

EGFR and HER2 signals play a salvage role in *MEK1*-mutated gastric cancer after MEK inhibition

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Abstract. Since the prognosis of unresectable advanced gastric cancer remains poor, novel therapeutic strategies are needed. Somatic *MEK1* gene mutations have been reported as oncogenic activating mutations in gastric cancer, and MEK inhibitors can be effective against such gastric cancers. In the present study, however, activated EGFR and HER2 signals after treatment with a MEK inhibitor (trametinib) were found in a *MEK1*-mutated gastric cancer cell line (OCUM-1 cell line) using a phospho-receptor tyrosine kinase array. The phosphorylation of EGFR and HER2 reactivated ERK1/2, which had been inhibited by trametinib, and EGF stimulation led to resistance to trametinib in this cell line. Lapatinib, an EGFR and an HER2 inhibitor, reversed the activation of ERK1/2 by inhibiting the phosphorylation of EGFR and HER2 and cancelled the resistance. The combination of trametinib and lapatinib synergistically inhibited the cell growth of the OCUM-1 cell line and strongly induced apoptosis by inhibiting the activated EGFR and HER2 signals. These results suggest that the EGFR and HER2 signals play a salvage role and are related to resistance to MEK inhibitors in *MEK1*-mutated gastric cancer. Moreover, combination therapy with trametinib and lapatinib can exhibit a synergistic effect and may contribute to overcoming the resistance to MEK inhibitors.

Introduction

Gastric cancer (GC) is one of the most common malignant tumors and the third most common cause of death from malignancies worldwide (1). Despite intensive investigations of anticancer treatments for GC, the prognosis of unresect-

able advanced or recurrent GC remains poor. The median overall survival of GC patients has reached only ~1 year using conventional cytotoxic chemotherapy (2-4). Trastuzumab, a monoclonal antibody against human epidermal growth factor receptor (EGFR) 2 (HER2), used in combination with chemotherapy has shown a survival benefit when used as a first-line treatment in patients with HER2-positive advanced GC (5). However, HER2 overexpression has only been reported in 13-23% of GC cases (6-8). *Fibroblast growth factor receptor 2* amplification, *MET* amplification and *RhoA* mutations have been anticipated as new molecular targets in GC, but these aberrations are relatively infrequent (9-12). Therefore, new therapeutic modalities are still needed.

The mitogen-activated protein kinase (MAPK) signal pathway cascade and its downstream factors promote cancer cell proliferation, differentiation and survival. The three-tiered kinase cascade consisting of RAF, mitogen-activated protein kinase kinase (MEK), and extracellular signal-regulated kinase (ERK) is frequently dysregulated in malignancies as a result of activating mutations in the upstream RAS (*KRAS*, *NRAS* and *HRAS*) (13,14). The activating *BRAF* mutation has been detected in a variety of human cancers (15), and the success of RAF inhibitors in *BRAF*-mutated melanoma has revealed a new modality and has substantiated the contribution of the MAPK signal to carcinogenesis (16,17). Somatic oncogenic *MEK1* mutations have also been identified in several human malignancies (18-21). In addition, our previous study demonstrated that *MEK1* mutations in poorly differentiated GC cell lines that are hypersensitive to MEK inhibitors have transformational abilities and that the growth of these cancer cells is dependent on these mutations (22). Considering the addition of cancer cells to active *MEK1* mutations for proliferation, GC with such oncogenic *MEK1* mutations might be suitable for targeted therapy with MEK inhibitors.

A persistent problem is the development of resistance to molecular-targeted therapies. A series of resistance mechanisms for MEK inhibition that operate ERK-dependently or ERK-independently have been reported, including *MEK* mutations, elevated RAS or RAF protein levels, and the activation of an alternative PI3K/AKT or STAT3 pathway (23-25). Currently, the phosphorylation of EGFR after the inhibition of the MAPK signal has been suggested as another potential

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mechanism responsible for the resistance to MEK inhibition in various cancers, and combination therapy with EGFR inhibitors yielded synergistic effects (26,27). In this study, we observed the phosphorylation of EGFR and HER2 after MEK inhibition in a *MEK1*-mutated GC cell line. Then, the relevancy of this mechanism to MEK inhibitor resistance was validated, and combinations with other tyrosine kinase inhibitors were tested to develop new therapeutic possibilities.

Materials and methods

Reagents and ligand. Trametinib (GSK1120212) and lapatinib (GW572016) were purchased from Selleck Chemicals (Houston, TX, USA) and were dissolved in dimethyl sulfoxide (DMSO) for the *in vitro* experiments. Recombinant human epidermal growth factor (EGF) was obtained from R&D Systems (Minneapolis, MN, USA) and was constituted in sterile phosphate-buffered saline (PBS).

Antibodies. Rabbit-antibodies specific for ERK1/2, EGFR, HER2, phospho-ERK1/2 (pERK1/2), phospho-EGFR (pEGFR), phospho-HER2 (pHER2), poly (ADP-ribose) polymerase (PARP), caspase-3, cleaved PARP (cPARP), cleaved caspase-3 (cCaspase-3), and β -actin were obtained from Cell Signaling Technology (Beverly, MA, USA).

Cell cultures. A poorly differentiated GC cell line harboring the *MEK1* Q56P mutation, OCU-M-1, was propagated in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA) and 1% Penicillin-Streptomycin Mixed Solution (Nacalai Tesque, Kyoto, Japan) in a humidified atmosphere of 5% CO₂ at 37°C.

Human phospho-receptor tyrosine kinase (RTK) array. The screening of 49 phosphorylated tyrosine kinases in the OCU-M-1 cell line was performed using the Human Phospho-RTK Array kit (R&D Systems). Whole lysates of OCU-M-1 cells incubated in the absence (DMSO) or presence of 1 nM trametinib for 72 h were compared in accordance with the manufacturer's recommendation. Protein detection was accomplished using an anti-phospho-tyrosine-HRP detection antibody and an enhanced chemiluminescence system (Image Quant LAS4000; GE Healthcare Life Science, Buckinghamshire, UK).

Growth inhibition assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the growth inhibitory effect of the drugs, as previously described (28). When the effect of EGF was evaluated, 1% FBS was used.

Real-time reverse transcription polymerase chain reaction (RT-PCR). A total of 1 μ g of RNA was isolated from the cells using ISOGEN reagent (Nippon Gene, Tokyo, Japan) and then converted to cDNA using GeneAmp RNA-PCR kit (Applied Biosystems, Foster City, CA USA). Real-time PCR was performed using SYBR Premix Ex Taq and Thermal Cycler Dice (Takara, Shiga, Japan), as described previously (29). The glyceraldehyde 3-phosphate dehydrogenase (*GAPD*,

NM_002046) gene was used to normalize the expression levels in subsequent quantitative analyses. To amplify the target genes encoding amphiregulin (AREG), EGF, heparin-binding EGF-like growth factor (HB-EGF), neuregulin 1 (NRG1), transforming growth factor α (TGFA), EGFR, HER2, HER3, and HER4 (*AREG*, *EGF*, *HB-EGF*, *NRG1*, *TGFA*, *EGFR*, *ERBB2*, *ERBB3* and *ERBB4* genes, respectively), the following primers were used: AREG-F, GTCGCTCTTGATAC TCGGCTCAG; AREG-R, TCCCAGAGTAGGTGTCATTG AGGTC; EGF-F, CAACCAGTGGCTGGTGAGGA; EGF-R, GAGCCCTTATCACTGGATACTGGAA; HB-EGF-F, CAA GGTGATTTCAGACTGCAGAGG; HB-EGF-R, TTTGGCA CTTGAAGGCTCTGG; NRG1-F, GCCAGGAATCGGCTG CAGGT; NRG1-R, AGCCAGTGATGCTTTGTAAATGCGA; TGFA-F, CTTTGGAAACCAGCAGGTCTGA; TGFA-R, CCCAAATAAGCCAGGCTGTTCTA; EGFR-F, CATCCA GGCCCAACTGTGAG; EGFR-R, CAGTGGAAGCCCTT GAAGCAGAA; ERBB2-F, TGGGAGCCTGGCATTCTCTG; ERBB2-R, CGGCCATGCTGAGATGTATAGGTA; ERBB3-F, GGGAGCATTTAATGGCAGCTA; ERBB3-R, GAATGGAATTGTCTGGGACTGG; ERBB4-F, GCAGCT AACTTTGAATGCCTGTCTC; ERBB4-R, GCAGCTA ACTTTGAATGCCTGTCTC; GAPD-F, GCACCGTCAAG GCTGAGAAC; and GAPD-R, ATGGTGGTGAAGACG CCACT.

Western blot analysis. The western blot analysis was performed as described previously (28). Briefly, subconfluent cells were washed with cold 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 chloride, 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. When the cells were stimulated using EGF, the cells were incubated with 1% FBS medium.

Statistical analysis. The results are shown as the mean value \pm standard deviation (SD) for three replicate independent experiments. The statistical analyses were performed using the Student's t-test (two-tailed). A P-value <0.05 was considered statistically significant. The statistical analyses were two-tailed and were performed using Microsoft Excel (Microsoft, Redmond, WA, USA).

Results

MEK inhibition led to the phosphorylation of EGFR and HER2 in the *MEK1*-mutated OCU-M-1 cell line. To address the phosphorylation of RTK after MEK inhibition, we used a phospho-RTK array. The arrangement of the RTK array is summarized in Fig. 1B. The phosphorylation levels of EGFR and HER2 were elevated at 72 h after treatment with a MEK

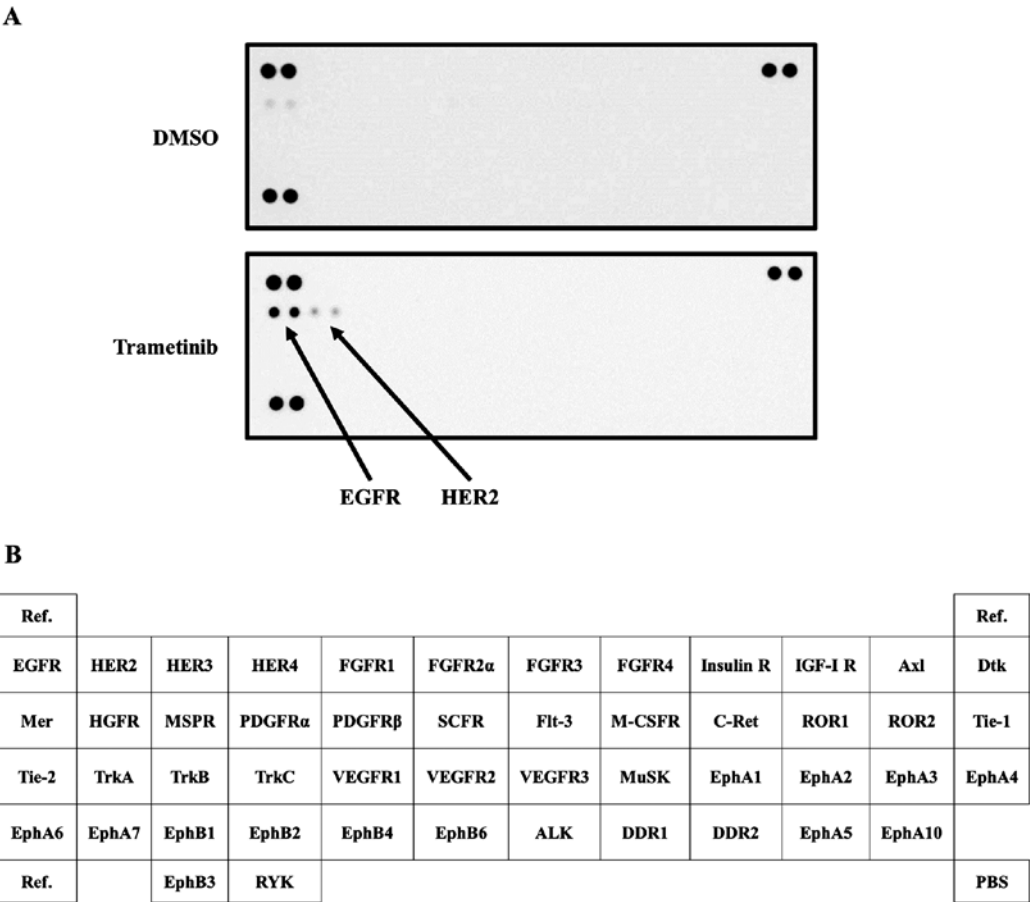


Figure 1. Phosphorylation of RTKs after MEK inhibition in the OCUM-1 cell line. (A) Phospho-RTKs in *MEK1*-mutated OCUM-1 cells incubated in the absence (DMSO) or presence of 1 nM of trametinib for 72 h were compared using a human phospho-RTK array. Each pair of horizontal spots represents one RTK. The densities of two pairs of spots, representing the phosphorylation levels of EGFR and HER2, were greatly increased after MEK inhibition with trametinib. (B) Arrangement of phospho-RTK array. The paired spots in 3 of the 4 corners are phosphorylated tyrosine controls used as references, and the spots in the lower right corner represent PBS, which was used as a negative control.

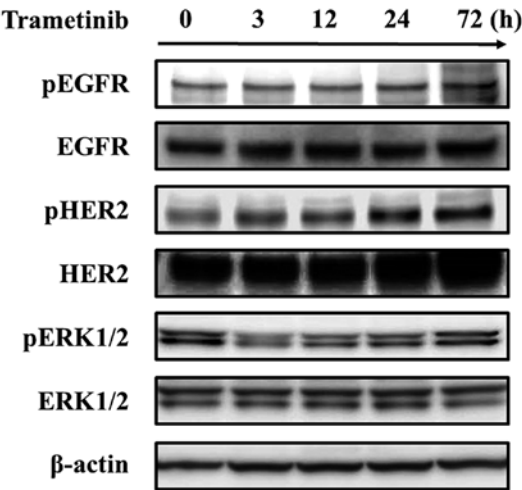


Figure 2. Time course for the phosphorylation levels of EGFR, HER2 and ERK1/2 after MEK inhibition. The OCUM-1 cells were incubated with 1 nM of trametinib for each of the indicated time periods and were then evaluated using a western blot analysis. The phosphorylation levels of EGFR and HER2 were increased at 24 and 72 h after trametinib exposure. Trametinib antecedently inhibited the phosphorylation levels of ERK1/2 from 3 h, and inhibited ERK1/2 was re-activated in accordance with the elevation of the phosphorylation levels of EGFR and HER2. β -actin was used as an internal control. pEGFR, phospho-EGFR; pHER2, phospho-HER2; pERK1/2, phospho-ERK1/2.

inhibitor (trametinib) (Fig. 1A). Next, to validate the results of the RTK array, we evaluated the phosphorylation level of EGFR and HER2 using a western blot analysis. In addition to the inhibitory effect on the ERK signal, trametinib led to the activation of EGFR and HER2 signals similar to the results of the RTK array. In addition, the phosphorylation of ERK1/2, which had been inhibited by trametinib, was reactivated following the activation of the EGFR and HER2 signals (Fig. 2). These results suggest that the EGFR and HER2 signals are activated under MEK inhibition in *MEK1*-mutated GC. To investigate the mechanism responsible for the activation of the EGFR and HER2 signals, real-time RT-PCR was performed. However, no significant changes in the mRNA expression levels of the molecules relative to the EGFR and HER2 signals were observed (*AREG*, *EGF*, *HB-EGF*, *NRG1*, *TGFA*, *EGFR*, *HER2*, *HER3* and *HER4*; data not shown).

Ligand-induced EGFR and HER2 activation led to the acquisition of resistance to a MEK inhibitor. To verify the contribution of EGFR and HER2 signals to MEK inhibitor resistance, the experiments were performed using recombinant human EGF (10 ng/ml) as the ligand. After the EGF-induced activation of the EGFR and HER2 signals, the OCUM-1 cell line became resistant to trametinib (Fig. 3A). Using a western

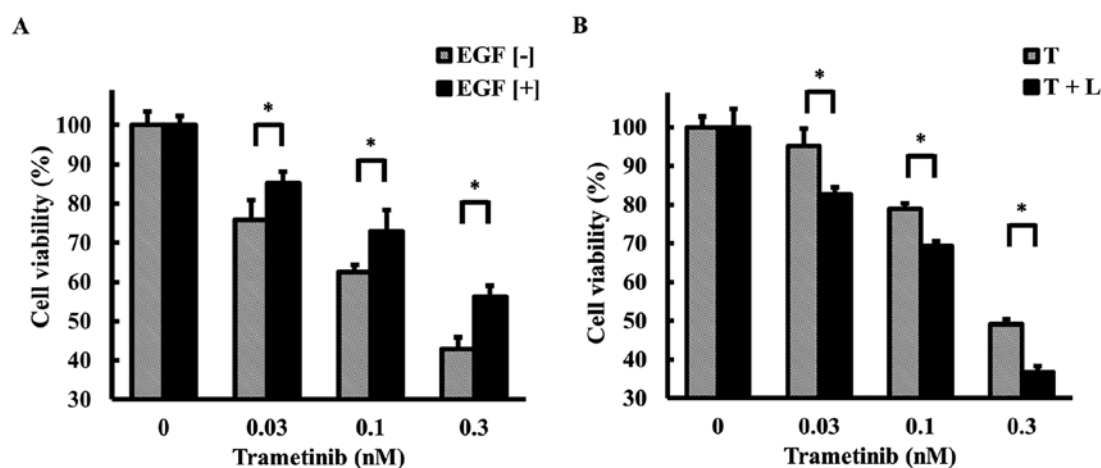


Figure 3. Growth inhibitory effect of trametinib in the OCUM-1 cell line. (A) Influence of EGF on the sensitivity to trametinib. Cells were exposed to each concentration of trametinib for 72 h with or without 10 ng/ml of EGF [EGF (+), solid bars; EGF (-), bars with hatch lines] and were evaluated using an MTT assay using 1% FBS. The growth inhibitory effect at every concentration of trametinib was strongly weakened by EGF stimulation. Columns, mean of independent triplicate experiments; error bars, SD; *P<0.05. (B) Growth inhibitory effect of trametinib monotherapy and combination with lapatinib. The cells were exposed to each concentration of trametinib for 72 h with or without 10 μ M of lapatinib (T + L, solid bars; T, bars with hatch lines) and were evaluated using an MTT assay. The combination of trametinib and lapatinib synergistically inhibited the cell growth in a significant manner. Columns, mean of independent triplicate experiments; error bars, SD; *P<0.05.

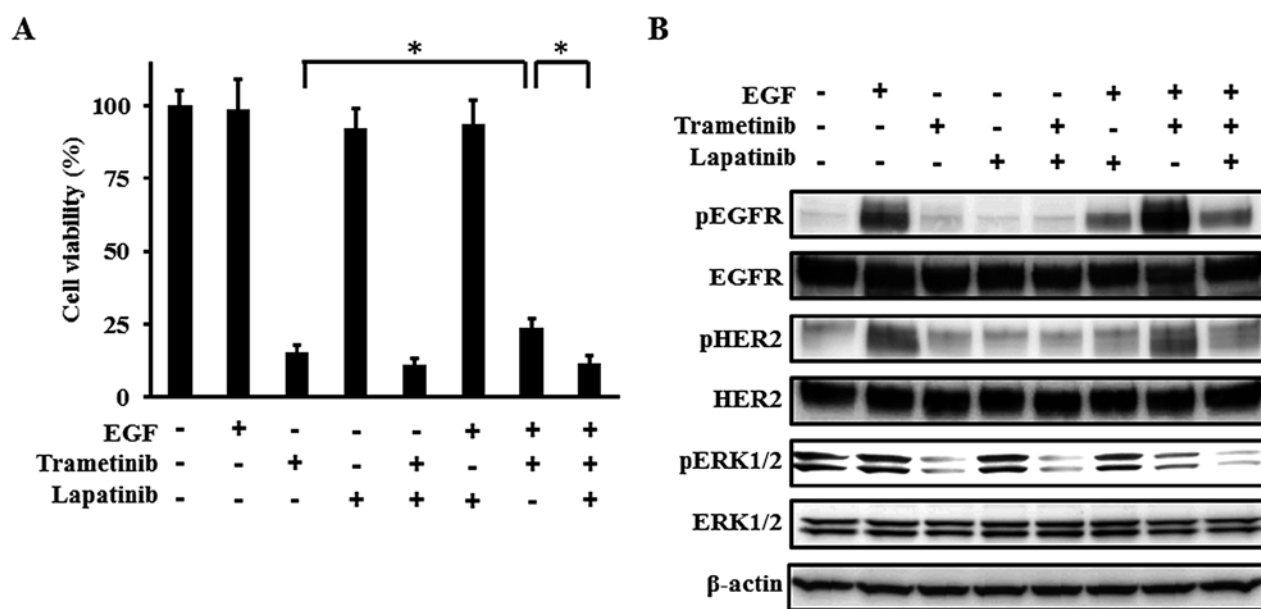


Figure 4. Effect of lapatinib on the resistance to trametinib induced by EGF. (A) Growth inhibitory effect of combination with trametinib and lapatinib in the presence of EGF. The cells were exposed to 1 nM of trametinib, 10 μ M of lapatinib, and/or 10 ng/ml of EGF for 72 h and the cell growth was evaluated using an MTT assay with 1% FBS. In the presence of EGF, the inhibitory effect of trametinib was weakened significantly, while the combination of trametinib and lapatinib abolished the resistance induced by EGF. EGF or lapatinib monotherapy did not influence the cell growth. Columns, mean of independent triplicate experiments; error bars, SD; *P<0.05. (B) Phosphorylation of EGFR, HER2, and ERK1/2. When the phosphorylation levels were examined using a western blot analysis, the samples were collected 3 h after stimulation. The following concentrations were used: 1 nM of trametinib, 10 μ M of lapatinib, and 10 ng/ml of EGF. EGF stimulation increased the phosphorylation levels of EGFR and HER2, resulting in the activation of ERK1/2. The decreased phosphorylation levels of ERK1/2 induced by trametinib were re-activated by EGF stimulation, and lapatinib abolished the re-activation of ERK1/2 by inhibiting the phosphorylation of EGFR and HER2. β -actin was used as an internal control. pEGFR, phospho-EGFR; pHER2, phospho-HER2; pERK1/2, phospho-ERK1/2.

blot analysis, EGF stimulation was shown to increase the phosphorylation levels of EGFR and HER2, and ERK1/2, which had been suppressed by trametinib, was re-phosphorylated (Fig. 4B).

Lapatinib abolished the resistance to trametinib induced by EGF. Next, to investigate the effect of lapatinib (an EGFR and

HER2 dual tyrosine kinase inhibitor) on the EGF-induced resistance, a combination therapy was tested. The EGF-induced resistance to trametinib was abolished by lapatinib (Fig. 4A). The suppression of the phosphorylation levels of ERK1/2 through the inhibition of EGFR and HER2 by lapatinib can explain the restored response to trametinib (Fig. 4B). These results suggest that the activation of EGFR and HER2 signals

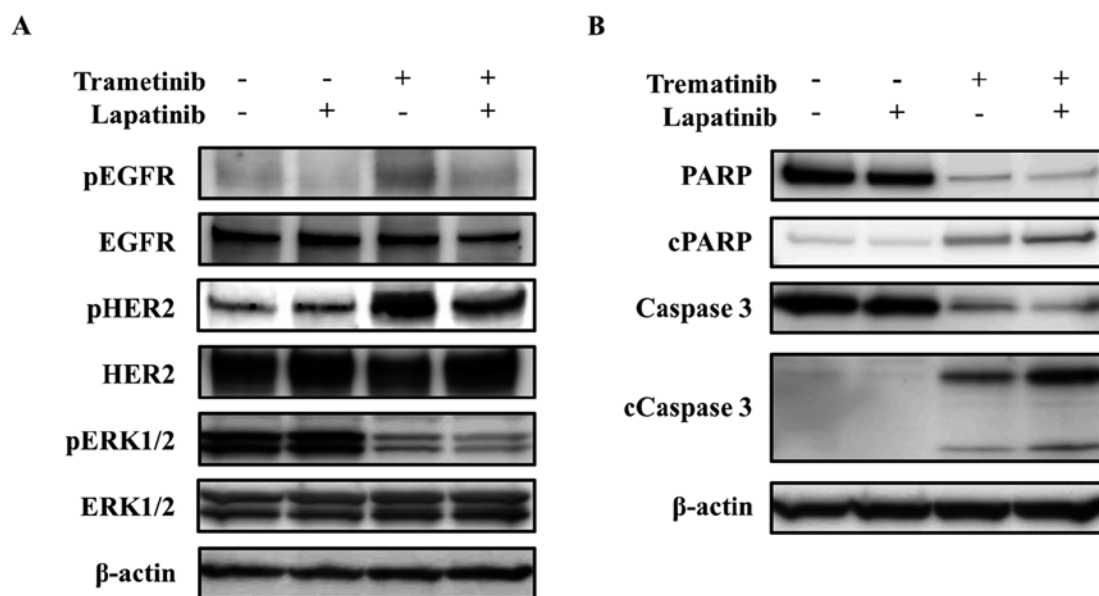


Figure 5. Synergistic effect of trametinib and lapatinib on EGFR, HER2, and ERK signals and apoptosis-related molecules. (A) Phosphorylation of EGFR, HER2, and ERK1/2. For the analysis of the phosphorylation levels using a western blot analysis, the samples were collected at 72 h after exposure. The following concentrations were used: 1 nM of trametinib and 10 μ M of lapatinib. The phosphorylation levels of EGFR and HER2 were elevated at 72 h after treatment with trametinib, similar to the results obtained using the phospho-RTK array. Lapatinib reduced the phosphorylation level of ERK1/2 by inhibiting the EGFR and HER2 signals, which were activated after trametinib treatment. β -actin was used as an internal control. pEGFR, phospho-EGFR; pHER2, phospho-HER2; pERK1/2, phospho-ERK1/2. (B) Expressions of apoptosis-related molecules. The cells were exposed to the inhibitors for 72 h, and apoptosis-related molecules were then examined using a western blot analysis. The following concentrations were used: 1 nM of trametinib and 10 μ M of lapatinib. Trametinib increased the expressions of cleaved PARP and cleaved caspase-3 to a greater extent when used in combination with lapatinib, compared with the results for trametinib monotherapy. β -actin was used as an internal control. cPARP, cleaved PARP; cCaspase-3, cleaved caspase-3.

potentially plays an important role in MEK inhibitor resistance and that lapatinib may abolish such resistance.

Combination of trametinib and lapatinib synergistically inhibits cell growth. To investigate the role of the activated EGFR and HER2 signals during treatment with trametinib, we examined the synergistic anticancer effect of trametinib and lapatinib in the OUCM-1 cell line using an MTT assay. The combination of trametinib and lapatinib showed a synergistic effect on the inhibition of cell proliferation (Fig. 3B). Furthermore, lapatinib decreased the phosphorylation levels of EGFR and HER2, which had been activated after the treatment with trametinib, and the phosphorylation level of ERK1/2 was inhibited to a greater degree by the combination treatment than by trametinib monotherapy (Fig. 5A). Cleaved PARP and cleaved caspase-3, which are hallmarks of apoptosis, had also increased at 72 h after the trametinib and lapatinib combination therapy, compared with after trametinib monotherapy (Fig. 5B). These results suggest that activated EGFR and HER2 signals are involved in salvage pathways under MEK inhibition in *MEK1*-mutated GC and that trametinib and lapatinib exert a synergistic effect by inhibiting the salvage signals.

Discussion

In this study, we demonstrated the reactivation of ERK1/2 via the activation of EGFR and HER2 signals after MEK inhibition with trametinib, and EGF-induced resistance in the *MEK1*-mutated GC cell line. Our data also identified the receptor tyrosine kinase family members EGFR and HER2 as potent targets for overcoming resistance to MEK inhibi-

tors. Moreover, the combination of trametinib and lapatinib was more effective against a *MEK1*-mutated GC cell line than trametinib monotherapy. To the best of our knowledge, this is the first report to show the activation of EGFR and HER2 after MEK inhibition, the potential of combination therapy as a therapeutic strategy, and the possibility of overcoming resistance in *MEK1*-mutated GC.

The serine threonine kinases BRAF, MEK, and ERK are major regulators of the MAPK signal and are frequently dysregulated in malignancies. Given the role of these kinases in cancer, the MAPK signal has become an attractive target for molecular targeted drugs (30). While a number of MAPK signal inhibitors, as well as RAF inhibitors, have been developed and are in clinical use, the acquisition of resistance has hindered the continuation of treatment, resulting in a limited survival benefit. Resistance to MAPK inhibitors is mediated by many mechanisms, including the reactivation of RAS/RAF/MEK/ERK signals, *MEK* mutations, and increases in the RAS or RAF protein levels, as well as increased signals through alternative pathways, including the PI3K/AKT and STAT3 pathways (31). Recently, BRAF inhibition leading to a feedback activation of EGFR and a strong synergistic effect of BRAF and EGFR inhibition were described in *BRAF*-mutated colorectal cancer cell lines (32). Furthermore, the combination of a MEK inhibitor and a dual EGFR and HER2 inhibitor produced a synergistic effect by inhibiting the activation of ERK signals both *in vivo* and *in vitro* in *KRAS*-mutated colon and lung cancer cell lines (27). These results suggest that the activation of HER family members is an important salvage signal during MAPK inhibition. Similarly, in our data, the EGFR and HER2 signals were activated after treatment with

a MEK inhibitor, resulting in the re-activation of the ERK signal. In addition, EGF stimulation also activated the ERK signal via the activation of EGFR and HER2 in the *MEK1*-mutated GC cell line, which led to MEK inhibitor resistance. The effects of the EGFR and HER2 signals were abolished by the inhibitor. These findings indicate the possibility of a novel combination therapy comprised of a MEK inhibitor and a HER inhibitor for GC patients, similar to results observed for other malignancies.

To address how MEK inhibition by trametinib activates EGFR and HER2, we investigated the mRNA expression of the corresponding genes using real-time RT-PCR. However, no significant change in mRNA expression was observed. MEK inhibition reportedly leads to MYC degradation and HER2 and HER3 upregulation in *KRAS*-mutated colon and lung cancer (27). Another study has shown that CDC25c, which can bind to EGFR, may be involved in the activation of EGFR in a *BRAF*-mutated colorectal cancer cell line (32). However, the detailed mechanism responsible for the activation of HER signals after MEK inhibition remains unclear. Furthermore, the activation of HER signals has been reported to result not only from MAPK inhibition, but also from exposure to cytotoxic agents, including 5-FU, oxaliplatin in colorectal cancer cells, and pemetrexed in non-small cell lung cancer (33,34). These findings suggest that the activation of HER signals plays a primary salvage role in many cancers, promising that resistance to many anticancer therapies can be overcome. Therefore, further research on approaches to overcoming such resistance is needed.

In conclusion, the present study revealed that the activation of EGFR and HER2 signals after MEK inhibition may serve as a potential mechanism responsible for the resistance to a MEK inhibitor in *MEK1*-mutated GC. Additionally, combined treatment with trametinib and lapatinib exhibited a synergistic effect by inhibiting the activation of the EGFR and HER2 signals. These data may contribute to the development of novel therapeutic strategies for *MEK1*-mutated GC.

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