor receptor,

EGFR)이 활성화되어 있음을 확인할 수 있었으며 이를 유도하는 인자를 찾기 위하여 리간드(Ligand)들의 세포내의 DNA, RNA, protein양을 측정하였다. 주목할 만하게, 저항성세포주에서 EGFR 리간드들 중 TGF-α 유전자의 증폭(Amplification)을 확인하였으며, 분비(Secretion)또한 증가하였음 확인하였다. 이는 TGF-α 에 의한 EGFR 활성증가는 crizotinib 저항성을 야기시킴을 제시하는 결과이다. 그래서, 이를 저해하기 위하여 EGFR 저해제인 세툭시맵 (cetuximab)을 처리하였다. 크리조티닙과 세툭시맵을 병용처리하였을 시 저항성 세포주에서 단백질 발현 및 세포 성장이 저해되는 것을 확인할 수 있었으나, 그 하부 유전자들의 신호전달을 효과적으로는 저해하지 못하였음을 확인하였다. 또한 저항성 세포주는 EGFR에 의해 조절되는 Thymidylate synthase(TS) 유전자의 발현이 증가하였을을 확인하였고, 이는 저항성간의 상관관계가 있을 것이라 가설을 세운 후 실험을 진행하였다. 진행폐암(Advanced lung cancer)에 대한 치료제로 쓰이는 TS저해제인 페

메트랙스(pemetrxed)과 cetuximab을 병용처리시 crizotinib과 cetuximab의 병용처리시보다 세포성장 저해효과 및 그 하부 유전자들의 신호전달을 효과적저해함을 확인할 수가 있었다. .

결론적으로, 본 연구에서는 EGFR과 TS 유전자를 함께 저해할 경우 crizotinib 저항성 세포주에서 더욱 효과적인 항암효과를 나타낼 수 있음을 알 수 있었으며 향후 crizotinib 저항을 가지는 비소세포성폐암 환자게 효과적인 치료방안이 될 수 있음을 시사하였다.

주요어 : EML4-ALK, crizotnib 내성, 비소세포성폐암, TGF-α, EGFR, Cetuximab, pemetrxed.

학번: 2012-24143