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Acquired resistance mechanisms to capmatinib, a MET inhibitor in *MET*-amplified non-small cell lung cancer cells

MET 이 증폭된 비소세포폐암에서 capmatinib 에 대한 내성획득 관련 분자적 변화 연구

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ABSTRACT

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Purpose: Amplified mesenchymal-epithelial transition factor, MET, is a receptor tyrosine kinase (RTK) that has been considered a druggable target in non-small cell lung cancer (NSCLC). Although multiple MET tyrosine kinase inhibitors (TKIs) are being actively developed for MET-driven NSCLC, the mechanisms of acquired resistance to MET-TKIs have not been elucidated. Capmatinib (INC280, Novartis) is a highly potent and selective small molecule MET-TKI. To understand the mechanisms of resistance to MET-TKIs and establish therapeutic strategies, we developed an *in vitro* model using capmatinib-resistant cell lines (EBC-CR1, CR2, and CR3) derived from the *MET*-amplified NSCLC cell line EBC-1.

Methods: We established capmatinib-resistant NSCLC cell lines from the *MET*-amplified NSCLC cell line EBC-1 and identified alternative signaling pathways using 3'mRNA sequencing and human phospho-RTK arrays. Copy number alterations were evaluated by quantitative PCR and cell proliferation assay; activation of RTKs and downstream effectors were compared between the parental cell line EBC-1 and the EBC-CR1, -CR2, and -CR3 resistant cell lines.

Results: We found that epidermal growth factor (*EGFR*) mRNA expression and protein activation were increased in EBC-CR1–3 cells compared to EBC-1 cells. EBC-CR1 cells showed EGFR-dependent growth and sensitivity to

afatinib, an irreversible EGFR TKI. EBC-CR2 cells, which overexpressed the EGFR-MET heterodimer, responded dramatically to the combination of capmatinib and the phosphoinositide-3 kinase catalytic subunit α (PIK3CA) inhibitor afatinib. In addition, EBC-CR3 cells, which had activated EGFR along with amplified *PIK3CA,* were sensitive to the combination of afatinib and the PI3Kα inhibitor BYL719.

Conclusions: Our *in vitro* studies suggested that activation of EGFR signaling and/or genetic alteration of downstream effectors like PIK3CA were alternative resistance mechanisms used by capmatinib-resistant NSCLC cell lines. In addition, combined treatments with MET, EGFR, and PI3Kα inhibitors may be an effective therapeutic strategy in MET-TKI-resistant NSCLC patients.

Keywords: non-small cell lung cancer, acquired resistance, *MET* amplification, capmatinib

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INTRODUCTION

Receptor tyrosine kinases (RTKs) and their ligands are genetically altered and overexpressed in various human cancers and therefore, are considered therapeutic targets (1). The RTK mesenchymal-epithelial transition factor (MET) and its ligand, hepatocyte growth factor (HGF), are required for organ regeneration and tissue damage repair, which resemble the pathological process of tumor invasion (2). Accordingly, aberrant activation of HGF/MET signaling cascades including multiple downstream effector pathways such as signal transducer and activator of transcription 3 (STAT3), rat sarcoma (RAS)/mitogen-activated protein kinase (MAPK), and phosphoinositide-3-kinase (PI3K)/AKT, occurs in many types of cancer; receptor crosstalk with other RTKs also has been observed (3). *MET* can be inappropriately activated via mutations, amplification and/or overexpression, or by elevated HGF levels (4-6).

Lung cancer is the leading cause of cancer-related death worldwide. Genetic alteration of MET has been detected in non-small cell lung cancer (NSCLC) and MET amplification has been reported in epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI)-naïve NSCLC patients, with a prevalence of 0.4% to 21% (7-11). In NSCLC, amplification of *MET* results in constitutive kinase activity in the absence of its ligand; overexpression acts as an oncogenic driver and activator of downstream signaling pathways such as the PI3K/AKT pathway (5, 12).

MET presents an attractive therapeutic target for cancers including NSCLC and amplification of *MET* is an excellent predictive marker of sensitivity to MET-TKIs (6, 9, 13-17). Capmatinib (INC280) is a highly potent and selective small molecule inhibitor of MET. The selectivity of capmatinib is >10,000-fold for MET in human kinase assays (18). In addition,

capmatinib demonstrated potent inhibition of cell growth and MET-dependent survival signaling activity in MET-dependent cell lines and patient tumors (6, 19). Although a dramatic response to capmatinib in *MET*-amplified NSCLC (19) was observed, acquired resistance to capmatinib is inevitable. Therefore, *in vitro* NSCLC cell line models are useful to investigate the molecular mechanisms of resistance to capmatinib and establish strategies to overcome it.

In this study, we established *MET*-amplified NSCLC cell lines that showed acquired resistance to capmatinib to evaluate resistance mechanisms. We demonstrated that capmatinib-resistant NSCLC cells were dependent on the main survival signal of EGFR via ligand and receptor overexpression and receptor heterodimerization. Moreover, *PIK3CA* amplification was observed in one capmatinib-resistant cell line, which suggested an additional mechanism of resistance to afatinib via activation of the PI3K/AKT pathway. Despite acquired resistance, the combination of inhibitors of EGFR or PIK3CA dramatically suppressed cell proliferation and the downstream signaling in capmatinib-resistant NSCLC cell lines, which suggested that this combination could be an effective therapeutic strategy against MET-TKI-resistance in NSCLC patients.

MATERIALS AND METHODS

1. Cell lines and drugs

The EBC-1 cell line, which is an NSCLC cell line that harbors a *MET* amplification, was purchased from the JCRB Cell Bank (Osaka, Japan). Capmatinib-resistant EBC-1 cell lines (EBC-CR1, CR2 and CR3) were established via stepwise exposure to capmatinib at final concentrations of 1.5, 2.2, and 2.4 μmol/L, respectively. EBC-CR1, -CR2, and -CR3 cells were maintained in 1 μmol/L of capmatinib over 2 months; these are referred to as capmatinib-resistant cell lines. All cell lines were incubated in RPMI1640 medium (Gibco, Carlsbad, CA) with 10% FBS, 2 mmol/L L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco) in a 37˚C incubator. Capmatinib (MET inhibitor), crizotinib (ALK/MET inhibitor), afatinib (irreversible EGFR inhibitor), BYL719 (PI3Kα inhibitor), PD173074 (FGFR inhibitor), and BGJ398 (FGFR inhibitor) were purchased from Selleck Chemicals (Boston, MA). Capmatinib was prepared at 5 mmol/L and the other drugs were prepared as 10 mmol/L stock solutions in 100% dimethyl sulfoxide. Recombinant heparin-binding EGF-like growth factor (HBEGF) was purchased from Prospec (East Brunswick, NJ).

2. Cell viability assay

To determine the sensitivity of the cell lines to these inhibitors *in vitro*, cells were seeded into 96-well microplates at 5,000 cells/well and incubated in medium for 24 hrs prior to exposure to the drugs. The cells were incubated with serial dilutions of the inhibitors in a final volume of 200 μL/well for 72 hrs. After the 72-hr incubation, Ez-cytox (Dogen, Korea) was added to each well and the plates were incubated for 3 hours in a 37˚C incubator. The

optical density was measured at a test wavelength of 450 nm using an Eon™ Microplate Spectrophotometer (Biotech, Winooski, VT). The IC_{50} values were calculated by Sigma Plot 12 (Hulinks; Tokyo, Japan).

3. Western blot analysis

EBC-1 parental and EBC-CR1, -CR2, and -CR3 cell lines, grown to adequate densities in 6-well plates, were treated for 2 hrs with capmatinib alone, 24 hrs with the combination of capmatinib and afatinib, or 24 hrs with BYL719. Rabbit monoclonal antibodies (mAbs) to detect phospho-MET (Tyr1234/1235), phospho-EGFR (Tyr1068), phospho-STAT3 (Tyr705), phospho-AKT (Ser473), total MET, total EGFR, total AKT, total p44/42 MAPK, and GAPDH, and mouse mAb to detect total STAT3 were purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-phospho-p44/42 MAPK (Thr202/Tyr204) was purchased from Santa Cruz Biotechnology (Dallas, TX). Western blotting chemiluminescence reagents were purchased from GE Healthcare (Chicago, IL). All antibodies and reagents were used according to the manufacturers' instructions.

4. Co-immunoprecipitation

Cells were washed once with Modified Dulbecco's phosphate-buffered saline and lysed in buffer containing 0.025 M Tris, 0.15 M NaCl, 0.0001 M EDTA, 1% NP-40, and 5% glycerol (pH 7.4). Protein G/A agarose resin (Thermo Fisher Scientific, Waltham, MA) was incubated for 2 hrs with rabbit monoclonal anti-MET antibody (Cell Signaling Technologies), anti-EGFR antibody (Cell Signaling Technologies) or rabbit IgG control (Invitrogen, Carlsbad, CA) as a negative control and lysates were mixed with antibody-coupled resin (Cell Signaling Technologies) overnight at 4˚C. Co-

immunoprecipitation was performed using the Pierce Co-IP Kit (Thermo Fisher Scientific). Immunoprecipitates were washed, eluted, and separated by SDS-polyacrylamide gel electrophoresis. Western blot was performed as described above.

5. Human phospho-RTK array

Phospho-RTK was detected using the Human Phospho-RTK Array kit (R&D Systems, Minneapolis, MN). An LAS-3000 imaging system (Fuji Photo Film Co., Stanford, CT) was used for image analysis.

6. Genomic DNA analysis

Gene copy numbers were determined by quantitative PCR. Genomic DNA (gDNA) was extracted from cell lines using the Exgene™ Cell SV Kit (Geneall Biotechnology, Korea). Quantitative PCR was used to determine gene copy number as follows: gDNA was amplified with specific primers (Table 1) and SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA) using the GeneAmp® PCR System (Applied Biosystems); images were captured with the Gel Logic 200 Imaging System (Kodak, Rochester, NY). Gene copy numbers were extrapolated from an external standard curve generated by serial dilutions of human genomic DNA (Merck Millipore, Darmstadt, Germany). The PCR products were purified using the PCR Purification Kit (Invitrogen), and sequenced by bidirectional Sanger sequencing by sequencing service (Macrogen, Korea).

7. Quantitative reverse transcription PCR and 3'-mRNA sequencing

Total RNA was extracted from EBC-1 parental and resistant cell lines using the RNA Mini Kit (Invitrogen) and reverse transcribed using

Superscript III Reverse Transcriptase (Invitrogen). The StepOnePlus Real-Time PCR System (Applied Biosystems) was used for amplification of cDNA and qRT PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) with specific primers (Table 2). Relative gene expression was calculated using the ΔΔCt (Cycle threshold) method with β-actin as the reference gene. Sequencing of the 3'-mRNA was performed using Quantseq® technology (Lexogen, Vienna, Austria). RNA was extracted from EBC-1 and resistant cell lines as described above. Hierarchical clustering was analyzed on $\log_2$-fold changes in gene expression using the MultiExperiment Viewer (Dana Farber Cancer Institute, Boston, MA). Normalized read counts were calculated as $(\log_2)>6$, and genes with an adjusted $p<0.05$ were considered significantly differentially expressed.

Table 1. Primers used in quantitative PCR for analysis of genomic amplification

Gene	Primer	Sequence
MET	MET-F	5'- ATCAACATGGCTCTAGTTGTC -3'
	MET-R	5'- GGGAGAATATGCAGTGAACC -3'
EGFR	EGFR-F	5'- CAAGGCCATGGAATCTGTCA -3'
	EGFR-R	5'- CTGGAATGAGGTGGAGGAACA -3
PIK3CA	PIK3CA-F	5'- ATCTTTTCTCAATGATGCTTGGCT -3'
	PIK3CA-R	5'- CTAGGGTCTTTCGAATGTATG -3'
Line1	Line1-F	5'- AAAGCCGCTCAACTACATGG -3'
	Line1-R	5'- TGCTTTGAATGCGTCCCAGAG -3'

Table 2. Primers used in quantitative real-time PCR for analysis relative gene expression

Gene	Primer	Sequence
HBEGF	HBEGF-F	5'- GAAGACTTCCATCTAGTCACAAAGA -3'
	HBEGF-R	5'- GGGAGGCCCAATCCTAGA -3'
β-actin	bactin-F	5'- CAATGAGCTGCGTGTGGCT -3'
	bactin-R	5'- TAGCACAGCCTGGATAGCAA -3

RESULTS

Establishment of EBC-1 cells with acquired resistance to capmatinib

EBC-1 cells were derived from NSCLC cell lines harboring *MET* amplification and showing high sensitivity to MET-TKIs. To explore the molecular mechanisms of resistance to MET-TKIs, we used capmatinib, a MET inhibitor that is currently being studied in clinical trials. Using the EBC-1 cell line, we established three cell lines resistant to capmatinib (EBC-CR1, CR2, and CR3) by stepwise exposure to capmatinib concentrations from 10 nM to 2.5 μM. The EBC-CR3 cell line was derived from the EBC-CR1 cell line by continuous exposure to a higher concentration of capmatinib (Fig. 1A). First, we evaluated the sensitivity of the resistant cell lines to capmatinib by cell viability assay. The MET-TKIs were not toxic to the resistant cell lines (IC_{50}, >10 μmol/L on EBC-CR1–3 cells and 3.70±0.10 nmol/L on EBC-1 cells) (Fig. 2A). Next, we confirmed the decrease of MET gene copy number by qPCR in resistant cell lines, which was accompanied by downregulation of MET phosphorylation (Fig. 1B). These results suggested that capmatinib strongly affected not only kinase activity but also *MET* copy number, which indicated its potent efficacy against MET-dependent tumors. Also, there were no additional MET-activating mutations identified by Sanger sequencing (data not shown).

In resistant cell lines, complete inhibition of MET phosphorylation by capmatinib demonstrated that there were no MET mutations that prevented the binding of capmatinib. Although complete inhibition of MET kinase was observed, phosphorylation of AKT and ERK1/2 were not completely inhibited in the resistant cell lines by a single treatment with capmatinib (Fig. 2B).

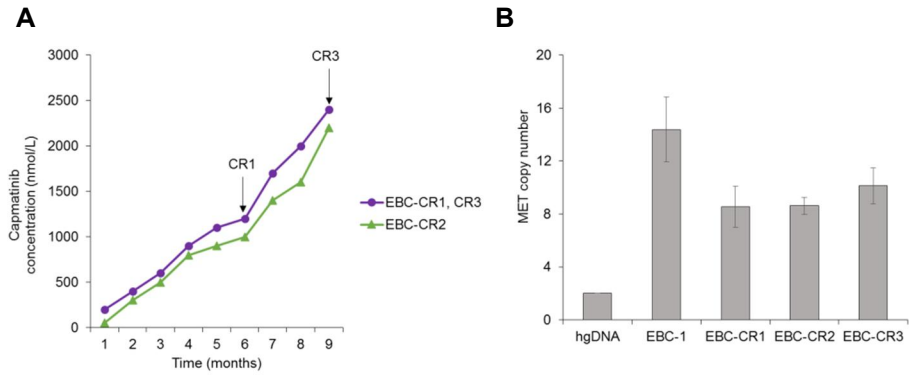


Figure 1. Establishment of capmatinib-resistant cells from EBC-1. A, EBC-1 cells were cultured in increasing concentrations of capmatinib to establish resistance. The resistant cell lines derived from EBC-1 were cultured in increasing concentrations of capmatinib from 10 nmol/L to 2.4 μmol/L and were maintained at 1 μmol/L over 2 months. The EBC-CR3 cell line was derived from EBC-CR1 cells by treatment with a stepwise higher concentration of capmatinib over 3 additional months; this cell line had different molecular characteristics compared to the EBC-CR1 cell line. **B,** The resistant cell lines showed significant *MET* copy number loss after long-term treatment with capmatinib ($p<0.05$). *MET* copy number was confirmed by qPCR. hgDNA: human genomic DNA

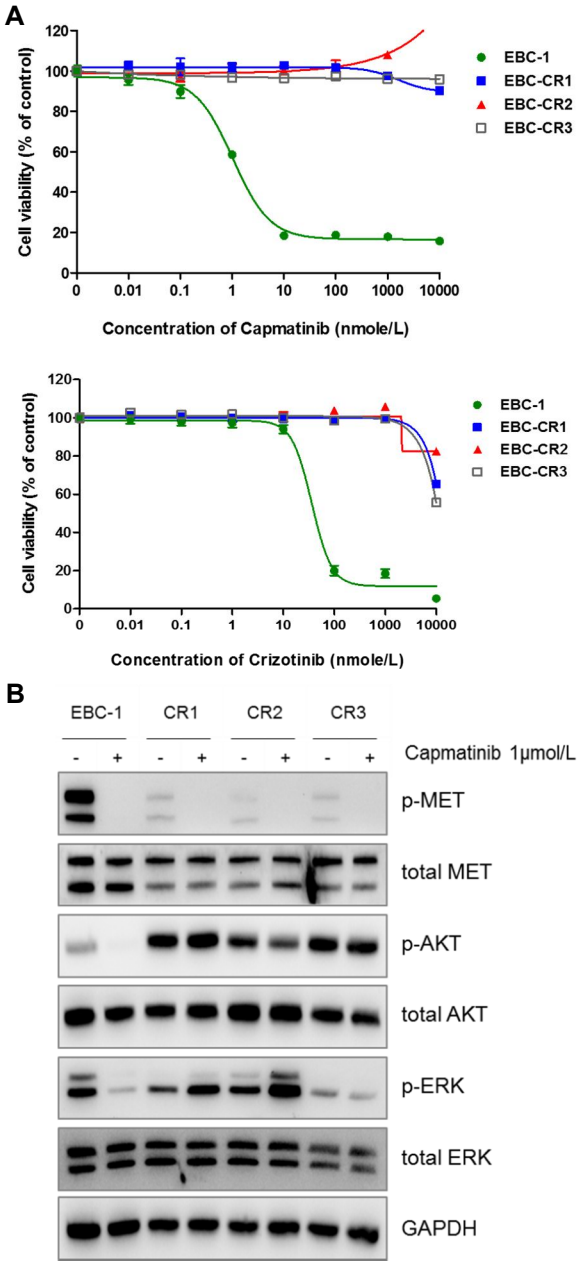


Figure 2. Confirmation of resistance to capmatinib. A, Capmatinib-resistant cell lines (EBC-CR1, EBC-CR2, and EBC-CR3) were derived from EBC-1, which is an NSCLC cell line that harbors *MET* amplification. Cell lines were treated with capmatinib and crizotinib for 72 hrs and growth inhibition was determined by cell viability assay (Ez-cytox). Tests were performed as three independent experiments.

The EBC-CR1, -CR2, and -CR3 cell lines showed resistance to capmatinib and cross-resistance to crizotinib. **B,** Capmatinib-resistant cells had persistent expression of phosphorylated ERK1/2 and AKT in the presence of capmatinib. EBC-1 and the resistant cell lines were treated with capmatinib at 1 μmol/L for 2 hrs then analyzed by Western blot. GAPDH was used as the loading control for the Western blot.

Acquired resistant mechanisms to capmatinib were associated with the EGFR kinase pathway

To identify alternative survival pathways of capmatinib resistance, EBC-1, EBC-CR1, CR2, and CR3 were screening for gene expression by 3'-mRNA sequencing. First, we analyzed expression of RTK-related genes by hierarchical clustering. RTK-related genes were stratified into several clusters and almost 20% of RTK-related genes were significantly upregulated in the resistant cell lines (Fig. 3). *MET* expression was slightly decreased and EGFR expression was increased in EBC-CR1, CR2, and CR3 compared to the parental cell line (Table 3). We then confirmed increased EGFR protein phosphorylation compared to multiple RTKs in the resistant cell lines, but observed no change in *EGFR* copy number (Fig. 4A and B). These results suggested that the EGFR signaling pathway might be an alternative pathway for MET kinase. Although EGFR activation was increased in all resistant cell lines, cell viability assay to afatinib (EGFR inhibitor) showed that only EBC-CR1 has sensitivity to afatinib alone (Fig. 5A). Interestingly, capmatinib inhibited EGFR kinase phosphorylation in EBC-1 cells in a dose-dependent manner. To determine the mechanism of EGFR pathway activation in the EBC-CR1 cell line, we tested the expression of multiple EGFR ligands (including epidermal growth factor (EGF), amphiregulin, epiregulin, transforming growth factor-α (TGFα), and HBEGF) and observed that *HBEGF* expression was elevated in EBC-CR1 cells (Fig 5B). To further explore the role of HBEGF in EGFR activation, we added exogenous HBEGF to the EBC-1 and EBC-CR1 cell lines and found that HBEGF was sufficient to rescue to MET inhibition in the MET-dependent NSCLC cell line, EBC-1 (Fig. 6A). In EBC-CR1, cultures with HBEGF were more resistant to EGFR inhibition than without ligand (Fig. 6B). Unlike the parental cell line, a single

treatment with afatinib effectively inhibited phosphorylation of EGFR, AKT, and ERK1/2 despite persistent MET kinase activation (Fig. 5C). As a result, EGFR activation was highly dependent on HBEGF as the main survival signal in EBC-CR1 cells.

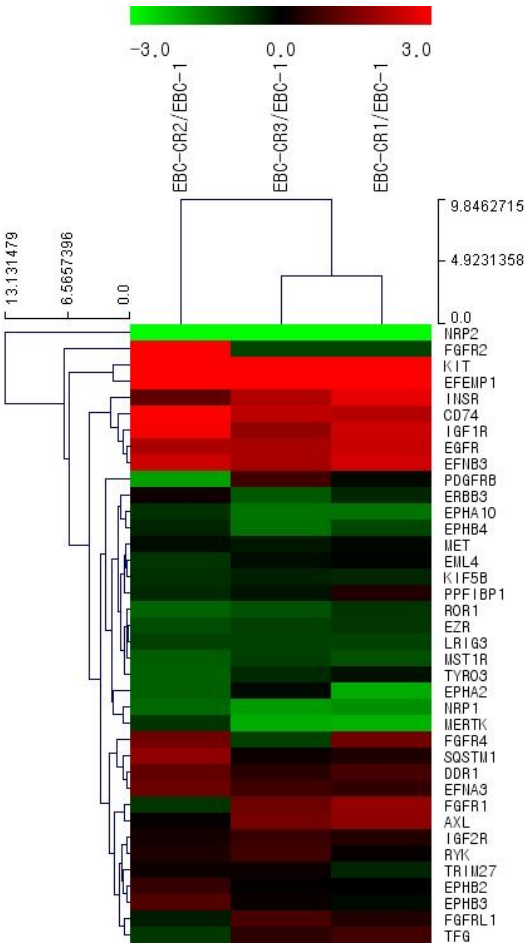


Figure 3. Hierarchical clustering analysis of the resistant cell lines revealed a cluster containing seven RTKs that was increased in all resistant cell lines. The values for clustering analysis fulfilled the requirements of normalized read counts (log_2)>6 and $p<0.05$. The fold changes in expression of these RTKs are indicated in Table 3. Each color represents relative gene expression with the highest expression as red, lowest expression as green, and median expression as black.

Table 3. Differential expression of genes encoding RTKs between EBC-1 and resistant cells

Gene	Fold change		
	EBC-CR1/EBC-1	**EBC-CR2/EBC-1**	**EBC-CR3/EBC-1**
KIT	24.250	73.500	28.250
EFEMP1	10.200	32.369	17.131
INSR	4.252	2.194	6.600
CD74	4.767	13.317	4.481
IGF1R	5.546	7.636	3.350
EGFR	4.001	4.160	5.189
MET	0.824	0.900	0.936

*Abbreviation: EFEMP1, EGF Containing Fibulin like Extracellular Matrix Protein1; INSR, Insulin Receptor; IGF1R, Insulin like Growth 1 Receptor

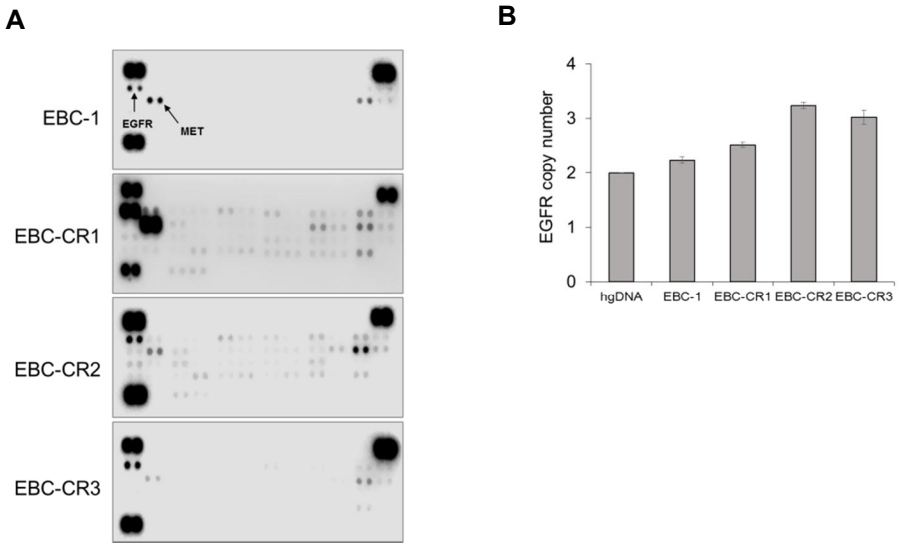
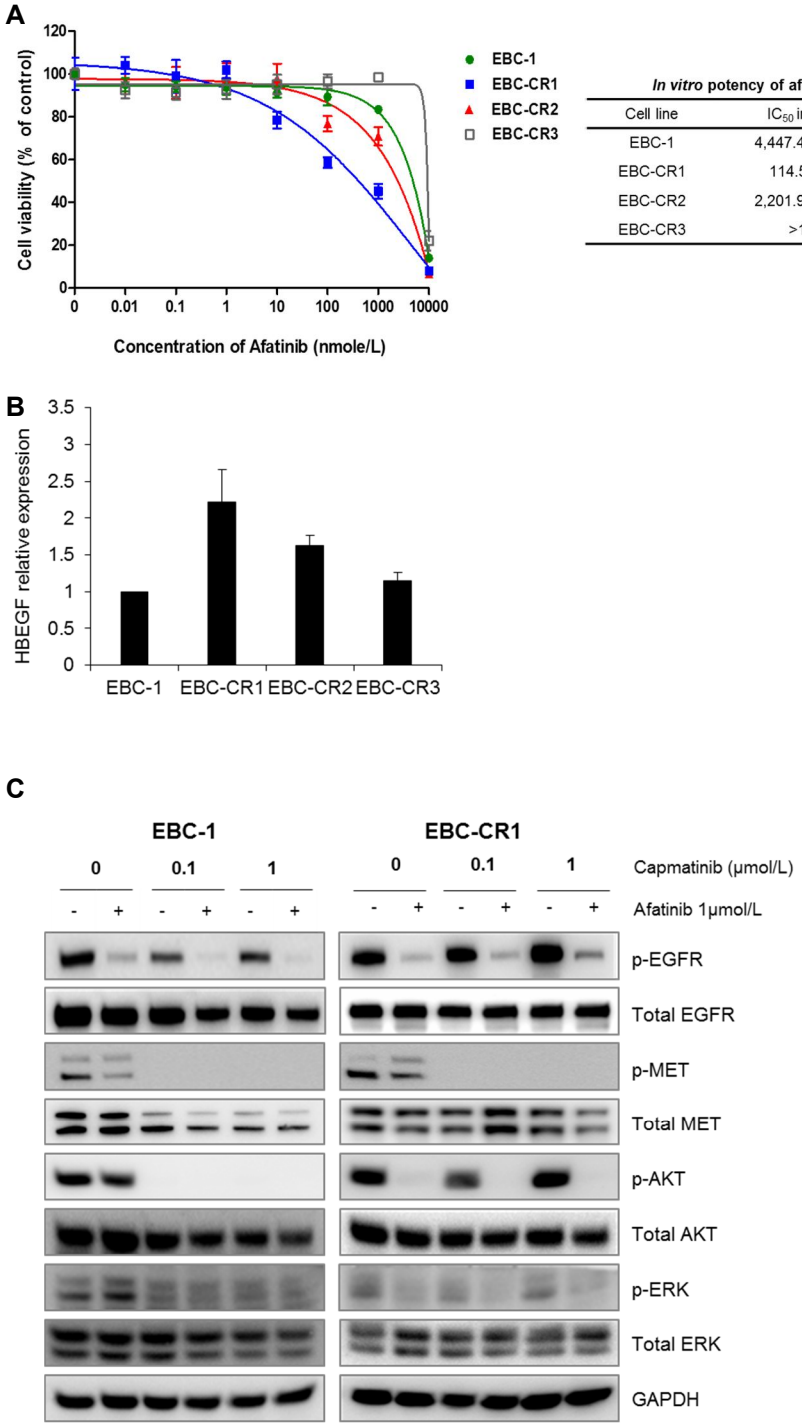


Figure 4. EGFR activation in capmatinib-resistant cells. A, EGFR phosphorylation was increased in all resistant cell lines, especially in EBC-CR1. Human Phospho-RTK arrays were used to compare the activation of multiple RTKs between parental and resistant cell lines. **B,** *EGFR* copy number was not altered in the resistant cell lines.



In vitro potency of afatinib

Cell line	IC_{50} in nmol/L
EBC-1	4,447.45±240.05
EBC-CR1	114.58±2.75
EBC-CR2	2,201.90±212.46
EBC-CR3	>1,000

Figure 5. Shift from MET to EGFR kinase pathway in EBC-CR1. A, EBC-1 and resistant cell lines were treated with afatinib for 72 hrs. The 50% inhibitory

concentrations (IC_{50}) were calculated using Sigma Plot 12.0; results are indicated as mean±SD. **B,** *HBEGF* expression was measured by quantitative reverse transcription PCR (RT-PCR) in three independent experiments. *HBEGF* expression was increased in the EBC-CR1 cell line compared to the parental and other capmatinib-resistant cell lines. **C,** For Western blot, EBC-1 and EBC-CR1 cells were treated with serial 10-fold dilutions ranging from 0.1 to 1μmol/L of capmatinib with or without afatinib at 1 μmol/L for 24 hrs. EBC-CR1 showed almost complete dependency on downstream signaling due to afatinib treatment with or without capmatinib. Phosphorylation of AKT and ERK1/2 was efficiently inhibited by capmatinib in EBC-1 cells.

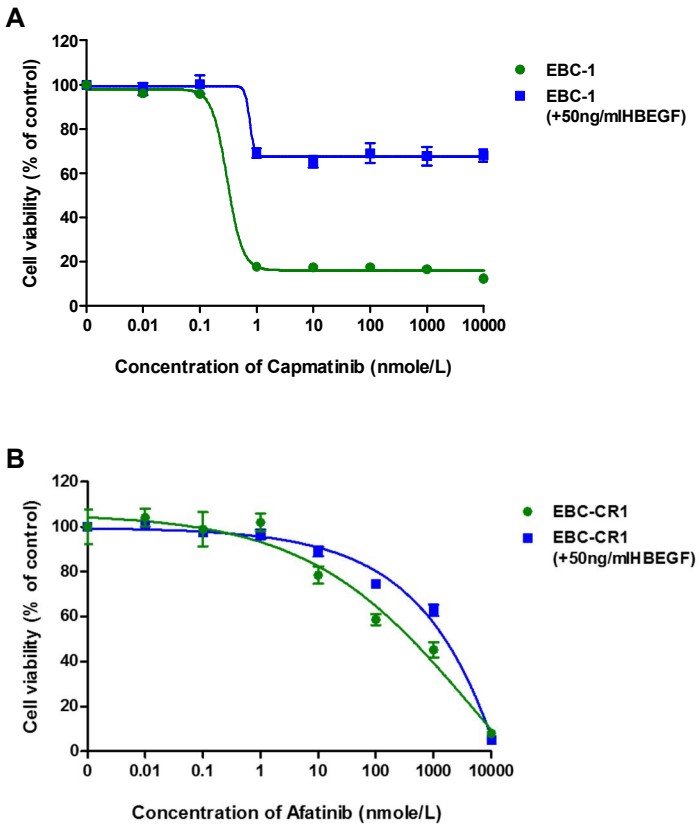


Figure 6. Ligand-dependent activation of EGFR in EBC-CR1. A, Addition of HBEGF in EBC-1 was sufficient to acquire resistance to capmatinib. EBC-1 cell treated with capmatinib ± HBEGF for 72hr. **B,** EBC-CR1 with HBEGF was more resistant to EGFR inhibition than without HBEGF. EBC-CR1 cell treated with afatinib ± HBEGF for 72hr.

Increased MET and EGFR heterodimerization caused acquired resistance to MET-TKI

Treatment of cells with capmatinib alone effectively inhibited phosphorylation of MET, AKT, and ERK1/2 in EBC-1 cells. As previously mentioned, phosphorylation of these signaling in EBC-CR1 was almost completely inhibited by treatment with afatinib alone; this was not observed in EBC-CR2 cells. EBC-CR2 cells had strongly increased fibroblast growth factor receptor 2 (*FGFR2*) expression and mildly increased *FGF1* expression compared to the other resistant cell lines according to the 3' mRNA sequencing results (Table 4). However, expression of the FGFR2-specific ligand *FGF7* was likely underestimated (data not shown). We hypothesized that because of its significant fold change, *FGFR2* overexpression could be a mechanism for capmatinib resistance. Kunii and colleagues showed that the FGFR inhibitor PD173074 effectively inhibited FGFR2 phosphorylation and proliferation in *FGFR2*-amplified cell lines and activated FGFR2, which resulted in indirect activation of EGFR (20). BGJ398, a pan-FGFR inhibitor, potently inhibited FGFR2 activation with a single-digit nanomolar IC_{50} and inhibited proliferation of an *FGFR2*-amplified cell line and in a xenograft tumor model (21). Although *FGFR2* expression was strongly increased, the EBC-CR2 cell line was completely resistant not only to the FGFR inhibitors BGJ398 and PD173074, but also to the combination of capmatinib and the FGFR inhibitors (Fig. 7A). Because the copy number of *FGFR2* was

unchanged according to the qPCR results (Fig. 7B), the increase in *FGFR2* expression occurred without amplification in EBC-CR2 cells.

In contrast to EBC-CR1 cells, in EBC-CR2 cells, the combined blockade of MET and EGFR kinase significantly abolished phosphorylation of the downstream signaling molecules, AKT and ERK1/2 (Fig. 8A). While activation of both MET and EGFR kinase was diminished in the parental cell line, EGFR phosphorylation was increased in EBC-CR2 cells by capmatinib treatment and MET phosphorylation was increased by afatinib. Intriguingly, EBC-CR2 displayed more MET/EGFR heterodimers than the parental cell line (Fig. 8B). To determine the efficacy of combined treatment with capmatinib and afatinib, we performed the cell viability assay to compare the effect of capmatinib, afatinib, and their combination in EBC-CR2 cells. We observed that the IC_{50} was lower in the cells treated with the combination compared to either compound alone, which suggested that capmatinib and afatinib synergistically inhibited cell proliferation (Fig. 8C). These results indicated that double blockade of MET and EGFR obstructed the MET-mediated EGFR survival signal caused by MET and EGFR heterodimerization. Accordingly, the MET/EGFR heterodimer is a much more dominant form of activated EGFR kinase, which is the main survival signal in EBC-CR2 cells.

Table 4. Differential expression of genes encoding FGFR2 and FGF ligand family in EBC-CR2

Gene	Relative expression
	EBC-CR2/EBC-1
FGFR2	86.286
FGF1	2.708
FGF2	1.374
FGF11	1.221
FGF12	1.361
FGF13	0.133

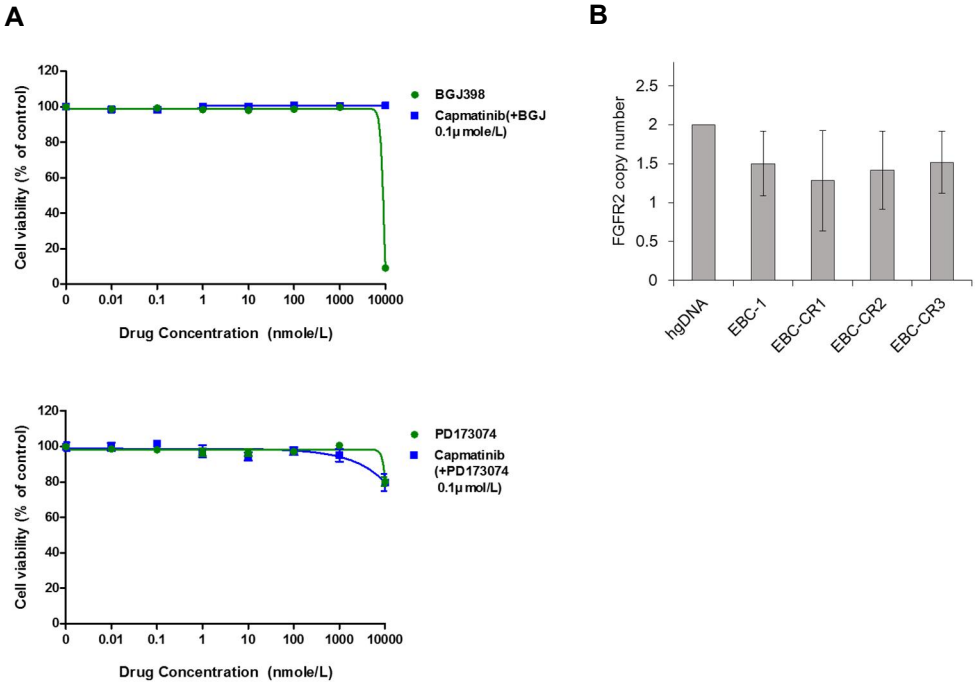


Figure 7. Confirmation of FGFR2 activity in EBC-CR2 cells. A, EBC-CR2 cells showed resistance to the FGFR2 inhibitors, BGJ398 and PD173074, after a single treatment combined with capmatinib. **B,** *FGFR2* copy number did not show any notable change in the resistant cell lines.

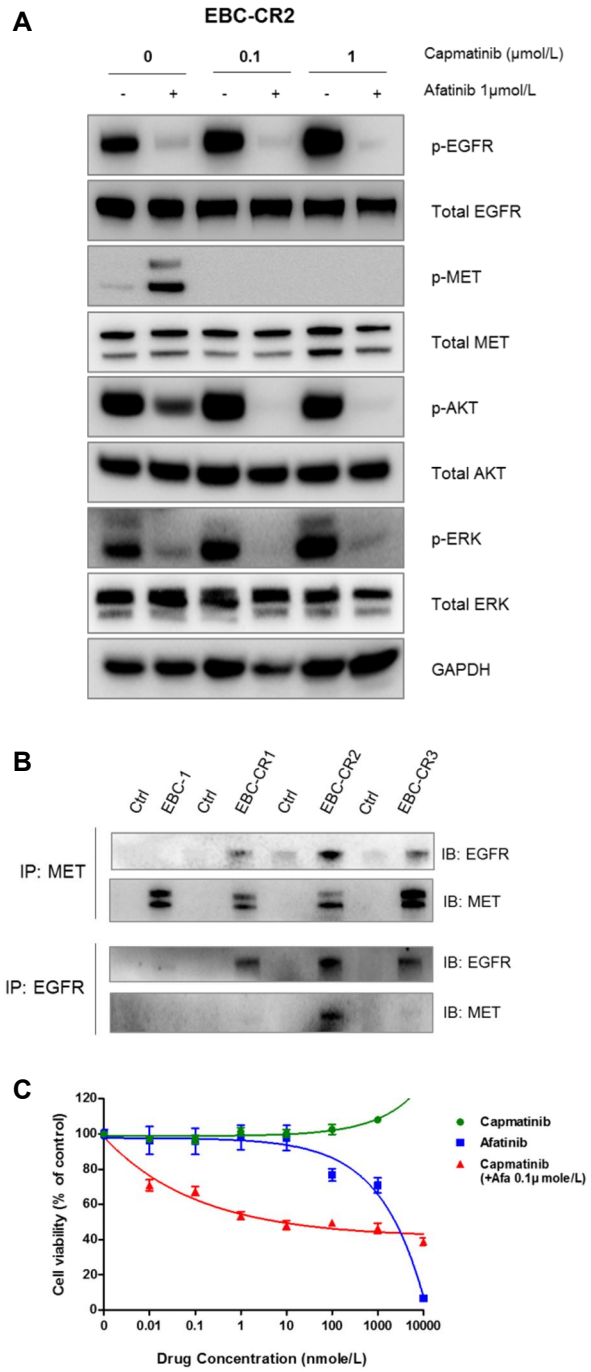


Figure 8. Combined treatment with capmatinib and afatinib effectively inhibited phosphorylation of downstream signaling and proliferation in EBC-CR2 cells. A, EBC-CR2 cells were treated with increasing concentrations of capmatinib with or

without afatinib at 1 μmol/L for 24 hrs. Capmatinib significantly increased EGFR phosphorylation in a dose-dependent manner. The combination of capmatinib with afatinib at 1 μmol/L effectively inhibited phosphorylation of AKT and ERK at a low concentration of capmatinib, 100 nmol/L. **B,** The heterodimerization of RTKs was analyzed by co-immunoprecipitation (co-IP). EBC-CR2 cells had more EGFR-MET heterodimers than the other resistant cell lines. **C,** EBC-CR2 cells treated with capmatinib, afatinib, and capmatinib with afatinib at 0.1 μmol/L, respectively, for 72 hrs. Treatment with the combination of capmatinib with afatinib synergistically inhibited EBC-CR2 cell proliferation.

Acquired resistance to EGFR-TKI beyond MET-TKI resistance

Despite continuous exposure of the EBC-CR3 cell line (derived from EBC-CR1 cells) to MET-TKI but not EGFR-TKI resulted in the loss of sensitivity to afatinib in the cell viability assay (Fig. 9A). According to the 3' mRNA sequencing results, DNA repair-related gene expression was significantly downregulated in EBC-CR3 cells compared to EBC-CR1 cells as shown in the gene ontology graph (Fig. 10A and Table 5). In Western blot, EBC-CR3 showed a complete block of EGFR or MET activation, but persistent AKT phosphorylation after afatinib treatment (Fig. 10B). We performed qRT PCR and Sanger sequencing to investigate the mechanism underlying EGFR-dependent resistance to afatinib. We found that *PIK3CA* expression was increased and gene copy number was amplified in CR3 cells, which was unlike what we observed in the other cell lines (Fig. 9B and 9C); however, we did not find any hot spot mutations in *PIK3CA* (data not shown). *PIK3CA* amplification was observed frequently and might play a pathogenic role in lung cancer, especially squamous cell carcinoma (22); thus, we hypothesized that *PIK3CA* amplification was an acquired resistance mechanism against afatinib. To confirm this hypothesis, we tested the efficacy of BYL719 (PI3Kα inhibitor) on *PIK3CA* expression in EBC-CR3 cells, because this molecular target of BYL719 had been reported previously (23). As a result, we showed afatinib resistance after a single treatment with BYL719; however, the cells remained sensitive to BYL719 in the presence of afatinib in the cell viability assay (Fig. 9A). In addition, the combination of BYL719 with afatinib effectively inhibited phosphorylation of AKT, despite the persistence of the MET signal (Fig. 9D), and inactivation of RAS/MAPK pathway (data not shown). These results demonstrated that wild type (wt) *PIK3CA* amplification could be an additional mechanism of resistance to EGFR-TKI in

MET-TKI-resistant cancers; however, the combined therapy effectively overcame the resistance to TKIs in a MET-independent manner in the presence of additional genetic alterations.

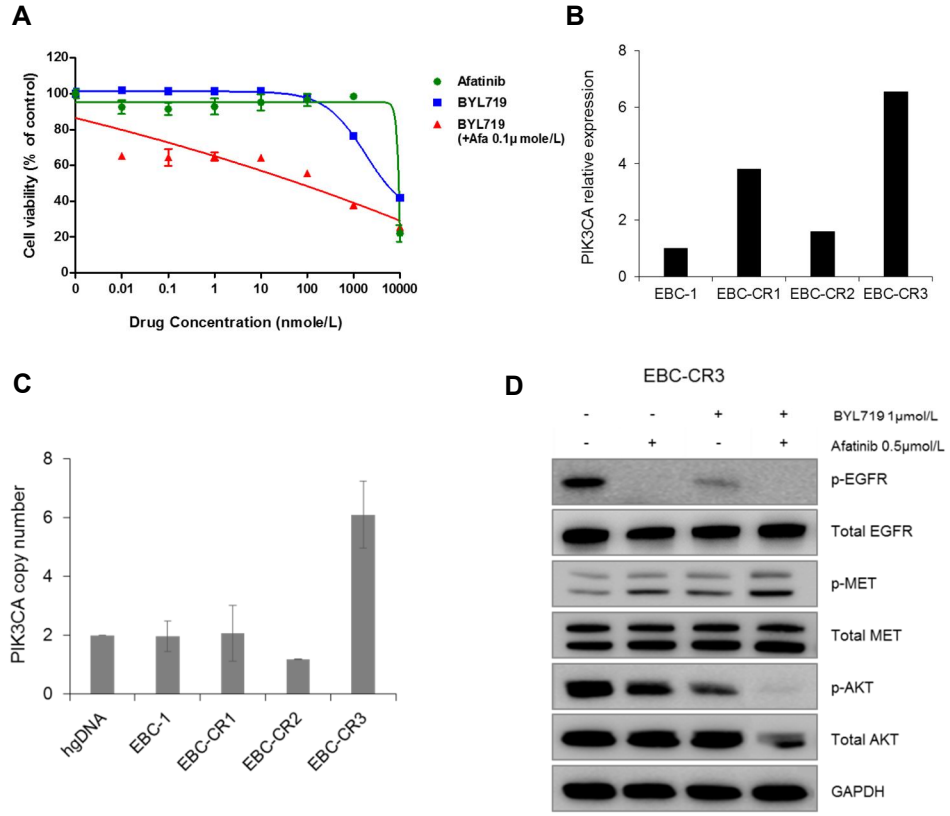


Figure 9. *PIK3CA* amplification in EBC-CR3 cells resulted in afatinib resistance. **A,** Treatment of EBC-CR3 cells with afatinib, BYL719, and BYL719 with afatinib at 0.1 μmol/L, respectively, for 72 hrs. BYL719 with afatinib synergistically inhibited proliferation of EBC-CR3 cells. **B,** The relative expression of *PIK3CA* in the cells lines was analyzed by 3' mRNA sequencing. *PIK3CA* was strongly expressed in EBC-CR3 cells. **C,** *PIK3CA* copy numbers in the cell lines were measured by qPCR. Compared to the other cell lines and the human genomic DNA control, *PIK3CA* was amplified in the EBC-CR3 cell line. **D,** Treatment of EBC-CR3 cells with afatinib 100 nmol/L, BYL719 1 μmol/L, or BYL719 with afatinib at 100 nmol/L, respectively, for 24 hrs. The combination of BYL719 with afatinib completely inhibited AKT phosphorylation.

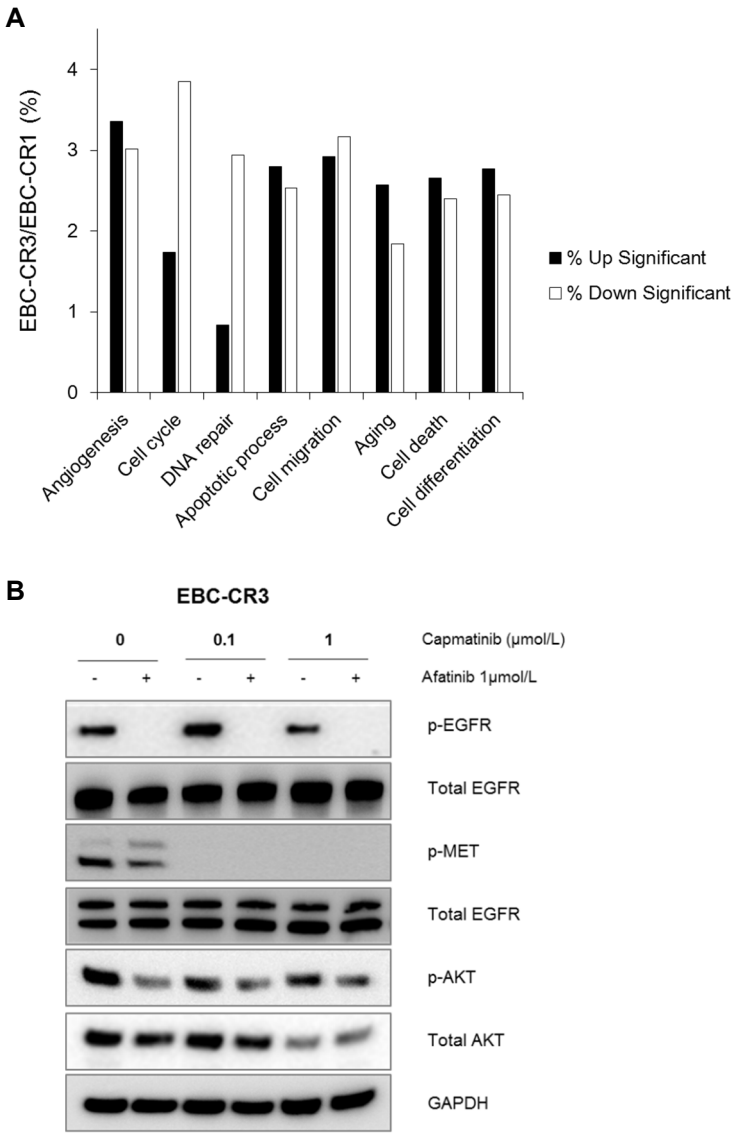


Figure 10. Comparison of molecular changes between EBC-CR3 and EBC-CR1 cells. A, DNA repair-related gene expression was downregulated in EBC-CR3 cells compared to EBC-CR1 cells. Gene expression results were analyzed by gene ontology. **B,** Phosphorylation of AKT was decreased in EBC-CR3 despite the presence of afatinib and/or capmatinib.

Table 5. Fold change of downregulated DNA repair gene expression on gene ontology analysis

Gene	Relative expression		
	EBC-CR1/EBC-1	EBC-CR2/EBC-1	EBC-CR3/EBC-1
ASTE1	1.124	0.587	0.497
DCLRE1A	0.928	0.329	0.419
DNA2	0.482	0.086	0.210
ESCO2	1.025	0.113	0.229
EXO1	1.055	0.500	0.500
INO80C	0.371	0.424	0.181
PCNA	0.879	0.360	0.425
PPP5C	1.216	0.622	0.437
RPS3	2.433	1.232	1.157
TP73	0.343	0.054	0.086
TRIP13	1.091	0.130	0.481
UBE2NL	1.721	0.318	0.604
XRCC3	0.633	0.284	0.302
UNG	0.771	0.235	0.329

*Abbreviation: ASTE1, Asteroid Homolog1; DCLRE1A, DNA Cross-Link Repair 1A; DNA2, DNA Replication Helicase/Nuclease2; ESCO2, Establishment Of Sister Chromatid Cohesion N-Acetyltransferase2; EXO1, Exonuclease1; INO80C, INO80 Complex Subunit C; PCNA, Proliferating Cell Nuclear Antigen; PPP5C, Protein Phosphatase 5 Catalytic Subunit; RPS3, Ribosomal Protein S3; TP73, Tumor Protein P73; TRIP13, Thyroid Hormone Receptor Interactor 13; UBE2NL, Ubiquitin Conjugating Enzyme E2 N Like; XRCC3, X-Ray Repair Cross Complementing 3; UNG, Uracil DNA Glycosylase

DISCUSSION

Previous clinical research reported *de novo MET* amplification in NSCLC patients. *MET* amplification and/or overexpression may be druggable targets for MET-TKIs on the basis of preclinical and clinical findings (24-26). Therefore, preclinical and clinical trials of MET-TKIs have been reported for several cancers. Capmatinib, which is currently in clinical trials, is a very selective and potent MET-specific agent for MET-dependent cancer (6, 14, 19). However, rapid development of acquired resistance to MET-TKIs has been a crucial limitation to efficacy. Nonetheless, only a few resistance mechanisms of MET-TKIs have been elucidated up until now. Therefore, it is important to identify the underlying mechanisms of MET-TKIs resistance through molecular studies and establish effective therapeutic strategies to manage acquired resistance. *In vitro* cancer cell lines can provide a valuable model system to investigate resistance mechanisms. In this report, we have used this *in vitro* strategy to identify molecular resistance mechanisms of capmatinib in *MET*-amplified NSCLC cell lines.

First, we established resistant cell lines derived from the EBC-1 NSCLC cell line by long-term drug exposure. To identify acquired resistance mechanisms to capmatinib in the setting of *MET*-amplified NSCLC cell lines, we performed cDNA and gDNA analysis. We observed increased *EGFR* expression without the presence of an activating mutation or amplification, as described above. These findings suggested that common resistance to capmatinib occurred via activation of signaling pathways outside of MET kinase, rather than via genetic alteration of specific genes. Alternative pathway activation has been reported as resistant mechanisms in TKI-resistant NSCLC (27), including NSCLC cells harboring *ALK* and *ROS1* translocation, or *EGFR* mutation (Table 6). Also, partial or complete engagement of the

EGFR signal was one resistance mechanism against capmatinib, which indicated a preferable alternative signaling pathway in MET-dependent cancer cells.

MET positively regulated the EGFR signaling pathway via multiple mechanisms; in particular, EGFR phosphorylation was decreased by the selective MET-TKI, capmatinib, in MET-amplified NSCLC cell lines (6). The EGFR signal has been reported as a preferable bypass mechanism for MET kinase, and the combination of EGFR-TKI with MET-TKI renders resistant cells sensitive, as described in several studies (28-30). One of our resistant cell lines (EBC-CR2) also was sensitive to the combined treatment. Interestingly, phosphorylation of EGFR in the EBC-CR2 cell line was increased by capmatinib in a dose-dependent manner, in contrast to the parental EBC-1 cell line. Therefore, we assumed that MET and EGFR kinase may be activated via heterodimer formation and the ability of the main cell signaling pathway to freely alternate between MET and EGFR. The EGFR pathway was mainly activated in the presence of MET-TKI and the MET pathway was activated in the presence of EGFR-TKI because of heterodimer interactions. EBC-CR2 cells displayed the most MET/EGFR heterodimerization compared to the parental and other resistant cell lines, as previously mentioned. In addition, effective suppression of cell proliferation in response to combined treatment supports combined therapy with MET and EGFR inhibitors as a potential therapeutic strategy for MET-TKI acquired resistance in cancer patients.

EGFR phosphorylation was inhibited by capmatinib in the parental EBC-1 cell line, but not in the EBC-CR1 cell line. This result suggested that the EGFR signal of EBC-CR1 was independent of the MET signal. The survival signal in EBC-CR1 cells had completely shifted from MET to EGFR, because a single treatment with afatinib effectively inhibited downstream

signaling and cell proliferation. Intriguingly, EBC-CR1 cells, which were completely EGFR-dependent, became resistant to afatinib via *PIK3CA* amplification by culture in continuous high concentrations of capmatinib. Genetic alteration of *PIK3CA* frequently co-occurs with other oncogenic alterations including MET. Concurrent *PIK3CA* mutation and the *MET* exon 14-skipping mutation, which results in constitutive activation of *MET*, result in primary resistance to MET-TKIs (31, 32). In the case of EBC-CR3 cells, *PIK3CA* amplification was an acquired resistance mechanism to EGFR-TKI beyond MET-TKI resistance, which was demonstrated by the independence of EBC-CR1 cells from the MET kinase signal.

Despite exposure to similar concentrations of capmatinib, EGFR/MET co-dependent EBC-CR2 cells consistently showed sensitivity to the combination of capmatinib with afatinib. Based on this observation, a possible explanation for resistance is that EGFR/MET heterodimerization increased the cells' genetic stability or required a higher threshold concentration to induce a genetic change; thus, a genetic alteration was not required. Anticancer drugs promote chromosomal instability by inducing fragile sites in chromosomes that lead to amplification events (33). In addition, EBC-CR3 cells had less DNA repair-related gene expression than EBC-CR1, which affected gene copy numbers. Capmatinib had less of an effect on the unconstrained survival signal change in response to RTK inhibitors in EBC-CR2 cells. In contrast to EBC- CR2 cells, EBC-CR1 cells relied on ligand-dependent activation, which resulted from high levels of expression of ligand and receptor. Therefore, a secondary event was required to increase the stability of the cells against potent MET inhibition, and shift the cells toward a MET-independent survival signal. Afatinib combined with BYL719 to block amplified *PIK3CA* effectively inhibited AKT signaling and proliferation in

EBC-CR3 cells because the amplified downstream effector, PIK3CA, was constitutively activated despite inhibition of RTKs.

Ligand-dependent EGFR activation was the mechanism of resistance to MET-TKI in EBC-CR1 cells. In contrast to EBC-CR1 cells, EBC-CR2 showed resistance to FGFR-TKIs despite receptor overexpression. The overexpression of FGFR2 and FGFR3 has been reported as an acquired resistance mechanism to EGFR-TKI via rapid transcriptional induction without gene amplification in NSCLC cell lines, and exogenous FGF2 or FGF7 rescued the proliferation of cells under EGFR-TKI treatment (34). Capmatinib may directly affect EGFR signaling resulting in *FGFR2* overexpression in EBC-CR2 because of the physical relationship between MET and EGFR. Based on the 3' mRNA sequencing results, expression of *FGF1,* but not *FGF2* or *FGF7,* was increased in EBC-CR2 cells. Considered together, these results suggested that receptors require specific ligands to act as a cell survival signal in EBC-CR1 cells in the absence of an activating mutation or copy number alteration.

In conclusion, our findings suggested that a single cell line can develop several types of EGFR-dependent mechanisms of resistance to MET-TKIs in *MET*-amplified NSCLC cell lines. One mechanism involved an increase in ligand and receptor expression. Indeed, we found increased EGFR and HBEGF expression or MET/EGFR heterodimer formation, which resulted in sensitivity of EBC-CR2 cells to combined therapy, as mechanisms to promote acquired resistance. The most interesting finding was that capmatinib treatment resulted in a shift from capmatinib resistance to afatinib resistance via *PIK3CA* amplification (Fig. 11). Because cancers become resistant to MET-TKI in clinical settings, it is important to understand TKI-resistance mechanisms in preclinical and clinical studies. Therefore, our results highlighted the relationship between MET and EGFR and additional altered

oncogenes such as *PIK3CA* in the context of therapeutic strategies involving kinase inhibitors.

Table 6. Acquired resistance mechanisms in TKI-resistant NSCLC cell lines

Cell lines			Drugs	Resistance mechanisms	References
Genetic alteration	**Parental cell line**	**Resistant cell line**			
***MET* amplification**	EBC-1	EBC-CR1 EBC-CR2 EBC-CR3	Capmatinib	EGFR ligand-dependent EGFR activation EGFR-MET heterodimer EGFR activation with PIK3CA amplification	(Unpublished data)
***ALK* translocation**	NCI-H3122	NCI-H3122CR1	Crizotinib	EGFR ligand-dependent EGFR activation	(35)
		NCI-H3122CH1	Alectinib	Alternative pathway activation	(Unpublished data)
		NCI-H3122LR1	Ceritinib	EGFR ligand-dependent EGFR activation	
		NCI-H3122CR1LR1	Crizotinib † Ceritinib	EGFR ligand-dependent EGFR activation	
		NCI-H3122CR1CH1	Crizotinib † Alectinib	Alternative pathway activation	
***ROS1* translocation**	HCC78	HCC78CR1 HCC78CR2 HCC78CR3	Crizotinib	ROS1 secondary mutation (L2155S) EGFR ligand-dependent EGFR activation	(36)
***EGFR* mutation**					
exon 19 del	PC-9	PC-9GR1 PC-9GR2	Gefitinib	Disappeared EGFR exon 19 del, MET activation, EMT	(37)
L858R, T790M	NCI-H1975	NCI-H1975AR	Osimertinib	EGFR ligand-dependent EGFR activation	(38)
exon 19 del	HCC4006	HCC4006AR1 HCC4006AR2		FGFR ligand-dependent FGFR activation	

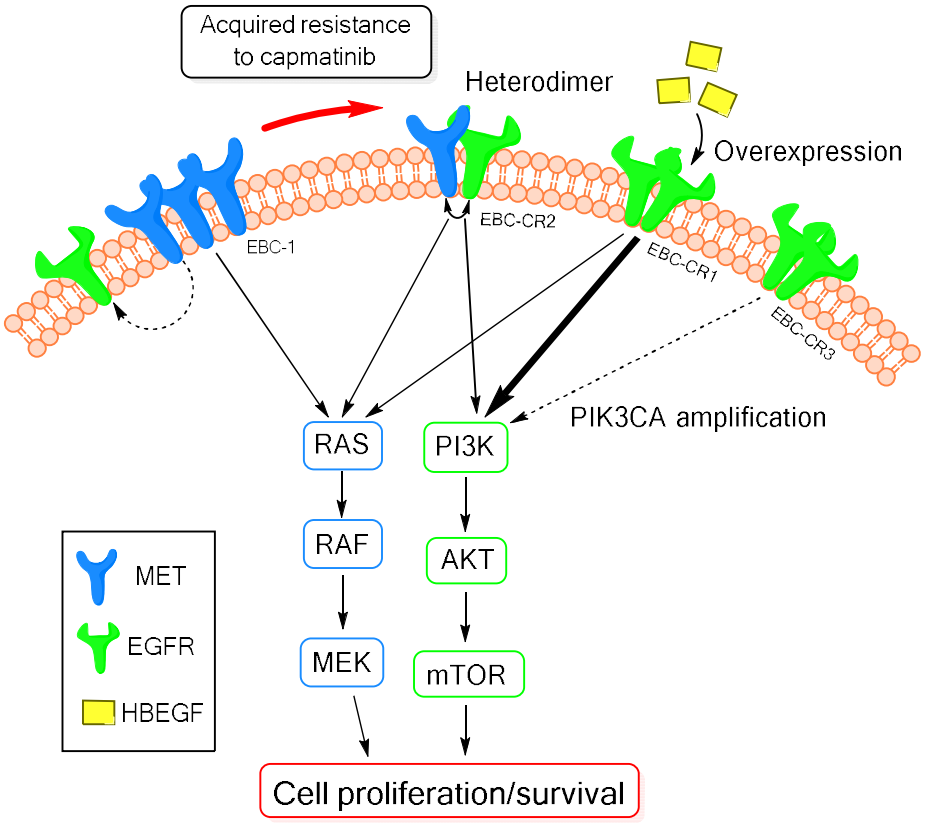


Figure 11. Schematic model of resistance mechanisms to capmatinib in *MET*-amplified NSCLC cell lines. In a drug-sensitive cell line harboring *MET* amplification, cell proliferation and survival signals are highly dependent on constitutively activated MET kinase without ligand binding and/or homodimerization. In addition, MET positively regulates the EGFR signal pathway via multiple mechanisms. In contrast, the MET pathway cannot act as a survival signal and alternative pathways must be activated in capmatinib-resistant cells. We confirmed three different mechanisms of resistance to capmatinib in MET-dependent NSCLC cell lines.

1: Heterodimerization between MET and EGFR, 2: Increased EGFR and HBEGF expression, 3: *PIK3CA* amplification via activated EGFR-dependent PIK3CA stimulation, resulting in afatinib resistance. Each mechanism has different molecular characteristics and therapeutic strategies.

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국문초록

연구 목적: *MET* 증폭은 비소세포성 폐암에서 MET 저해제의 효과적인 표적으로 여겨져 왔다. 또한 다양한 MET 저해제가 활발히 개발되고 있음에도 불구하고, 내성획득기전은 명확히 밝혀진 바가 많지 않다. Capmatinib (INC280) 은 강력하고 선택적으로 MET kinase 를 억제하는 MET 저해제 중 하나로, 본 연구에서는 capmatinib 을 사용하여 MET 이 증폭된 비소세포성 폐암 세포주인 EBC-1 를 가지고 내성을 보이는 세포주 3 가지를 만들어 capmatinib 에 대한 내성 기전과 이를 극복할 수 있는 치료적 전략을 세우고자 하였다.

연구 방법: 먼저 세 가지 capmatinib 내성 세포주로부터 3'mRNA sequencing 과 human phospho-RTK array 를 통해 MET 을 대체하여 활성화되는 신호경로를 확인하였다. 또한 유전자의 copy number 는 quantitative PCR (qPCR)을 통해 확인하였다. 그리고 약제에 대한 세포증식, receptor tyrosine kinase (RTK) 및 하위 신호 전달계의 활성화 정도를 EBC-1 과 세 가지 내성 세포주에서 비교하였다.

연구 결과: Crizotinib 에 획득 내성을 보이는 세 가지 세포주 모두 EBC-1 에 비해 EGFR 의 mRNA 발현 정도 및 EGFR 단백질 활성화가 증가되어 있음을 확인할 수 있었다. 또한, EBC-CR1 의 경우 EGFR 에 의존적인 신호 전달이 일어나며 EGFR TKI 인 afatinib 에 세포증식 억제효과가 있었으며, EBC-CR2 는 EGFR 과 MET 사이의 이질이합체를 형성하며 이러한 경우 capmatinib 과 afatinib 의 병용이 효과적으로 작용하였다. 마지막으로

EBC-CR3 의 경우 EGFR 의 활성과 더불어 *PIK3CA* 증폭이 일어나 PI3Kα 저해제인 BYL719 와 afatinib 의 병용이 효과가 있음을 검증하였다.

결론: 이번 *in vitro* 실험 결과는 비소세포성 폐암 세포주에서 capmatinib 에 대한 획득내성에는 EGFR 신호와 더불어 하위 신호전달계인 *PIK3CA* 와 같은 유전자의 증폭에 의한 대체 신호경로의 활성화가 원인이 됨을 시사하고 있다. 또한, 이러한 MET, EGFR, PIK3CA 저해제의 병용은 MET 저해제 내성 환자들에게 치료적 전략으로 활용할 수 있을 것으로 생각된다.

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주요어 : 비소세포폐암, 획득 내성, *MET* 증폭, capmatinib
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