

MEK inhibitors against *MET*-amplified non-small cell lung cancer

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Abstract. Several receptor tyrosine kinases (RTKs) including EGFR, ALK, and MET have been identified as therapeutic targets in non-small cell lung cancer (NSCLC). Among the downstream pathways of RTKs, the MAPK pathway is particularly important for cancer cell proliferation, differentiation, and survival. In this study, the effects of MEK inhibitors (trametinib and PD0325901) in several NSCLC cell lines with driver gene alterations, especially RTK genes, were tested *in vitro* using an MTT assay, and a wide range of sensitivities was found. In particular, all the *EGFR*-mutated cell lines were resistant to MEK inhibitors, whereas all the *MET*-amplified cell lines were sensitive. A bioinformatics technique and western blot analyses showed that the PI3K/AKT pathway is more activated in *EGFR*-mutated NSCLC than in *MET*-amplified NSCLC, and a PI3K inhibitor enhanced the sensitivity to trametinib in the *EGFR*-mutated cell lines, suggesting that this pathway is associated with resistance to MEK inhibitors. Although the HCC827 cell line (*EGFR* mutation) was resistant to MEK inhibitors, the HCC827CNXR cell line, whose driver gene shifts from *EGFR* to *MET*, exhibited enhanced sensitivity to MEK inhibitors, indicating the biological importance of the MAPK pathway for *MET*-amplified NSCLC. Furthermore, a synergistic effect of crizotinib (a MET inhibitor) and trametinib was observed in *MET*-amplified NSCLC cell lines. Our findings indicate that the MAPK pathway is biologically important for *MET*-amplified NSCLC and strongly encourage

the development of combination therapy with a MET inhibitor and a MEK inhibitor against *MET*-amplified NSCLC.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, and ~90% of lung cancers are classified as non-small-cell lung cancer (NSCLC) (1). Although the prognosis of advanced NSCLC is very poor, the identification of epidermal growth factor receptor gene mutations (*EGFR* mutations) as oncogenic driver mutations in a subset of patients with NSCLC, coupled with the development of EGFR tyrosine kinase inhibitors (EGFR-TKIs), has opened the door to a new era in the treatment of this disease (2-7). Receptor tyrosine kinases (RTKs), including EGFR, have been shown to act not only as key regulators of normal cellular processes, but also to play a critical role in the development and progression of many cancers, and several RTKs including ALK, ROS1, RET, and MET have also been identified as therapeutic targets in NSCLC (8-10).

RTKs generally undergo autophosphorylation, which in turn promotes the recruitment of downstream effector proteins leading to the activation of multiple signal cascades, including the mitogen-activated protein kinase (MAPK), PI3K/AKT, and STAT pathways (11,12). Among these pathways, the MAPK pathway is particularly important for cancer cell proliferation, differentiation and survival (13,14). The three-tiered kinase cascade consisting of RAF, mitogen-activated protein kinase kinase (MEK), and extracellular signal-regulated kinase (ERK) is frequently dysregulated in many malignancies including NSCLC (13). In this study, we tested the effects of MEK inhibitors (trametinib and PD0325901) in several NSCLC cell lines with driver gene alterations, especially RTK genes, *in vitro* and found a wide range of sensitivities. Among them, MEK inhibitors were effective against all *MET*-amplified cell lines but were not effective against any *EGFR*-mutated NSCLC cell line. Next, the mechanism and synergistic effect of a MET inhibitor and a MEK inhibitor against *MET*-amplified NSCLC cell lines were also investigated.

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Materials and methods

Cell cultures and reagents. The A549 cell line (*KRAS* mutation) was maintained in DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Gibco BRL, Grand Island, NY, USA) in a humidified atmosphere of 5% CO₂ at 37°C. The H358 (*KRAS* mutation), H1299 (*NRAS* mutation), PC-9, HCC827, Ma-1, 11_18, PC-9/ZD, H1975 (*EGFR* mutation), EBC-1, H1993 (*MET* amplification), H2228, and H3122 (*ALK* fusion) cell lines were maintained in RPMI medium (Sigma-Aldrich) supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ at 37°C. The HCC827CNXR cell line whose driver gene shifts from an *EGFR* mutation to a *MET* amplification (oncogene swap) was established as described previously and was maintained in RPMI medium supplemented with 10% FBS (15). Trametinib, PD0325901 (MEK inhibitors), LY294002 (a PI3K inhibitor), and crizotinib (a *MET* inhibitor) were purchased from Selleck Chemicals (Houston, TX, USA).

Growth inhibition assay in vitro. The growth-inhibitory effects of drugs were examined using a 3,4,5-dimethyl-2H-tetrazolium bromide assay (MTT; Sigma-Aldrich) (16). The experiment was performed in triplicate.

Bioinformatics technique. The mutational profiles of known driver oncogenes such as *KRAS* mutation, *EGFR* mutation, *BRAF* mutation, *ALK*, *RET* or *ROS1* fusion, *MAP2K1*, *NRAS* or *HRAS* mutation, *MET* exon 14 skipping mutation, *MET* amplification, *ERBB2* mutation, *ERBB2* amplification, *RIT1* mutation, and *NF1* loss, for 230 lung adenocarcinomas were based on the previously published report (17) and the gene expression profiles of these samples were extracted from the Cancer Genome Atlas (TCGA) data portal (<https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp>). The average expression profile for the 12 mutational subclasses (17) was calculated and used for further analysis. In total, 2,320 genes with a median gene expression (log₂ value) of >7.0- and a 4-fold change among the 12 subclasses were extracted and used for clustering analysis. Cluster 3.0 was used for hierarchical clustering analysis, and JAVA TreeView was used for display. For the pathway analyses, genes involved in each specific pathway were extracted from the REACTOME database (<http://www.reactome.org/>). The Z-scores for each gene in the 12 subclasses were calculated and summarized for each subclass. A positive value indicated that the genes in the pathway were relatively activated.

Antibodies. Rabbit antibodies specific for EGFR, phospho-EGFR, phospho-MET, AKT, phospho-AKT, ERK1/2, phospho-ERK1/2, poly (ADP-ribose) polymerase (PARP), caspase-3, cleaved PARP, cleaved caspase-3, and β-actin, and a mouse antibody specific for MET were obtained from Cell Signaling (Beverly, MA, USA).

Western blot analysis. A western blot analysis was performed as described previously (16). Briefly, subconfluent cells were washed with cold phosphate-buffered saline (PBS) and harvested with lysis A buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.0), 5 mM EDTA, 50 mM sodium chlo-

ride, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and a protease inhibitor mix, Complete™ (Roche Diagnostics). Whole-cell lysates were separated using SDS-PAGE and were blotted onto a polyvinylidene fluoride membrane. After blocking with 3% bovine serum albumin in a TBS buffer (pH 8.0) with 0.1% Tween-20, the membrane was probed with the primary antibody. After rinsing twice with TBS buffer, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody and washed, followed by visualization using an ECL detection system and LAS-4000 (GE Healthcare, Buckinghamshire, UK). When the phosphorylation and apoptosis were examined after the drug exposure, the samples were collected 3 and 24 h after the exposure, respectively.

Statistical analysis. Continuous variables were analyzed using the Student t-test, and the results were expressed as the average and standard deviation (SD). The statistical analyses were two-tailed and were performed using Microsoft Excel (Microsoft, Redmond, WA, USA). A P-value of <0.05 was considered statistically significant.

Results

Sensitivity to MEK inhibitors in several NSCLC cell lines with driver gene alterations. To investigate the effects of MEK inhibitors, we used two MEK inhibitors (trametinib and PD0325901), and the growth inhibitory assay was performed using an MTT assay. The inhibitory curves for trametinib in the *EGFR*-mutated or *MET*-amplified NSCLC cell lines are shown in Fig. 1A, and the 50% inhibitory concentrations (IC₅₀) are summarized in Fig. 1B and Table I. Although the MAPK pathway is the same downstream pathway associated with cellular growth and survival among *RAS*-mutated, *EGFR*-mutated, *ALK*-fused, and *MET*-amplified NSCLC cell lines, a wide range of sensitivities to MEK inhibitors was observed (Fig. 1B and Table I). Among them, all the *EGFR*-mutated cell lines were resistant to MEK inhibitors (IC₅₀ ≥ 1 μM), whereas all the *MET*-amplified cell lines were sensitive (IC₅₀ ~0.1 μM) (Fig. 1 and Table I).

PI3K/AKT pathway is more activated in *EGFR*-mutated NSCLC than in *MET*-amplified NSCLC. To investigate the mechanism responsible for the difference in sensitivities, bioinformatics techniques were used based on the TCGA dataset for lung adenocarcinomas. Overall, 230 lung adenocarcinomas were clustered into 12 subclasses based on the mutational profiles of known driver oncogenes (17). The average expression profile for each subclass was calculated and used for further analysis. The average gene expression profiles of samples with *MET* amplification (*MET*-amp subclass) were clustered next to those of samples with mutations in *MAP2K1*, *NRAS* and *HRAS* (*MAP2K1* subclass). These two expression profiles were clustered in a different branch from the other types of expression profiles, suggesting their similarity compared with the other profiles. In addition, the expression profile of the *EGFR*-mutated samples (*EGFR* subclass) was clustered in a notably different branch from that of the *MET*-amp subclass (Fig. 2A). These findings suggest that the expression profile of the *MET*-amp subclass is similar to that

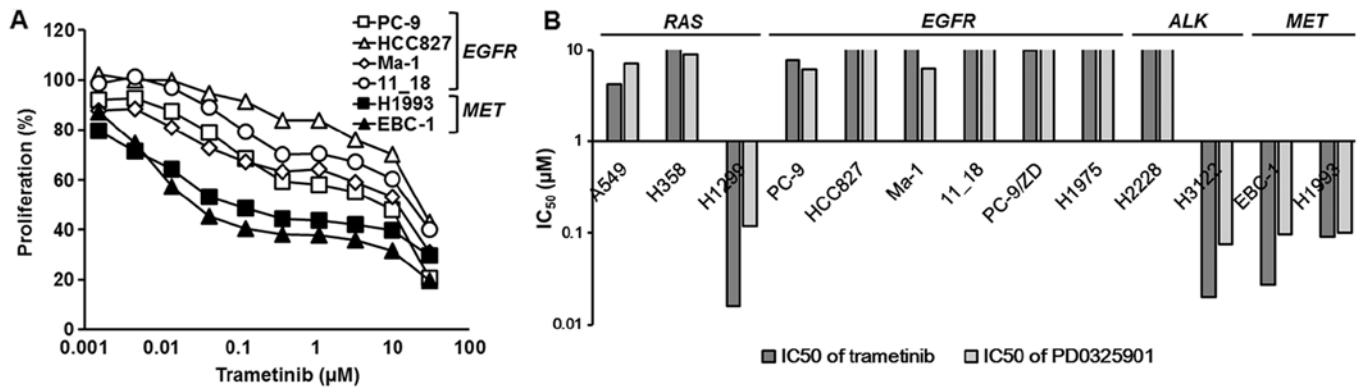


Figure 1. Sensitivities to MEK inhibitors (trametinib or PD0325901) in several NSCLC cell lines with driver gene alterations. (A) Growth inhibitory curves for trametinib in *EGFR*-mutated or *MET*-amplified NSCLC cell lines. All the *EGFR*-mutated cell lines were resistant to trametinib, whereas all the *MET*-amplified cell lines were sensitive. (B) The 50% inhibitory concentrations (IC_{50}) of MEK inhibitors (trametinib or PD0325901). A wide range of IC_{50} values was observed among the NSCLC cell lines.

Table I. The 50% inhibitory concentrations (IC_{50}) of MEK inhibitors in non-small cell lung cancer cell lines with driver gene alterations.

Driver gene	Cell line	Driver gene alterations	IC_{50}	
			Trametinib (μ M)	PD0325901 (μ M)
<i>RAS</i>	A549	<i>KRAS</i> G12S	4.17	7.23
	H358	<i>KRAS</i> G12C	11.3	8.90
	H1299	<i>NRAS</i> Q61K	0.016	0.12
<i>EGFR</i>	PC-9	<i>EGFR</i> exon 19 deletion	7.82	6.02
	HCC827	<i>EGFR</i> exon 19 deletion	22.7	>30
	Ma-1	<i>EGFR</i> exon 19 deletion	11.7	6.24
	11_18	<i>EGFR</i> exon 21 L858R	17.6	16.5
	PC-9/ZD	<i>EGFR</i> exon 19 deletion and exon 20 T790M	10.0	15.5
	H1975	<i>EGFR</i> exon 21 L858R and exon 20 T790M	12.4	25.6
<i>ALK</i>	H2228	<i>EML4-ALK</i>	>30	>30
	H3122	<i>EML4-ALK</i>	0.020	0.076
<i>MET</i>	EBC-1	<i>MET</i> amplification	0.027	0.097
	H1993	<i>MET</i> amplification	0.092	0.10
	HCC827CNXR	<i>MET</i> amplification	0.14	0.55

of the MAP2K1 subclass and notably different from that of the *EGFR* subclass. Next, the PI3K pathway was analyzed, since several reports have shown the activation of the PI3K/AKT pathway in *EGFR*-mutated NSCLC (18,19). Genes involved in the 'PI3K-cascade' were extracted from the REACTOME database. The Z-scores for each gene in the 12 subclasses were calculated and were summarized for each subclass. A positive value indicated that the genes in this pathway were relatively activated. As shown in Fig. 2A, the MAP2K1 subclass exhibited a particularly negative value, suggesting that the genes involved in the PI3K/AKT pathway play a less important role in this cluster. The *MET*-amp subclass, which was similar to the MAP2K1 subclass, also had a negative value, whereas the *EGFR* subclass had a positive value. These findings indicate

that the PI3K/AKT pathway is activated in *EGFR*-mutated NSCLC, compared with *MET*-amplified NSCLC. The higher expression of phospho-AKT in the *EGFR*-mutated NSCLC cell lines than that in the *MET*-amplified NSCLC cell lines was observed in western blot analyses, indicating the activated PI3K/AKT pathway in *EGFR*-mutated NSCLC (Fig. 2B). Furthermore, a western blot analysis also showed that the A549 cell line (*KRAS* mutation; *KRAS* subclass) had a significantly higher expression of phospho-AKT, compared with the H1299 cell line (*NRAS* mutation; MAP2K1 subclass) (Fig. 2B). These findings are consistent with our database analyses revealing that the *KRAS* subclass had a positive value and the MAP2K1 subclass had a negative value (Fig. 2A). In addition, the A549 cell line was resistant to MEK inhibitors, while the H1299 cell

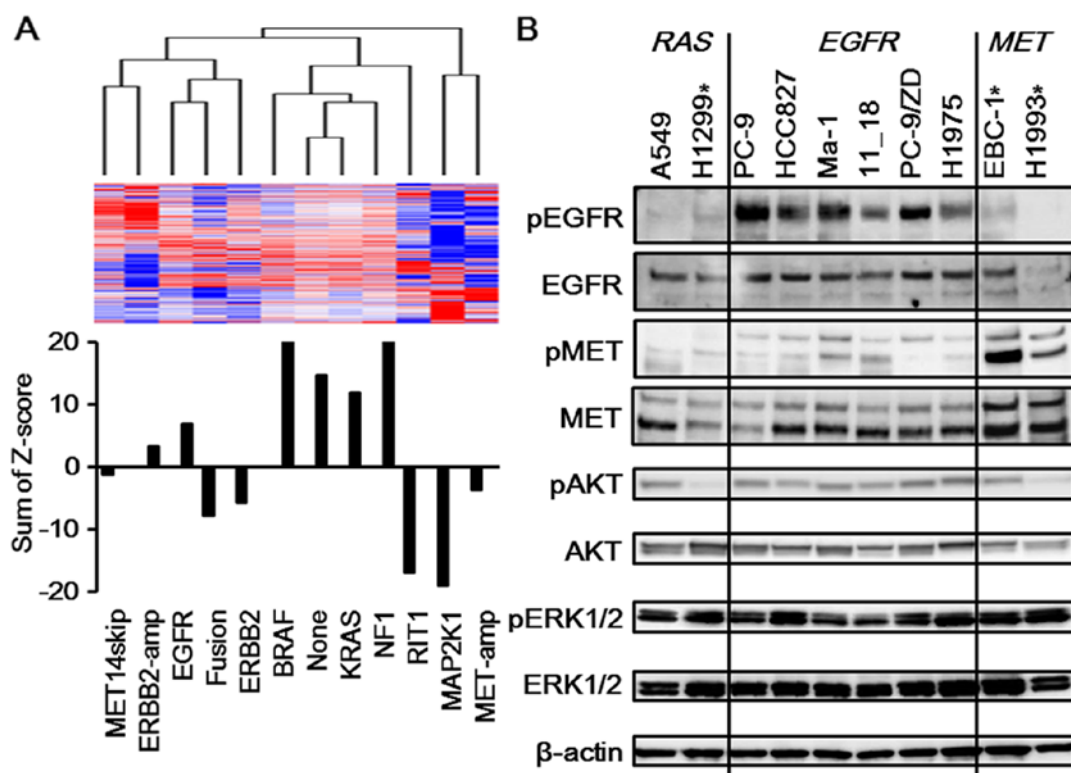


Figure 2. Activated PI3K/AKT pathway in *EGFR*-mutated NSCLC. (A) PI3K/AKT pathway in each NSCLC cluster. The mutational profiles of known driver oncogenes for 230 lung adenocarcinomas were based on The Cancer Genome Atlas (TCGA) dataset, and the expression profiles of those samples were extracted from the TCGA data portal. The average expression profile for each subclass was calculated and was used for further analysis. The average gene expression profile of the *MET*-amp subclass (n=5) was clustered next to that of the *MAP2K1* subclass (n=4). These two expression profiles were clustered together in a different branch from those of the other types of expression profiles. In addition, the expression profile of the *EGFR* subclass was clustered in a notably different branch from that of the *MET*-amp subclass. Next, genes involved in the ‘PI3K-cascade’ from the REACTOME database were extracted. The Z-scores for each gene in the 12 subclasses were calculated and were summarized for each subclass. A positive value indicated that the genes in the pathway were relatively activated. The *MAP2K1* subclass had a particularly negative value. The *MET*-amp subclass, which is similar to the *MAP2K1* subclass, also had a negative value, whereas the *EGFR* and *KRAS* subclasses had positive values. *MET*14skip, subclass of samples with *MET* exon 14 skipping mutations; *ERBB2*-amp, subclass of samples with *ERBB2* amplification; *EGFR*, subclass of samples with *EGFR* mutations; fusion, subclass of samples with *ALK*, *RET* or *ROS1* fusions; *ERBB2*, subclass of samples with *ERBB2* mutations; *BRAF*, subclass of samples with *BRAF* mutations; *KRAS*, subclass of samples with *KRAS* mutations; *NF1*, subclass of samples with *NF1* loss; *RIT1*, subclass of samples with *RIT1* mutations; *MAP2K1*, subclass of samples with *MAP2K1*, *NRAS* or *HRAS* mutations; *MET*-amp, subclass of samples with *MET* amplification; none, subclass of samples without any of the above driver gene alterations. (B) Western blotting for related pathways. To confirm the bioinformatics analyses, western blot analyses were performed using the *RAS*-mutated, *EGFR*-mutated or *MET*-amplified NSCLC cell lines. The phosphorylation levels of AKT were significantly elevated in the A549 (*KRAS* mutation) and *EGFR*-mutated cell lines (PC-9, HCC827, Ma-1, 11_18, PC-9/ZD, and H1975), compared with those in the H1299 (*NRAS* mutation) and *MET*-amplified cell lines (EBC-1 and H1993). The phosphorylation level of *EGFR* was elevated in the *EGFR*-mutated cell lines and that of *MET* was elevated in the *MET*-amplified cell lines. β -actin was used as an internal control. *These cell lines were sensitive to MEK inhibitors.

line was sensitive (Fig. 1B and Table I). Altogether, these findings suggest that the activated PI3K/AKT pathway might be associated with resistance to MEK inhibitors.

PI3K/AKT pathway is associated with resistance to MEK inhibitors in EGFR-mutated NSCLC cell lines. To elucidate the association between the PI3K/AKT pathway and resistance to MEK inhibitors, western blot analyses after treatment with trametinib were performed. The phosphorylation level of ERK1/2 was reduced after the treatment (Figs. 3A and 4B). When the PC-9 and HCC827 cell lines (*EGFR* mutation) were exposed to trametinib, the phosphorylation level of AKT was elevated with no change in *EGFR* or *MET* phosphorylation (Figs. 3A and 4B). In contrast, the phosphorylation of AKT was not changed in the EBC-1 and H1993 cell lines (*MET* amplification) (Fig. 3A). In addition, the expression levels of apoptosis-related molecules (cleaved caspase-3 and cleaved PARP) were elevated after treatment in the EBC-1 and H1993

cell lines, whereas these levels were not elevated in the PC-9 and HCC827 cell lines (Figs. 3B and 4B). Next, combination treatment with trametinib and a PI3K inhibitor (LY294002) was tested. The sensitivities to trametinib in the PC-9 and HCC827 cell lines were enhanced using LY294002 (Fig. 4A). The trametinib-induced elevation in the phosphorylation level of AKT was reduced and apoptosis was also significantly induced by the combined treatment with trametinib and LY294002 in these cell lines (Fig. 4B). These findings support the hypothesis that the activated PI3K/AKT pathway is associated with resistance to MEK inhibitors in *EGFR*-mutated NSCLC cell lines.

MEK inhibitors are effective for the HCC827CNXR cell line, in which the driver gene has shifted from EGFR to MET. We previously created a CNX-2006 (third-generation *EGFR*-TKI)-resistant HCC827 cell line (HCC827CNXR) (15). This HCC827CNXR cell line lost the *EGFR* mutations and instead

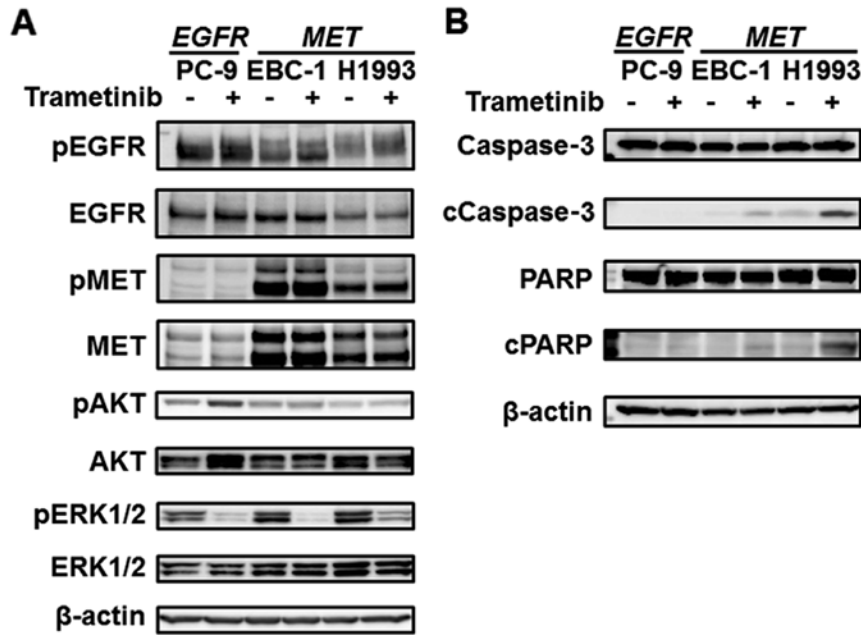


Figure 3. Western blotting after treatment with trametinib in *EGFR*-mutated or *MET*-amplified NSCLC cell lines. Western blot analyses for related pathways and apoptosis-related molecules were performed 3 and 24 h after treatment with trametinib (0.1 μM), respectively. (A) Western blotting for related pathways after treatment. The phosphorylation of ERK1/2 was suppressed by the treatment in all the cell lines. When the PC-9 cell line (*EGFR* mutation) was exposed to trametinib, the phosphorylation level of AKT was elevated with no change in *EGFR* or *MET* phosphorylation. In contrast, the phosphorylation level of AKT was not changed in the EBC-1 and H1993 cell lines (*MET* amplification). β-actin was used as an internal control. (B) Western blotting for apoptosis-related molecules after treatment. The expression levels of cleaved caspase-3 and cleaved PARP were elevated by the treatment in the EBC-1 and H1993 cell lines (*MET* amplification), whereas these levels were not elevated in the PC-9 cell line (*EGFR* mutation). β-actin was used as an internal control. cCaspase-3, cleaved caspase-3; cPARP, cleaved PARP.

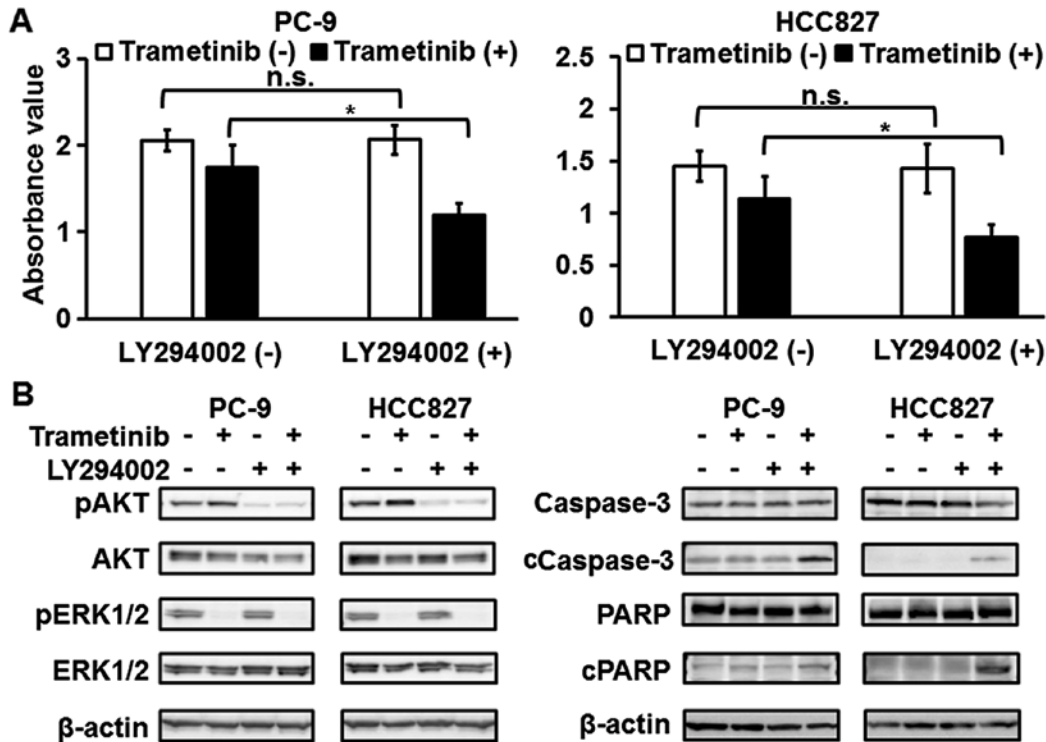


Figure 4. Combination therapy with trametinib and a PI3K inhibitor (LY294002) against *EGFR*-mutated NSCLC cell lines. (A) Efficacy of combination with trametinib and LY294002 against *EGFR*-mutated cell lines. A growth inhibitory assay was performed using an MTT assay with trametinib (0.1 μM) and LY294002 (1 μM). LY294002 alone did not inhibit the proliferation of the PC-9 and HCC827 cell lines. In contrast, LY294002 enhanced the sensitivity to trametinib in both cell lines. Columns, mean of independent triplicate experiments; bars, SD; NS, not significant; *P<0.05. (B) Western blotting for related pathways and apoptosis-related molecules. Western blot analyses for related pathways and apoptosis-related molecules were performed 3 and 24 h after treatment (trametinib, 0.1 μM; LY294002, 1 μM), respectively. The phosphorylation level of AKT elevated by trametinib was reduced by the combination treatment with LY294002 in the PC-9 and HCC827 cell lines. The expression levels of cleaved caspase-3 and cleaved PARP were also significantly elevated by the combination treatment. β-actin was used as an internal control. cCaspase-3, cleaved caspase-3; cPARP, cleaved PARP.

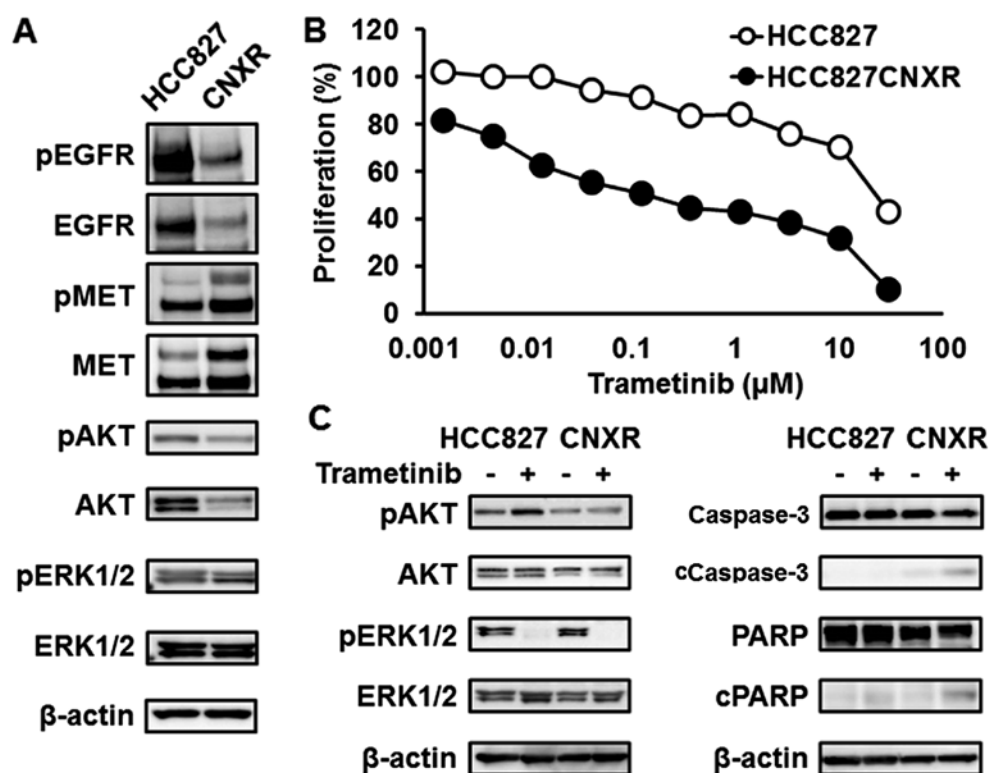


Figure 5. Trametinib against HCC827 (*EGFR* mutation) or HCC827CNXR (*MET* amplification) cell lines. (A) Western blotting for related pathways. The expression levels of EGFR and phospho-EGFR were elevated in the HCC827 cell line. In contrast, the expression levels of MET and phospho-MET were elevated, instead of a decrease in EGFR, in the HCC827CNXR cell line (oncogene swap). The expression levels of AKT and phospho-AKT were reduced along with the oncogene swap. β -actin was used as an internal control. (B) Inhibitory curves for trametinib in the HCC827 and HCC827CNXR cell lines. To investigate the efficacy of trametinib against the HCC827CNXR cell line, a growth inhibitory assay was performed using an MTT assay. The HCC827CNXR cell line was sensitive to trametinib, while the HCC827 cell line was resistant. Line, mean of independent triplicate experiments. (C) Western blotting for related pathways and apoptosis-related molecules. Western blot analyses for related pathways and apoptosis-related molecules were performed 3 and 24 h after treatment with trametinib (0.1 μ M), respectively. The phosphorylation level of AKT was elevated after treatment with trametinib in the HCC827 cell line, whereas a similar elevation was not observed in the HCC827CNXR cell line. The expression levels of cleaved caspase-3 and cleaved PARP were also significantly elevated by treatment in the HCC827CNXR cell line. β -actin was used as an internal control. cCaspase-3, cleaved caspase-3; cPARP, cleaved PARP.

harbored *MET* amplification (Fig. 5A) (15). HCC827CNXR was sensitive to MET inhibitors, meaning that the driver gene had shifted from the *EGFR* mutation to *MET* amplification (oncogene swap) (15). As is seen in other *MET*-amplified NSCLC cell lines, the phosphorylation level of AKT was reduced in the HCC827CNXR cell line, compared with that in the HCC827 cell line (Fig. 5A). In addition, the HCC827CNXR cell line was sensitive to MEK inhibitors, while the HCC827 cell line was resistant (Fig. 5B and Table I), meaning that the downstream RTK pathway had shifted to the MAPK pathway along with the oncogene swap. In the HCC827 cell line, the phosphorylation level of AKT was elevated after treatment with trametinib, whereas a similar elevation was not observed in the HCC827CNXR cell line (Fig. 5C). The expression levels of apoptosis-related molecules were also elevated in the HCC827CNXR cell line after treatment with trametinib (Fig. 5C). These findings suggest that the MAPK pathway is biologically important for *MET*-amplified NSCLC.

Synergistic effect of a MET inhibitor and a MEK inhibitor against MET-amplified NSCLC cell lines. Since our experimental findings suggest that the MAPK pathway is biologically important for *MET*-amplified NSCLC, the synergistic effect of a MET inhibitor (crizotinib) and trametinib was investigated.

As shown in Fig. 6A, trametinib enhanced the efficacy of crizotinib against *MET*-amplified NSCLC cell lines (EBC-1 and H1993). The IC_{50} of crizotinib in the EBC-1 and H1993 cell lines decreased to 0.0023 and 0.12 μ M from 0.010 and 0.58 μ M, respectively (Fig. 6A). The combination with trametinib significantly reduced the phosphorylation level of ERK1/2 and induced apoptosis, compared with crizotinib monotherapy (Fig. 6B).

Discussion

In this study, the sensitivities of several NSCLC cell lines with driver gene alterations to MEK inhibitors were investigated, and interesting results were obtained. MEK inhibitors were not effective against any of the *EGFR*-mutated cell lines but were effective against all the *MET*-amplified cell lines. Furthermore, combination therapy with a MET inhibitor and a MEK inhibitor was particularly effective against *MET*-amplified NSCLC cell lines. To the best of our knowledge, this is the first study to demonstrate such a difference in the sensitivity to MEK inhibitors among NSCLC cell lines, the biological importance of the MAPK pathway in *MET*-amplified NSCLC, and the synergistic effect of a MET inhibitor and a MEK inhibitor against *MET*-amplified NSCLC.

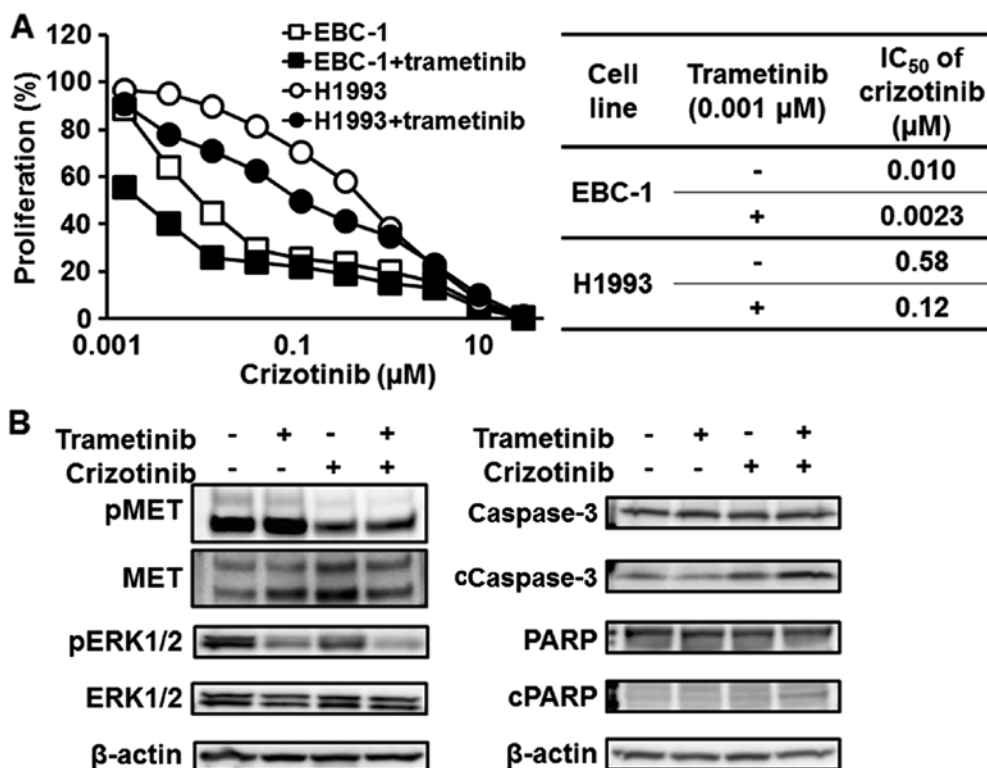


Figure 6. Synergistic effect of crizotinib and trametinib against *MET*-amplified NSCLC cell lines. (A) Inhibitory curves for crizotinib in *MET*-amplified NSCLC cell lines with or without trametinib. The growth inhibitory assay for crizotinib was performed using an MTT assay with or without trametinib (0.001 μM). Trametinib enhanced the sensitivity to crizotinib in the EBC-1 and H1993 cell lines. The IC₅₀ of crizotinib in the EBC-1 and H1993 cell lines decreased to 0.0023 and 0.12 μM from 0.010 and 0.58 μM, respectively. (B) Western blotting for related pathways and apoptosis-related molecules in the EBC-1 cell line. Western blot analyses for related pathways and apoptosis-related molecules were performed 3 and 24 h after treatment (crizotinib, 0.01 μM; trametinib, 0.001 μM), respectively. The phosphorylation of ERK1/2 was particularly suppressed by the combination treatment, compared with each monotherapy. The expression levels of cleaved caspase-3 and cleaved PARP were also significantly elevated using the combination treatment, compared with each monotherapy. β-actin was used as an internal control. cCaspase-3, cleaved caspase-3; cPARP, cleaved PARP.

The MAPK pathway includes RAS, RAF, MEK, and ERK. The constitutive activation of this pathway can lead to uncontrolled cell growth and survival, ultimately resulting in oncogenic transformation and progression (13,14). Reflecting the central role of the MAPK pathway in cell proliferation, activated mutants of RAS family members are among the oncoproteins most frequently detected in human malignancies (20). In addition, among the downstream targets of RTK pathways, the MAPK pathway is particularly important for cancer cell proliferation and survival (11-14). Therefore, MEK inhibitors are expected to be effective against NSCLC cell lines in which cell growth or survival is dependent on oncogenic driver RTK gene alterations, i.e., *EGFR*-mutated, *ALK*-fused or *MET*-amplified NSCLC cell lines. However, a wide range of sensitivities to MEK inhibitors was observed. Especially, MEK inhibitors were effective against all the *MET*-amplified NSCLC cell lines, whereas they were not effective against any of the *EGFR*-mutated cell lines. Furthermore, the established HCC827CNXR cell line, in which the driver gene had shifted from *EGFR* to *MET* (oncogene swap), exhibited enhanced sensitivity to MEK inhibitors. These findings suggest that the MAPK pathway is biologically important for *MET*-amplified NSCLC. The mechanism responsible for this difference was investigated using a bioinformatics technique, which showed an association with the activated PI3K/AKT pathway in *EGFR*-mutated NSCLC. Indeed, the phosphorylation level of AKT

in the *EGFR*-mutated cell lines was significantly elevated, compared with that in the *MET*-amplified cell lines, and this phosphorylation increased after treatment with trametinib, suggesting that the PI3K/AKT pathway plays a salvage role in *EGFR*-mutated NSCLC treated with MEK inhibitors. In addition, a PI3K inhibitor enhanced the sensitivity to a MEK inhibitor in the *EGFR*-mutated NSCLC cell lines. These experimental findings support the association between the activated PI3K/AKT pathway and resistance to MEK inhibitors in *EGFR*-mutated NSCLC. Although further mechanisms remain unclear, similar reports showing the activated PI3K/AKT pathway in *EGFR*-mutated NSCLC have been previously reported (18,19). Differences in adoster protein binding might exist between *EGFR* and *MET*, and further research is needed.

MET is overexpressed in 22-67% of NSCLCs, and one type of *MET* abnormality is amplification (21-24). The prevalence of *MET* amplification is ~5% in untreated patients and 20% in those with acquired resistance to *EGFR*-TKIs (21-31). *MET* positivity via IHC and an increased *MET* gene copy number are both significantly associated with poor overall survival (21-24,32). Although some preclinical studies, phase I/II trials, and subgroup analyses of phase III trials of small molecules targeting *MET* (used either singly or in combination) for *MET*-amplified NSCLC have yielded favorable results, the efficacy of such agents has not yet been confirmed in large clinical trials (10,33-35). Furthermore, two large phase III

trials using anti-MET drugs in combination with EGFR-TKIs have failed (36,37). A previous report showed the synergistic effect of a MET inhibitor and a MEK inhibitor in *RAS*-mutated cell lines and the role of *RAS* mutations in resistance to a MET inhibitor (38). These findings indicate the importance of the MAPK pathway in treatment with a MET inhibitor, and our present study also showed the importance of this pathway for *MET*-amplified NSCLC and the efficacy of MEK inhibitors against such NSCLC. In addition, our experimental findings revealed the synergistic effect of a MET inhibitor and a MEK inhibitor against *MET*-amplified NSCLC, suggesting that combination therapy with a MET inhibitor and a MEK inhibitor might be a promising treatment strategy for *MET*-amplified NSCLC.

In conclusion, we observed that MEK inhibitors were not effective against any of the *EGFR*-mutated NSCLC cell lines that were examined, but were effective against all the *MET*-amplified NSCLC cell lines. Although this difference seems to have been caused by the activation of the PI3K/AKT pathway in *EGFR*-mutated NSCLC, the detailed molecular mechanism remains unclear and further research is needed. In addition, the importance of the MAPK pathway for *MET*-amplified NSCLC has been shown, and combination therapy consisting of a MET inhibitor and a MEK inhibitor was particularly effective against *MET*-amplified NSCLC cell lines. These experimental findings strongly encourage the development of this combination therapy against *MET*-amplified NSCLC.

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