A Novel Molecular Target in *EGFR*-mutant Lung Cancer Treated With the Combination of Osimertinib and Pemetrexed

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Abstract. *Background/Aim:* Synergistic effects of epidermal growth factor receptor tyrosine kinase inhibitors and chemotherapy have been reported. Here, we evaluated the therapeutic potential of combining osimertinib with pemetrexed and investigated the molecular mechanisms. *Materials and Methods:* We analyzed the antitumor effects of osimertinib±pemetrexed in PC-9 and H1975 cells. Gene expression on exposure to osimertinib±pemetrexed was assessed in these cultured cells. Cell lines resistant to osimertinib±pemetrexed were established to explore mechanisms of resistance. *Results:* Osimertinib+pemetrexed treatment delayed the emergence of resistance relative to monotherapy in vitro and in vivo. Expression of the anti-apoptotic gene *PLK1* was down-regulated in PC-9 and H1975 exposed to osimertinib+ pemetrexed, whereas it was up-regulated in resistant cells. Furthermore, inhibition of *PLK1* induced apoptosis and inhibited proliferation of resistant cells. *Conclusion:* Blocking *PLK1* contributes to mediating the synergistic anti-proliferative effect of osimertinib+pemetrexed. *PLK1* over-expression may be a critical mechanism for acquired resistance to osimertinib+pemetrexed.

Recently developed anti-cancer treatments such as molecular-targeted drugs and immunotherapeutic agents have elicited dramatic responses in some advanced non-small cell lung cancer (NSCLC) patients (1). In particular, NSCLC

patients with epidermal growth factor receptor (*EGFR*) activating mutations, such as the in-frame deletion [del(E746-A750)] in exon 19 or mutated exon 21 (L858R), show high sensitivity to *EGFR*-tyrosine kinase inhibitors (TKIs) (2-6). The FLAURA phase III trial reported that progression-free survival (PFS) and overall survival (OS) were significantly longer in treatment-naïve *EGFR*-mutant NSCLC patients treated with the 3rd generation *EGFR*-TKI osimertinib relative to those treated with first-generation drugs of this type (7). Osimertinib is an effective and well-tolerated treatment for NSCLC patients with *EGFR*-mutations, including elderly patients, and is currently used as a standard first-line therapy in clinical practice (8). However, as with early-generation *EGFR*-TKI treatment, initially susceptible NSCLCs inevitably acquire resistance to osimertinib treatment and thus, there is an urgent unmet need for novel treatment strategies to overcome or delay such acquired resistance to *EGFR*-TKIs (9, 10).

Previous studies have documented synergistic effects when treating NSCLC with a combination of *EGFR*-TKI and cytotoxic agents (11-13). Phase III clinical trials indicated that combining gefitinib with concurrent chemotherapy resulted in improved PFS of patients with untreated advanced NSCLC harboring the appropriate *EGFR* mutations (14, 15). Currently, ongoing phase II and III trials are comparing osimertinib with osimertinib+chemotherapy such as cisplatin/carboplatin and pemetrexed (16). The molecular mechanisms responsible for the synergistic effects of combination treatments in NSCLC are not completely understood, but could result in improved therapeutic approaches. We have previously contributed to pivotal phase II-III clinical trials conducted by the North East Japan Study Group in *EGFR*-mutant NSCLC patients (2, 14, 16). In addition, our previous studies have identified promising therapeutic targets for overcoming resistance to targeted inhibitors in NSCLC patients with driver oncogenes (17-20).

In the present study, we employed two different NSCLC cell lines harboring *EGFR* mutations to investigate possible

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mechanisms responsible for the superior efficacy of combination therapy with osimertinib+pemetrexed. Here, we undertook gene expression profiling to identify molecular mechanisms contributing to the success of combination therapy. To this end, we established NSCLC cell lines resistant to osimertinib alone or to the combination of osimertinib and pemetrexed as models to investigate potential therapeutic strategies for overcoming resistance to combination therapy.

Materials and Methods

Cell culture. Two human lung adenocarcinoma cell lines were employed in the present study. PC-9 has an *EGFR* exon 19 deletion. It was provided by Immuno-Biological Laboratories (Gunma, Japan). NCI-H1975 (H1975) with an exon 21 L858R mutation and secondary exon 20 T790M mutation was purchased from the American Type Culture Collection (Manassas, VA, USA). These cell lines were cultured in RPMI-1640 (FUJIFILM, Osaka, Japan) containing 10% fetal bovine serum (FBS; Biowest, Nuaille, France) and 1% penicillin and streptomycin (FUJIFILM) at 37°C in a 5% CO₂ incubator. The cell lines were obtained in 2011 and 2014, expanded and cryopreserved, and one aliquot of each was thawed for this study. No cell line authentication was performed by the authors. All cells were routinely screened for the absence of mycoplasma.

Drugs and cell viability assays. Osimertinib, pemetrexed, and the Polo-like kinase 1 (PLK1) inhibitor volasertib were purchased from Selleck Chemicals (Houston, TX, USA). To evaluate their sensitivity to drugs *in vitro*, cells were plated in 96-well tissue culture plates at 5,000 cells/well and incubated at 37°C for 24 h. Cells were then incubated with titrated concentrations of the drugs (0.001, 0.01, 0.1, 1, 10 µM) or vehicle (DMSO) at 37°C for 72 h. Cell numbers were estimated using the Counting Kit-8 (Dojindo, Kumamoto, Japan). The half maximal inhibitory concentration (IC₅₀) value for the drugs tested was defined as the concentration of osimertinib or pemetrexed or combinations thereof required for a 50% reduction of cell growth. Each experiment was performed independently three times. The corrected absorbance of each sample was calculated and compared with that of the untreated control, according to the protocol provided by the manufacturer of the kits.

Western blotting. Protein extraction and western blotting were performed as previously described (17, 18). Primary antibodies used were as follows: anti-PLK1 (4513, Cell Signaling Technology, Danvers, MA, USA; 1:1,000); anti-TS (9045, Cell Signaling Technology; 1:1,000); anti-EGFR (2232, Cell Signaling Technology; 1:1,000); anti-phosphorylated EGFR (p-EGFR) (2234, Cell Signaling Technology; 1:1,000); anti-AKT (9272, Cell Signaling Technology; 1:1,000); anti-p-AKT (9271, Cell Signaling Technology; 1:1,000); anti-ERK (9102, Cell Signaling Technology; 1:1,000); anti-p-ERK (9101, Cell Signaling Technology; 1:1,000); anti-BIM (2933, Cell Signaling Technology; 1:1,000); anti-cleaved PARP (5625, Cell Signaling Technology; 1:1,000); and anti-GAPDH (sc-47724, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1,000). Horseradish peroxidase-linked anti-rabbit IgG (4030-05, Southern Biotech, Birmingham, AL, USA; 1:3,000) or anti-mouse IgGκ (sc-516102, Santa Cruz; 1:3,000) were used as the secondary antibodies. The ImageJ software was used for quantitative image analysis.

Apoptosis assays. Apoptosis assays were performed using the Annexin V-FITC Apoptosis Detection Kit (Nacalai Tesque, Inc, Kyoto, Japan) and measured by flow cytometry. Cells were harvested by treating with trypsin-EDTA, washed with PBS, and centrifuged at 1,500 rpm for 3 min. The cell pellets (5.0×10⁵ cells) were resuspended in 100 µl of binding buffer containing 5 µl of Annexin V-fluorescein isothiocyanate and 5 µl of propidium iodide, incubated for 15-30 min in the dark on ice, and then fluorescence was acquired using a BD FACSVerser and FACSuite software (Becton Dickinson, Franklin Lakes, NJ, USA). The flow cytometry data were analyzed by FlowJo ver.10.7.1 software.

RNA extraction and quantitative real-time reverse transcription-PCR. Total RNA was extracted from cultured cells using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (21). The RNA was reverse-transcribed to cDNA using a ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The primers, cDNA, and THUNDERBIRD Probe qPCR Mix (Toyobo) were mixed together, and qPCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, San Francisco, CA, USA). *Plk1* (Hs00983227) and *TS* (Hs00426586) expression was measured using the TaqMan Gene Expression Assay (Thermo Fisher Scientific). *GAPDH* (Hs02786624) expression was considered as the standard against which to normalize relative expression of the genes of interest. Gene expression levels were quantified as 2^{-ΔΔC_t} values (21).

Microarray analysis. Gene expression microarray analysis was performed using the GeneChip Human Gene 2.0 Sense Target array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. Microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through the GEO series accession number GSE165029.

Transfection of small-interfering RNA (siRNA). siRNA experiments were performed using Silencer Select Negative Control siRNA as the negative control (4390844, Thermo Fisher Scientific). Pre-Designed Silencer Select siRNAs were used to knockdown PLK1 (4390824, Thermo Fisher Scientific) and TS (4392420, Thermo Fisher Scientific). siRNA and Lipofectamine R1MAX Reagent were dissolved in Opti-MEM Media (Thermo Fisher Scientific) at a final concentration of siRNA complexes of 20 nM. The transfection medium was replaced after 6 h, and cells were then incubated at 37°C for a further 48 h.

Tumor cell implantation experiments. Female severe combined immunodeficient (SCID)-Beige mice (CAnN.Cg-Foxn1^{nu}/CrJrlj) were purchased at 5 weeks of age from Charles River Laboratories Japan Inc., Kanagawa, Japan. A total of 5.0×10⁶ PC-9 cells was suspended in 200 µl of Matrigel (CORNING Inc., Corning, NY, USA)/PBS (Nacalai Tesque, Inc) and subcutaneously injected into the flanks of 6-week-old mice. When tumor volumes reached an average size of 200 mm³, the mice were randomized into the following four groups (n=6 for each group): vehicle control [per os (*p.o.*), once a day], pemetrexed [intraperitoneal (*i.p.*), at 100 mg/kg, twice a week], osimertinib (*p.o.*, at 1.0 mg/kg, once a day), or both osimertinib (*p.o.*, 1.0 mg/kg, once a day) and pemetrexed (*i.p.*, at 100 mg/kg, twice a week). Treatments were administered until the end of the 29-day observation period. Body weights were measured once a week. Tumor volumes (V) were calculated twice a week by

caliper measurements of the width (W) and length (L) of each tumor ($W^2 \times L/2$). Extraction of tumor proteins for immunohistochemical analysis was performed using samples from mice at the end of the 29-day treatment period. The study protocol was approved by the Ethics Committee on the Use of Laboratory Animals, KAC Co., Ltd (Shiga, Japan) (approval no. 20-0313). All experiments on live vertebrates were carried out in accordance with relevant guidelines and regulations. On the last observation day, the abdominal aorta of the mice was severed under 5% isoflurane anesthesia and the mice were euthanized by hemorrhage. The study was performed in compliance with the Animal research: Reporting of *in vivo* experiments (ARRIVE) guidelines.

Immunohistochemical analysis. PLK1 expression was detected in deparaffinized formalin-fixed, paraffin-embedded tissue sections (3 μ m) using a PLK/PLK1 antibody. Immunohistochemical analysis (IHC) was performed as previously described (22). The sections were stained overnight at 4°C with rabbit anti-human PLK/PLK1 antibody (P53350, ReyBiotech, Norcross, GA, USA) at a final dilution of 1:500. For human specimens, written informed consent was obtained from the patient, and the specimens were collected in accordance with the Declaration of Helsinki 2013. The study protocol was approved by the Ethics Committee Review Board at Nippon Medical School Hospital (approval no. 25-05-299 and B-2020-286).

Statistical analysis. Data were expressed as the mean $\pm$ standard error (SE) of three independent experiments. Differences between the mean values of the two groups were assessed with unpaired Student's *t*-test. Differences among more than two groups were assessed by one-way analysis of variance (ANOVA) followed by the Tukey test. A *p*-value of <0.05 was considered to be significant.

Results

Increased apoptotic activity caused by the combination of osimertinib and pemetrexed in NSCLC cells with EGFR mutations. We first examined the effects of osimertinib alone or pemetrexed alone on the proliferation of PC-9 and H1975 cells, which both harbor *EGFR* mutations. These cell lines were highly sensitive to osimertinib, with IC_{50} values of 0.069 ± 0.0079 and 0.085 ± 0.016 μ mol/l, respectively. Pemetrexed was also a potent inhibitor of proliferation with IC_{50} s of 0.080 ± 0.0053 μ mol/l and 0.080 ± 0.013 μ mol/l, respectively.

Next, we compared the anti-proliferative effects of the combined treatment with osimertinib and pemetrexed, using the IC_{50} doses established above. We found that the anti-proliferative effect of the combined treatment was significantly greater than that with either alone for both PC-9 and H1975 cells (Figure 1A). We examined the effects of drug combinations on apoptosis and found increased levels of cleaved PARP and BIM proteins in cells treated with osimertinib+pemetrexed (Figure 1B). Osimertinib+pemetrexed caused increased apoptotic activity as documented by increased proportions of annexin V-positive cells (Figure 1C). These results indicate that combining osimertinib with pemetrexed increases apoptosis and thus leads to enhanced anti-proliferative activity of NSCLC cells with *EGFR* mutations.

Long-lasting anti-tumor effect of osimertinib combined with pemetrexed in NSCLC cells with mutated EGFR. We assessed whether a combination of osimertinib with pemetrexed prevents the acquisition of resistance to the former. PC-9 and H1975 cells were exposed to osimertinib with or without pemetrexed at half the IC_{50} dose of the drugs, and then their concentrations were slowly increased with time in culture. After 2 months, the IC_{50} -values for osimertinib alone and the combination of osimertinib and pemetrexed in PC-9 cells were 3.34 ± 0.29 μ mol/l and <0.001 μ mol/l, respectively (Figure 2A). In H1975, these IC_{50} -values were 3.58 ± 0.82 μ M and 0.0072 ± 0.0041 μ M, respectively, after 3 months (Figure 2B). These results showed that combining osimertinib with pemetrexed significantly hinders the emergence of osimertinib resistance relative to monotherapy in these *EGFR*-mutant NSCLC cells.

We further investigated whether osimertinib+pemetrexed combination therapy enhanced cytotoxicity to PC-9 *in vivo*, in mouse xenograft models. The animals exhibited none of the toxicity otherwise seen with high doses and regularly gained body weight (data not shown). The combination of osimertinib and pemetrexed (group 3) inhibited tumor growth significantly more than pemetrexed alone (group 1). Group 3 also tended to inhibit tumor growth more than osimertinib alone (group 2) (Figure 2C). Thus, combining osimertinib with pemetrexed mediated enhanced *in vivo* antitumor effects against xenografts harboring the *EGFR* mutation, consistent with the results obtained *in vitro*.

Inhibition of thymidylate synthase does not result in apoptosis of EGFR-mutant NSCLC cells treated with pemetrexed. To investigate the molecular mechanisms of the synergistic effects of combined osimertinib and pemetrexed, we assessed the expression of the transcription factor thymidylate synthase (TS) and *EGFR* signal pathway mediators at the protein level in PC-9 and H1975 cells. The levels of phosphorylated *EGFR*, AKT, and ERK were markedly reduced by osimertinib, as determined by western blotting (Figure 3A). A previous study reported that *EGFR*-TKI irreversibly inhibit TS expression, which is well known as a therapeutic target of pemetrexed (23). Accordingly, we evaluated the effect of osimertinib on TS expression and found that it was significantly reduced at both the mRNA and protein level in PC-9 and H1975 cells in a dose-dependent manner (Figure 3A and B). Therefore, we hypothesized that single-agent osimertinib suppressed TS expression, resulting in enhanced anti-proliferative interactions with pemetrexed. We next examined whether the inhibition of TS protein expression enhanced the pro-apoptotic activity of pemetrexed in these cells. However, we found that TS-specific siRNA treatment did not enhance the apoptotic activity in PC-9 cells treated with pemetrexed, as assessed by the Annexin V flow cytometry assay (Figure 3C).

Figure 1. The effect of a combination of osimertinib and pemetrexed on inducing apoptosis of PC-9 and H1975 cells. (A) PC-9 and H1975 cells were incubated for 72 h with osimertinib or pemetrexed or a combination of both at doses equivalent to their IC_{50} values. The anti-proliferative effect of the combination treatment was significantly increased relative to osimertinib or pemetrexed alone. Data are means $\pm$ SEM from three independent experiments (* p <0.05, ** p <0.01, *** p <0.001). (B) Western blots of proteins representing apoptosis markers. Higher expression of cleaved PARP and BIM is seen in PC-9 and H1975 cells treated with the drug combination. (C) Apoptosis assay performed using an Annexin V-FITC Apoptosis Detection Kit. Percentages of apoptotic cells are higher with combination treatment (* p <0.05, ** p <0.01, *** p <0.001).

Figure 2. Osimertinib in combination with pemetrexed delays the appearance of drug resistance with long-lasting effects. (A) PC-9 and (B) H1975 cells were exposed to half IC_{50} doses of drugs, either osimertinib alone or both osimertinib and pemetrexed, and then treated with increasing concentrations of osimertinib. Time to resistance was significantly delayed by combination treatment. The mean of three biologically independent samples per time point is shown. (C) PC-9 cells were subcutaneously injected into mice and when the tumors had reached an average size of 200 mm^3 the animals were treated with vehicle alone, osimertinib alone, pemetrexed alone or both osimertinib and pemetrexed. Combination treatment enhanced cytotoxicity to PC-9 in vivo (** $p < 0.01$, *** $p < 0.001$). Data represented as the mean $\pm$ SEM ($n=6$).

Figure 3. Effects of thymidylate synthase (TS) on the anti-tumor effects of osimertinib in combination with pemetrexed. (A) Protein levels of p-EGFR, EGFR, p-AKT, AKT, p-ERK, ERK and TS were analyzed by western blotting. (B) TS expression by PC-9 and H1975 cells after exposure to osimertinib for 24 h was suppressed as determined by quantitative real-time reverse transcription PCR (qRT-PCR; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (C) TS expression was significantly decreased after TS-specific siRNA treatment. Percentages of apoptotic cells in PC-9 cells treated with TS-specific siRNA after treatment with pemetrexed at their half IC_{50} doses for 48 h.

The combination of osimertinib and pemetrexed enhances apoptosis via a PLK1 signaling pathway. To clarify the molecular mechanisms underlying the enhanced apoptotic activity of the combination therapy, we used GeneChip microarrays to profile gene expression in PC-9 and H1975 cells treated with osimertinib with or without pemetrexed (Figure 4A). This revealed that Polo-like kinase 1 (PLK1), AURKA and HIST1H2 were significantly down-regulated in both cell lines treated with osimertinib and pemetrexed. Network analysis was performed to provide a perspective on the apoptotic function of these three genes. *PLK1* as well as *AURKA* were shown to be important genes associated with apoptotic activity (Figure 4B). Previous studies indicated that PLK1 plays an important role in cell cycle control and apoptosis in human cancer cells (24-26). We confirmed that treatment with the combination of osimertinib and pemetrexed resulted in significant down-regulation of PLK1 in PC-9 and H1975, as documented both by qRT-PCR and western blotting (Figure 4C). Furthermore, we quantified PLK1 protein in PC-9 and H1975 after the cell lines had been treated with osimertinib and pemetrexed for 1-3 months. Reduced PLK1 protein expression and increased cleaved PARP was still seen in PC-9 and H1975 cells treated with both drugs for 2-3 months. In contrast, the levels of these two proteins were already increased in cells treated with osimertinib alone (Figure 5A). We next analyzed PLK1 expression by immunohistochemical staining in tumors derived from PC-9 in the mouse xenograft model. We found that the number of PLK1-negative tumor cells was significantly higher in the osimertinib and pemetrexed-treated group (Figure 5B). To elucidate the potential mechanisms of PLK1 involvement in regulating apoptotic activity, we evaluated whether knocking down PLK1 would induce apoptosis of these *EGFR*-mutant NSCLC cells. Indeed, treatment with *Plk1*-specific siRNA did enhance apoptosis as assessed by western blotting and flow cytometry (Figure 5C and D).

These findings suggest that treatment with a combination of osimertinib+pemetrexed delayed the up-regulation of PLK1 and resulted in enhanced induction of apoptosis, thereby delaying the emergence of drug-resistance relative to monotherapy.

PLK1 over-expression in osimertinib+pemetrexed-resistant EGFR-mutant cells and patients. We have established osimertinib-resistant PC-9 (PC-9 OsiR) and osimertinib+pemetrexed-resistant PC-9 (PC-9OsiPEMR) cell lines (Figure 6A). In both of these, we found that p-EGFR was markedly reduced, but p-AKT and p-ERK were expressed, suggesting the involvement of an *EGFR*-independent signaling pathway (Figure 6B). In contrast, PLK1 was strongly over-expressed in both PC-9OsiR and PC-9OsiPEMR (Figure 6B). Importantly, we also investigated PLK1 expression in *EGFR*-mutant NSCLC samples from a patient before and after osimertinib+pemetrexed therapy. This patient had previously received several different

EGFR-TKI and chemotherapies including gefitinib, erlotinib, afatinib, osimertinib, and pemetrexed (27). Interestingly, increased PLK1 expression was observed after resistance to osimertinib and pemetrexed had been acquired (Figure 6C). Thus, PLK1 up-regulation is implicated in the acquired resistance to osimertinib+pemetrexed in *EGFR* mutants, both in cell lines and in a patient, independent of *EGFR* signaling.

PLK1 inhibitors induce apoptosis of osimertinib-resistant cell lines, thus overcoming resistance to this drug. Finally, we tested whether PLK1 inhibition induced apoptosis of *EGFR* mutant NSCLC cells in order to evaluate the potential of PLK inhibitors as novel therapeutic agents. PLK1 knockdown by *Plk1*-specific siRNA resulted in increased expression of apoptotic markers and increased numbers of Annexin V-positive cells, suggesting enhanced apoptotic activity (Figure 7A and B). We next examined whether PLK1 inhibitors could overcome osimertinib+pemetrexed resistance in *EGFR*-mutant NSCLC cells. We selected volasertib (BI6727) as the PLK-1 inhibitor in this experiment. A previous study had reported that volasertib could overcome resistance to the *EGFR*-TKI erlotinib in NSCLC cells (28). We found that volasertib significantly inhibited the proliferation of both PC-9OsiR and PC-9OsiPEMR cells (Figure 7C). We also found increased levels of cleaved PARP proteins in cells treated with volasertib, reflecting the activation of apoptosis (Figure 7D). PLK1 inhibitors may therefore represent promising agents for treating *EGFR*-mutant NSCLC resistant to osimertinib+pemetrexed.

Discussion

In this study, we demonstrated that the combination of osimertinib and pemetrexed induced more apoptosis and enhanced anti-tumor effects compared to monotherapy in *EGFR*-mutant NSCLC *in vitro* and *in vivo*. Several *EGFR*-dependent or independent osimertinib resistance mechanisms are known, including *MET* amplification, C797S mutation, *HER2* amplification, *PIK3CA* and *RAS* mutations (29). However, specific therapeutic strategies to overcome resistance to osimertinib in the clinical setting for curative *EGFR*-mutant NSCLC treatment have not yet been established. It has been proposed that combining *EGFR*-TKIs including gefitinib and osimertinib with cisplatin/carboplatin and pemetrexed in NSCLC patients might represent a strategy to overcome acquired resistance (14-16). Our findings provide evidence for the feasibility of this approach in *EGFR*-mutant NSCLC patients.

We have ultimately identified PLK1 as a therapeutic target for combination therapy and as a promising treatment target for osimertinib-resistant tumors in *EGFR*-mutated NSCLC. PLK1 is highly over-expressed in various different malignancies including glioma, head and neck cancer, ovarian cancer, prostate cancer, breast cancer, and NSCLC (30-35). Increased PLK1 expression was correlated with poor

Figure 4. Down-regulation of polo-like kinase 1 (plk1) is associated with antitumor effects of the combination of osimertinib and pemetrexed. (A) Gene expression profiles of PC-9 and H1975 cells treated with osimertinib with or without pemetrexed. Heat map of genes whose expression fluctuated more than 3-fold. (B) Network analysis for apoptosis regulation showing the importance of PLK1 and AURKA genes associated with apoptosis. (C) Expression of plk1 in PC-9 and H1975 cells after exposure to osimertinib and pemetrexed for 24 h. Suppression compared with osimertinib or pemetrexed alone as determined by qRT-PCR is depicted (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). PLK1 protein was analyzed by western blotting.

Figure 5. Combination treatment delayed the up-regulation of PLK1 and resulted in enhanced induction of apoptosis. (A) PLK1 expression in cells treated by both osimertinib and pemetrexed before the emergence of resistance, relative to monotherapy. (B) PLK1 expression in the PC-9 xenografted mouse tumor was analyzed by immunohistochemistry (IHC). Hematoxylin and eosin (HE) staining and IHC staining of PLK1 are shown. Staining xenografted mouse tumor treated with osimertinib and pemetrexed for PLK1 (scale bar=100 μ m). (C) Levels of apoptosis markers in PC-9 and H1975 cells treated with *plk1*-specific siRNA. (D) Percentages of apoptotic cells in PC-9 and H1975 cells treated with *plk1*-specific siRNA (* p <0.05).

Figure 6. *PLK1* up-regulation is implicated in acquired resistance to osimertinib and pemetrexed in *EGFR*-mutant cells and in a patient. (A) MTT assays showing that PC-9OsiR and PC-9OsiPEMR are resistant to osimertinib. (B) Protein levels of p-*EGFR*, p-AKT, p-ERK, and *PLK1* in PC-9OsiR and PC-9OsiPEMR were analyzed by western blotting. (C) HE staining in *EGFR*-mutant lung cancer specimens from an NSCLC patient before (upper left) and after (upper right) osimertinib+pemetrexed therapy. Immunohistochemical staining for *PLK1* before treatment (lower left) and after osimertinib and pemetrexed therapy (lower right) (scale bar=100 μm).

prognosis in NSCLC and breast cancer (36, 37). *PLK1* is one of a family consisting of five members, *PLK1*, -2, -3, -4, and -5 and has a key role in cell-cycle regulation, including mitotic entry, centrosome maturation, bipolar spindle maturation, activation of cyclin and cyclin-dependent kinases, spindle assembly, and chromosome separation (38, 39). *PLK1* also modulates DNA damage responses, including recovery

from DNA damage checkpoints. *PLK1* over-expression emphasizes its tumor-promoting activity. *PLK1* inhibition has been shown to cause mitotic blockade and to result in apoptosis (26, 40, 41). Therefore, *PLK1* down-regulation induced by combination therapy of osimertinib+pemetrexed contributes to enhanced apoptosis and its inhibition might have therapeutic value for *EGFR*-mutant NSCLC patients.

Figure 7. Inhibition of PLK1 induced apoptosis in osimertinib-resistant cell lines and inhibition of proliferation. (A) Levels of apoptosis markers in PC-9OsiR and PC-9OsiPEMR cells treated with *plk1*-specific siRNA. (B) Percentage of apoptotic cells in PC-9OsiR and PC-9OsiPEMR cells treated with *plk1*-specific siRNA (* $p < 0.05$, ** $p < 0.01$). (C) Cell viability of PC-9OsiR and PC-9OsiPEMR in response to volasertib (25 nM) compared to osimertinib alone (100 nM) for 72 h (* $p < 0.05$, ** $p < 0.01$). (D) Volasertib-induced cleaved PARP expression in PC-9OsiR and PC-9OsiPEMR cells.

In this study, we showed that the PLK1 inhibitor volasertib has potential as a novel therapeutic agent for *EGFR*-mutant NSCLC patients. Volasertib was granted orphan drug designation for acute myeloid leukemia (AML) in 2014 after showing promising efficacy in clinical trials (42, 43). In lung cancer, erlotinib-resistant T790M-negative *EGFR*-mutated NSCLC cells with epithelial-mesenchymal transition properties had higher sensitivity to volasertib, which caused G₂/M arrest and apoptosis. Erlotinib-resistant T790M-positive *EGFR*-mutated NSCLC cells had a higher sensitivity to the combination of erlotinib and volasertib than to either agent alone. PLK1 inhibition together with erlotinib treatment could enhance DNA damage and apoptosis because of inhibiting DNA repair *via* EGFR inhibition (28). These results are consistent with our findings that EGFR-TKI-resistant cells are sensitive to volasertib independent of EGFR signaling. Our study demonstrates a possible pivotal role for volasertib in treating osimertinib-resistant or osimertinib+pemetrexed-resistant cells.

Our study had some limitations. First, we could evaluate PLK1 over-expression after osimertinib+pemetrexed therapy in only one clinical sample. In general, re-biopsy after EGFR-TKI therapy is difficult because of the small number of areas that can be biopsied. Further studies need to be performed to evaluate PLK1 expression after osimertinib+ pemetrexed treatment using more clinical samples. A second limitation is that although we showed that combination therapy delayed the development of resistance, eventually PLK1 expression increased and the tumor became drug resistant. Further studies are needed to determine the optimal timing of PLK1 inhibitor use and whether combining it with osimertinib may be curative.

In conclusion, we found that PLK1 down-regulation, associated with pro-apoptotic and anti-proliferative activity, is seminally involved in the synergistic effects of osimertinib+pemetrexed combination therapy for *EGFR*-mutated NSCLC both *in vitro* and *in vivo*. Furthermore, PLK1 over-expression may be a critical mechanism responsible for acquired resistance of *EGFR*-mutated NSCLC cells to osimertinib or both osimertinib and pemetrexed treatment. These results suggest that PLK1 inhibition might be a novel therapeutic strategy to overcome acquired resistance.

Conflicts of Interest

M. Seike has received a commercial research grant from Eli Lilly Japan K.K and has received speakers' bureau honoraria from AstraZeneca and Eli Lilly Japan K.K.

Authors' Contributions

Conception and design: N. Takano, M. Seike, T. Sugano; Acquisition of data: N. Takano, M. Seike, K. Matsuda; Analysis and interpretation of data: All Authors; Drafting the manuscript or revising it critically for important intellectual content: N. Takano, M. Seike, T. Sugano, A.

Gemma; Final approval of the version to be published: All Authors; Agreement to be accountable for all aspects of the work: All Authors.

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