ORIGINAL ARTICLE

Phosphoproteomic Analysis Identified Mutual Phosphorylation of FAK and Src as a Mechanism of Osimertinib Resistance in *EGFR*-Mutant Lung Cancer

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ABSTRACT

Introduction: Osimertinib is a standard treatment for patients with *EGFR*-mutant NSCLC. Although some osimertinib resistance mechanisms have been identified, nearly 50% of the mechanisms remain to be elucidated. This study was aimed at identifying non-genetic mechanisms underlying osimertinib resistance.

Methods: We established two osimertinib-resistant cell lines from *EGFR* mutation-positive PC-9 and HCC827 NSCLC cell lines (PC-9OR and HCC827OR, respectively) using a stepwise method. We compared the phosphoproteomic profiles of the osimertinib-resistant and parental cells using mass spectrometry. Upstream kinases were identified using the application Kinase Enrichment Analysis version 3.

Results: Phosphoproteomic analysis revealed 80 phosphorylation sites that were mutually up-regulated in PC-9OR and HCC827OR cells. The Kinase Enrichment Analysis version 3 analysis identified focal adhesion kinase (FAK) and proto-oncogene tyrosine-protein kinase Src (Src) as upstream kinases of these up-regulated phosphoproteins. The small-interfering RNA–mediated knockdown of FAK reduced Src phosphorylation and that of Src reduced FAK phosphorylation in both cell lines. Furthermore, FAK- or Src-specific small-interfering RNA treatments restored EGFR phosphorylation in PC-9OR and HCC827OR cells. The combination of FAK and Src inhibitors inhibited PC-9OR and HCC827OR cell proliferation in vitro and suppressed tumor growth in a xenograft mouse model. Immunohistochemistry of tumors from patients with *EGFR*-mutant NSCLC suggested that phosphorylated FAK and Src are involved in initial and acquired resistance to osimertinib.

Conclusions: Phosphoproteomic analysis may help elucidate the mechanisms of resistance to molecular-targeted therapies in lung cancer. Mutual phosphorylation of FAK and Src is involved in osimertinib resistance. Thus, FAK and Src inhibition may be novel treatment strategies for osimertinib-resistant NSCLC.

Keywords: Non–small cell lung cancer; EGFR; Osimertinib; Resistant; Src; FAK

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Introduction

Lung cancer is the leading cause of cancer-related death worldwide.[1] NSCLC accounts for 85% of all lung cancers and has a poor prognosis.[2] Nevertheless, the discovery of gene mutations and fusion genes, such as *EGFR* mutations and *ALK* rearrangement, and the development of molecular-targeted therapies have greatly improved the prognosis of NSCLC.[3]

In the FLAURA trial, osimertinib, a third-generation EGFR tyrosine kinase inhibitor (TKI), prolonged overall survival compared with first-generation EGFR TKIs (gefitinib or erlotinib) in patients with advanced *EGFR*-mutant NSCLC.[4] In the ADAURA trial, osimertinib markedly prolonged disease-free survival compared with placebo as adjuvant chemotherapy in patients with *EGFR*-mutant NSCLC.[5] Therefore, osimertinib has become the standard of treatment not only for advanced stage but also for postoperative treatment in patients with *EGFR*-mutant NSCLC. Nevertheless, treatment for disease recurrence and progression after osimertinib treatment remains limited. The resistance mechanism of osimertinib has been investigated, and treatments based on the findings are being considered.[6] To elucidate the resistance mechanism, however, most studies analyzed genetic mutations using next-generation sequencing, which has revealed several genetic resistance mechanisms, such as *EGFR* C797S, *EGFR* amplification, *HER2* mutation, and *MET* amplification.[7,8] Approximately 50% of the resistance mechanisms remain unclear, particularly the non-genetic ones.[7–11]

Recent advances in proteomic analysis have led to the identification of therapeutic targets for cancers.[12] Phosphoproteomics has made remarkable progress, making it possible to comprehensively analyze phosphorylation at approximately 20,000 sites in a single analysis.[13] Protein phosphorylation, a post-translational modification, directly affects several biological processes including cell growth and survival in cancer cells, and specific kinases that trigger protein phosphorylation could be therapeutic targets in cancer.[14] Hence, investigation of protein phosphorylation by phosphoproteomic analysis could be useful for identifying signaling cascades and specific kinases related to drug resistance.

Therefore, we performed a phosphoproteomic analysis of cancer cell lines with the aim to profile kinome activity and identify osimertinib resistance mechanisms and novel treatment targets.

Materials and Methods

Cell Lines and Reagents

Two types of parent adenocarcinoma cell lines with deletions in *EGFR* exon 19 (PC-9 and HCC827) were used in this study. PC-9 cells were obtained from Immuno-Biological Laboratories, and HCC827 cells were purchased from the American Type Culture Collection. Defactinib was used as a potent and selective inhibitor of focal adhesion kinase (FAK), whereas saracatinib was used as a potent and selective inhibitor of the proto-oncogene tyrosine-protein kinase Src (Src). Osimertinib, defactinib, and saracatinib were purchased from Selleck Chemical (Houston, TX).

Cell Culture

All cells were cultured in RPMI 1640 medium (FUJIFILM Wako Pure Chemical Co.) containing 10% fetal bovine serum (FBS; BioWest, Nuaille, France) and 1% penicillin and streptomycin (FUJIFILM Wako Pure Chemical Industries) at a temperature of 37°C and a CO_2 concentration of 5%.[9,10] All cells were routinely screened for the absence of mycoplasma.

Cell Viability Assay

Briefly, 2000 cells (100 μL) were plated in 96-well plates in six replicates.[9,10] After 72 hours of drug treatment, the cells were incubated with 10 μL of the Cell Counting Kit-8 (FUJIFILM Wako Pure Chemical Co.) for 90 minutes. Thereafter, absorbance was measured at 450 nm using a microplate reader. Data were plotted using GraphPad Prism software version 8.4.3 (GraphPad Software, La Jolla, CA), and the IC_{50} values were calculated using a sigmoidal dose-response curve. The combined effects of defactinib and saracatinib were evaluated using SynergyFinder Plus, a web application designed to statistically analyze the synergistic effects of dual anticancer drugs based on the results of cell viability assay.[15] The following four models of synergy were evaluated using SynergyFinder Plus: zero interaction potency (ZIP) model, Loewe additivity model, highest single-agent model (HSA), and Bliss independence model.

Establishment of Osimertinib-Resistant Cells

We established osimertinib-resistant PC-9 (PC-9OR) and osimertinib-resistant HCC 827 (HCC827OR) cell lines using a stepwise method, as previously reported.[9,10] Using the calculated IC_{50} values, we confirmed that the two cell lines were resistant to osimertinib. To sustain osimertinib resistance in PC-9OR and HCC827OR cells, osimertinib was administered periodically throughout the passage cycles of the cells.

Phosphoproteomic Analysis

Phosphoproteomic analysis was performed to compare the two *EGFR*-mutant cell lines (PC-9 and HCC827) and their respective osimertinib-resistant cell lines (PC-9OR and HCC827OR). Phosphoproteomic

analysis using liquid chromatography–tandem mass spectrometry was performed as previously reported[16] on an UltiMate 3000 Nano LC system (Thermo Fisher Scientific) and an HTC-PAL autosampler (CTC Analytics) connected to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). Buffer A (0.1% formic acid and 2% acetonitrile) was graded with 5% to 30% buffer B (0.1% formic acid and 90% acetonitrile) for 85 minutes for phosphoproteome and for 45 minutes for phosphotyrosine proteome analysis. The setup of the Q Exactive Plus mass spectrometer and MS data analysis was similar to that previously reported.[12,16]

Western Blotting Analysis

Cell collection, protein extraction, two-dimensional polyacrylamide gel electrophoresis, and transfer to immunoblotting polyvinylidene difluoride membranes were performed as previously described.[17,18] The membranes were incubated with primary antibodies overnight at 4°C. Thereafter, the membranes were incubated with a species-specific HRP-conjugated secondary antibody for 1 hour at room temperature.[17,18] The primary antibodies were as follows: EGFR (2232, Cell Signaling Technology; 1:1000); phospho-EGFR (p-EGFR) (phospho-Tyr1068) (2234, Cell Signaling Technology; 1:1000); AKT (9272, Cell Signaling Technology; 1:1000); phospho-AKT (phospho-Ser473) (4060S, Cell Signaling Technology; 1:1000); ERK (9102, Cell Signaling Technology; 1:1000); phospho-ERK (phospho-Thr202 and Tyr204) (9101, Cell Signaling Technology; 1:1000); Src (2108, Cell Signaling Technology; 1:1000); phospho-Src (phospho-Tyr527) (2105, Cell Signaling Technology; 1:1000); FAK (3285, Cell Signaling Technology; 1:1000); phospho-FAK (phospho-Tyr576 and 577) (3281, Cell Signaling Technology; 1:1000); cleaved PARP (5625, Cell Signaling Technology; 1:1000); and GAPDH (sc-47724, Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000). All antibodies were diluted in 5% bovine serum albumin (FUJIFILM Wako Pure Chemical Industries)/TBS-T (NACALAI TESQUE, Inc.) or 3% skim milk (Sigma-Aldrich)/TBS-T. Between each step, the membranes were washed thrice (10–30 min each time) with TBS-T. The immunoreactive bands were visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc., Class A Common Stock). Chemiluminescent signals were measured using an Amersham Imager 600 (Global Life Sciences Technologies Japan K.K.). ImageJ software (https://imagej.nih.gov/ij/download.html) was used for quantitative image analysis.

siRNA Transfection

Small-interfering RNA (siRNA)–targeting protein tyrosine kinase 2 (PTK2), Src, and negative control siRNA were purchased from Thermo Fisher Scientific. The siRNA complexes were transfected into cells at 40 nM and mixed with Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) in OPTI-MEM (1×) (Thermo Fisher Scientific), in accordance with the manufacturer's instructions. The transfection medium was replaced 24 hours after transfection, and the cells were incubated at 37°C for 60 hours.

Xenograft Mouse Model

Female mice with severe combined immunodeficiency were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan).[19] Approximately 5.0×10^6 PC-9OR cells were suspended in 200 μL of Matrigel (CORNING Inc., Corning, NY)/phosphate-buffered saline and then injected subcutaneously into the gluteal region of 6-week-old mice. When the tumor volume exceeded 200 mm^3, the mice were randomly assigned to one of the following four groups (n = 4 per group): vehicle control (per os [PO], once daily), defactinib (PO, 25 mg/kg once daily), saracatinib (PO, 25 mg/kg once daily), and defactinib (PO, 25 mg/kg once daily) plus saracatinib (PO, 25 mg/kg once daily). Tumor volume was calculated using the following formula: $L \times W^2/2$, where L is the long diameter and W is the short diameter of the tumor. All animal experiments were approved by the Laboratory Animal Center of the Nippon Medical School (approval number, 2022-042). Animal studies were performed according to the Animal Research Reporting of in vivo Experiment (ARRIVE) guidelines.

Immunohistochemistry

After deparaffinization of the tissue sections, antigens were retrieved by microwaving the tissue sections in Universal HIER antigen retrieval reagent (10×) (Abcam). After removing endogenous peroxidase using 3% hydrogen peroxide water/methanol, the tissue sections were incubated with the following primary antibodies at the appropriate dilution: phospho-Src (phospho-Tyr527) (2105, Cell Signaling Technology; 1:50) and phospho-FAK (phospho-Tyr576 and 577) (orb5211, Biorbyt Ltd.; 1:200). After incubation with a secondary antibody using a Histofine Simple Stain MAX-PO(MULTI) kit (Nichirei), peroxidase activity was visualized using the DAB reaction. Sections were counterstained with hematoxylin. All sections were stained with hematoxylin and eosin. The use of patient specimens was approved by the Ethics Committee of Nippon Medical School (approval number B-2022-603). Informed consent from individuals was obtained using the method of opt-out on the website according to instructions by the Ethics Committee of Nippon Medical School.

Kinase Enrichment Analysis

Upstream kinases were identified using Kinase Enrichment Analysis version 3 (KEA3). KEA3 is a web application that infers upstream kinases whose substrates are overrepresented in the list of phosphorylated proteins.[20]

Statistical Analysis

All data are presented as means ± SD. Statistical significance was determined using the standard Student's *t* test. Statistical significance was set at *p* less than 0.05.

Results

Phosphoproteomic Analysis of Parent Cells and Osimertinib-Resistant Cells

The sensitivity of PC-9 and HCC827 cells to osimertinib was confirmed using the MTS cell proliferation assay. The IC_{50} values were 0.007 to 0.001 μmol/L for PC-9 cells and 0.0002 to 0.0003 μmol/L for HCC827 cells (Fig. 1*A*). Osimertinib-resistant strains were established using a stepwise method, and both cell lines became resistant after 6 months. The IC_{50} values were 1.927 ± 0.124 μmol/L for PC-9OR cells and 2.807 ± 0.267 μmol/L for HCC827OR cells (Fig. 1*A*). Secondary mutations including T790M and C797S were not observed in the whole exome sequencing. In addition, EGFR minor mutations, such as EGFR L718Q and G796D, and novel mutations, such as MET amplification and BRAF V600E, were not observed in the two resistant NSCLC cells.[10]

To identify the mechanism of osimertinib resistance, phosphoproteomic analysis was performed to compare the phosphoprotein signal levels between the parental and resistant strains. Phosphoproteomic analysis included 17,853 phosphorylation sites of proteins (Supplementary Table 1). Figure 1*B* and *C* reveals the results of the phosphoproteomic analysis of parent and osimertinib-resistant cells. We extracted phosphorylation sites satisfying a false discovery rate less than 0.05 and a fold change greater than or equal to 2. Comparing the PC-9OR and PC-9 cells, 423 phosphorylation sites were identified, whereas 1329 phosphorylation sites were identified by comparing HCC827OR and HCC827 cells (Fig. 1*B* and *C*). Of these, there were 80 phosphorylation sites that were overlapping (Supplementary Table 2). We performed KEA to identify the upstream kinases responsible for the phosphorylation of the 80 proteins (Fig. 1*D*). Among the top 10 factors (Src, ABL1, MAPKB, PTK2, SYK, FYN, MAPK3, EGFR, PRKCD, and CDK5) identified using the mean rank method of KEA3, we focused on Src, which was the highest-ranked factor and PTK2 (i.e., FAK), which was also identified using the phosphoproteomic analysis. The top 50 upstream kinases identified using the mean-rank method are listed in Supplementary Table 3.

Mutual Phosphorylation of FAK and Src in Osimertinib-Resistant Cell Lines

Figure 2*A* reveals the results of protein expression analysis of the parental and resistant strains using Western blotting. Both PC-9OR and HCC827OR cells had decreased phosphorylation of EGFR and increased phosphorylation of FAK and Src compared with that in the parental strains.

Next, to investigate the reciprocal relationship between FAK and Src, we performed siRNA treatment of FAK and Src and evaluated the changes in protein expression using Western blotting (Fig. 2*B*). FAK inhibition suppressed FAK expression by almost 80% in the PC-9OR strain and by approximately 55% to 80% in the HCC827OR strain, as assessed using densitometry. FAK-specific siRNA treatment of PC-9OR and HCC827OR cells also reduced Src phosphorylation. Src inhibition suppressed Src expression by approximately 80% in the PC-9OR strain and by almost 55% to 80% in the HCC827OR strain, as assessed using densitometry. Src-specific siRNA treatment of PC-9OR and HCC827OR cells also reduced FAK phosphorylation. These results suggested that FAK and Src are mutually phosphorylated. Furthermore, treatment with Src-specific siRNA restored EGFR phosphorylation in PC-9OR and HCC827OR cells.

We also evaluated the effects of FAK and Src inhibitors on the viability of PC-9OR and HCC827OR cells. Defactinib and saracatinib were used as inhibitors of FAK and Src, respectively. Combined treatment with defactinib and saracatinib markedly inhibited the phosphorylation of FAK and Src compared with treatment with defactinib or saracatinib alone (Fig. 2*C*). Moreover, the combined treatment with defactinib and saracatinib inhibited the phosphorylation of AKT and ERK and increased the expression of cleaved PARP (Fig. 2*C*). In PC-9OR cells, defactinib and saracatinib monotherapy suppressed cell viability to 60.7% and 63.8%, respectively, compared with the vehicle group (Fig. 2*D*). In HCC827OR cells, defactinib and saracatinib monotherapy suppressed cell viability to 60.8% and 71.8%, respectively, compared with the vehicle group (Fig. 2*D*). The combined treatment with defactinib and saracatinib markedly suppressed cell viability to 38.8% in PC-9OR cells and to 24.6% in HCC827OR cells (Fig. 2*D*). Src knockdown using siRNA enhanced the inhibitory effect of defactinib on the cell viability of PC-9OR and HCC827OR cells (Fig. 2*E*). FAK knockdown using siRNA enhanced the inhibitory effects of saracatinib on PC-9OR and HCC827OR cells (Fig. 2*E*).

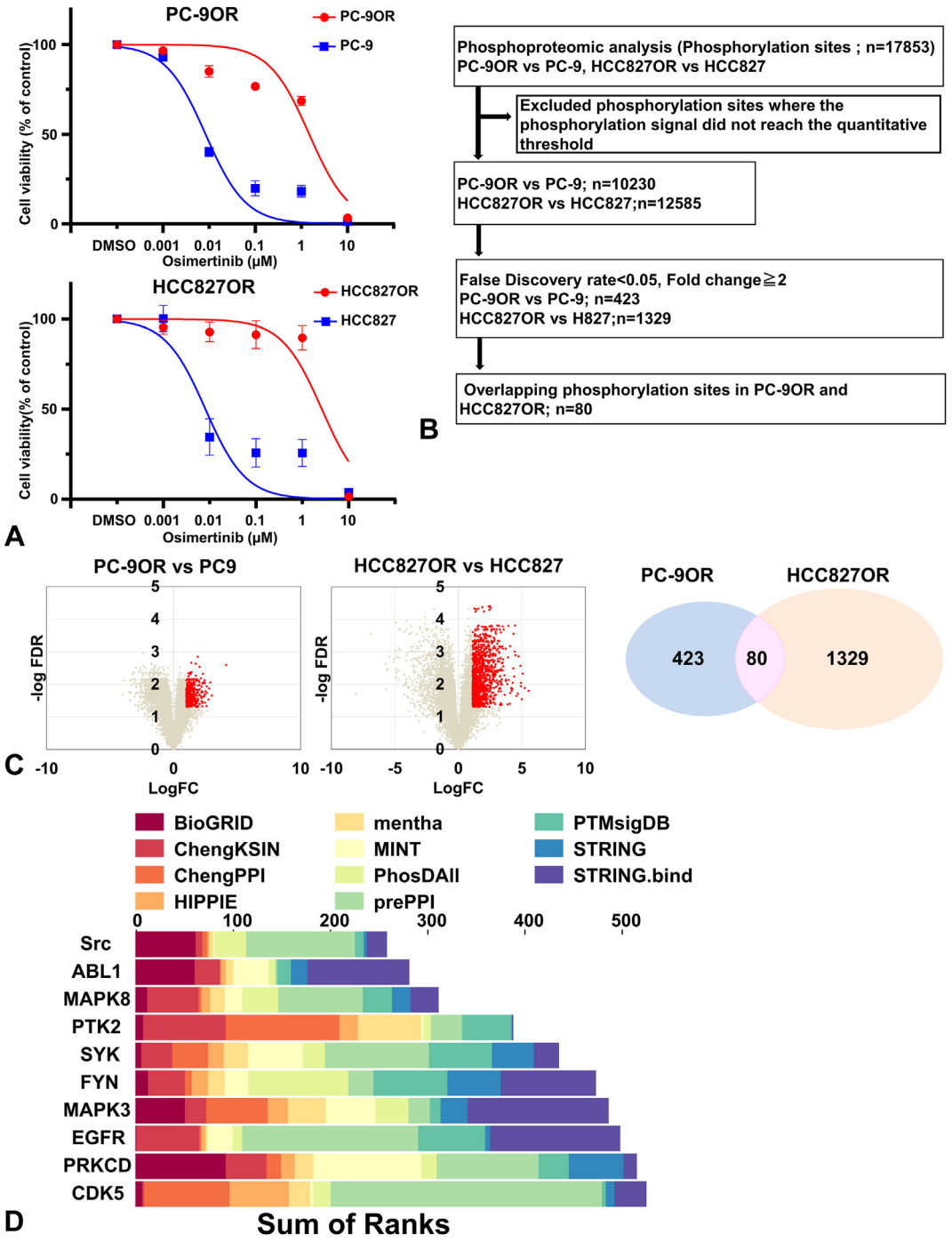

Figure 1. Phosphoproteomic analysis in parent cells (PC-9 and HCC827) and osimertinib-resistant cells (PC-9OR and HCC827OR). (*A*) MTS cell proliferation assay of PC-9, HCC827, PC-9OR, and HCC827OR cells. The assay was performed after 120 hours of incubation. Error bars: SD. (*B*) Flow chart of the phosphoproteomic analysis. (*C*) Phosphorylation sites with phosphorylation signals of false discovery rate less than 0.05 and fold change greater than or equal to 2 (PC-9OR versus PC-9; 423 factors, HCC827OR versus HCC827; 1329 factors, overlapping phosphorylation sites; 80). (*D*) Upstream kinase estimated using Kinase Enrichment Analysis version 3.

Next, to investigate whether the combined effects of defactinib and saracatinib were additive or synergistic, we performed a cell viability assay using various drug concentrations and calculated synergy scores using SynergyFinder plus (Supplementary Fig. 1). With the increasing concentration of saracatinib, the inhibitory effect of defactinib on cell viability revealed an increasing trend at the same concentration. For PC-9OR cells, all four synergy scores were more than 0 (ZIP = 3.7; Loewe = 11.71; HSA = 14.16; Bliss = 3.72).

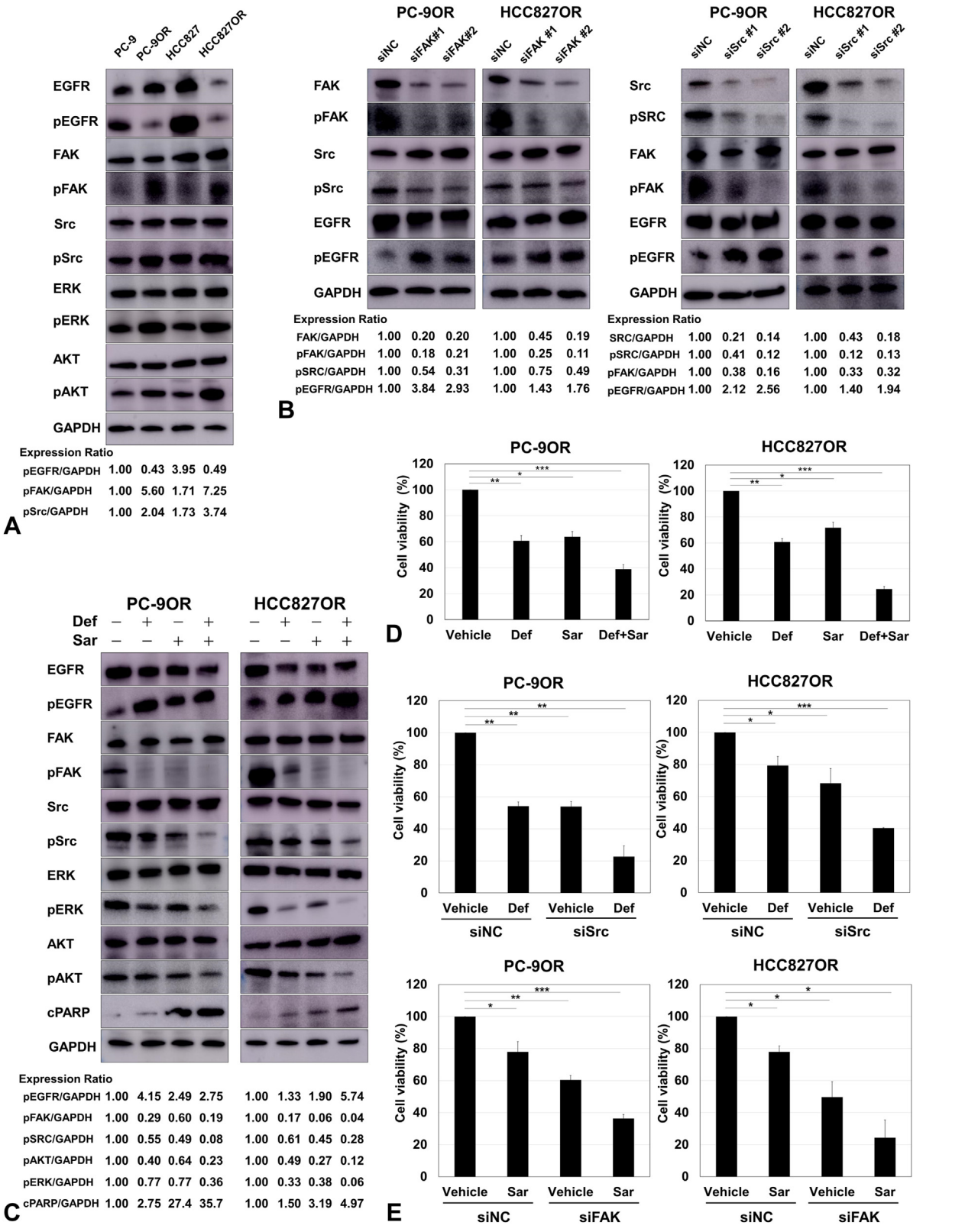

Figure 2. Mutual phosphorylation of FAK and Src is involved in osimertinib resistance in PC-9OR and HCC827OR cells. (*A*) Immunoblotting of EGFR, FAK, and Src signaling pathway molecules. (*B*) Immunoblotting after siRNA treatment. siNC, siRNA negative control. (*C*) Immunoblotting of indicated proteins in PC-9OR and HCC827OR cells treated with DMSO, 2 μmol/L Def, 1 μmol/L Sar, or a combination of both for 24 hours. (*D*) Cell viability assay of osimertinib-resistant cells (PC-9OR and HCC827OR) treated with 2 μmol/L Def, 1 μmol/L Sar, or a combination of both. Cell viability is illustrated as a percentage compared with control. The assay was performed after 120 hours of incubation. Error bars: SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (*E*) Cell viability assay of PC-9OR and HCC827OR cells treated with 2 μmol/L Def or 1 μmol/L Sar after siRNA treatment. Def, defactinib; Sar, saracatinib. Cell viability is illustrated as a percentage compared with control. The assay was performed after 120 hours of incubation. Error bars, SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

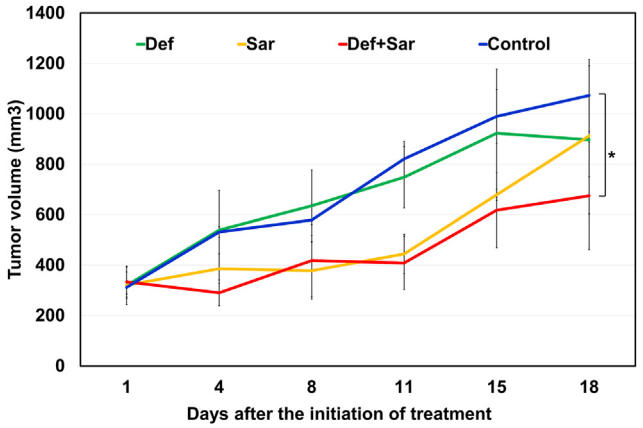

Figure 3. Effect of combined treatment with defactinib and saracatinib on tumor growth in vivo. PC-9OR-derived tumor-bearing mice were randomized into control, Def, Sar, or Def plus Sar combination treatment groups. Tumor size was measured to calculate the tumor volume (mm^3). Values indicate the average tumor volume plus SD in each group. *$p < 0.05$ for the combination of Def plus Sar versus control. Def, defactinib; Sar, saracatinib.

Similarly, for HCC827OR cells, all four synergy scores were more than 0 (ZIP = 4.4; Loewe = 13.6; HSA = 14.86; Bliss = 4.76). These results suggested a weak synergistic effect of defactinib and saracatinib. Moreover, we evaluated the inhibitory effect of osimertinib in combination with defactinib and saracatinib on cell proliferation. In PC-9OR cells, treatment with osimertinib in combination with defactinib and saracatinib did not result in any significant difference in cell growth inhibition compared with that in the treatment with defactinib and saracatinib (34.0% versus 31.4%, $p = 0.218$) (Supplementary Fig. 2). Similarly, in HCC827OR cells, treatment with osimertinib in combination with defactinib and saracatinib did not result in any significant difference in cell growth inhibition compared with that in the treatment with defactinib and saracatinib (34.9% versus 34.5%, $p = 0.947$) (Supplementary Fig. 2).

Combination of Defactinib and Saracatinib Suppresses Tumor Growth in a Xenograft Mouse Model

Next, we evaluated the antitumor effects of defactinib and saracatinib in vivo using a xenograft mouse model. No apparent toxicity or weight loss was observed in the animals. Combined treatment with defactinib and saracatinib markedly inhibited tumor growth compared with treatment with defactinib or saracatinib alone (Fig. 3). Thus, combined treatment with defactinib and saracatinib enhanced the antitumor effects of the drugs in xenograft mouse models established using osimertinib-resistant cells.

Elevated Phosphorylation of FAK and Src in Patient-Derived Tumor Specimens After Osimertinib Resistance

We also evaluated the FAK and Src phosphorylation status using immunohistochemical staining in seven patients with *EGFR*-mutant NSCLC whose paired tumor specimens were available before the initiation of osimertinib treatment and after the establishment of osimertinib resistance. Figure 4 reveals the relationship between FAK and Src phosphorylation and the efficacy of osimertinib. Patients with elevated FAK phosphorylation in tumor specimens before osimertinib treatment initiation (patient no. 1–4) had progression-free survival (PFS) of less than 8 months in all cases. In contrast, the PFS of patients whose tumor specimens did not have FAK or Src phosphorylation before treatment initiation (patient no. 5–7) tended to be longer. In two of these three patients, both phosphorylated FAK and Src expression levels were elevated after osimertinib resistance. Figure 5A–D reveals the increased phosphorylation of FAK and Src in tumor tissues after osimertinib resistance compared with that before osimertinib treatment. Data from another patient with elevated phosphorylation of FAK and Src in tumors after osimertinib resistance are also revealed (Supplementary Fig. 3).

Discussion

We investigated the non-genetic mechanism of EGFR TKI resistance, specifically osimertinib resistance, and identified novel targets using phosphoproteomic analysis of osimertinib-resistant lung cancer cell lines. Previous studies on the mechanisms underlying EGFR TKI resistance have mainly focused on DNA and RNA. Unlike previous studies, we conducted a comprehensive analysis of phosphoproteins to identify involved kinases, which may directly reflect therapeutic targets after EGFR TKI resistance. This study revealed that the mutual phosphorylation of FAK and Src is involved in osimertinib resistance and could be a therapeutic target after osimertinib resistance in patients with *EGFR*-mutant NSCLC. Both osimertinib-resistant cell lines used in this study had suppressed EGFR phosphorylation, suggesting that FAK and Src are activated through EGFR-independent mechanisms.

Although genetic analysis has been widely performed to identify drug resistance mechanisms in lung cancer, many resistance mechanisms remain unclear.[6] Phosphoproteomics analysis can assess dynamic changes caused by treatment and post-translational modifications that are difficult to assess using genetic analysis.[21,22] Phosphoproteomic analysis has revealed novel therapeutic targets against cetuximab-resistant colorectal cancer cells by revealing active kinase candidates

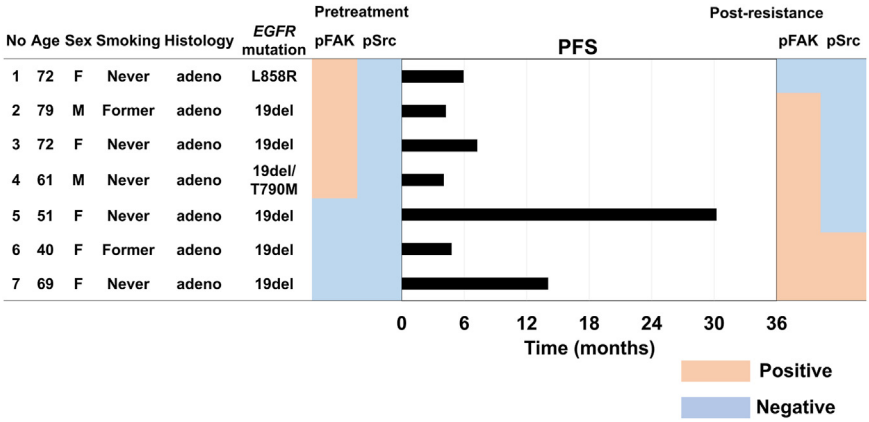

Figure 4. Phosphorylation of FAK and Src before and after osimertinib therapy and the efficacy of osimertinib treatment. Patient characteristics, status of phosphorylation of FAK and Src in tumor tissues before and after treatment and progression-free survival in patients with NSCLC who received osimertinib.

from the comparison of cetuximab-sensitive and -resistant colorectal cancer cells.[12] Thus, phosphoproteomic analysis could be useful to identify drug resistance mechanisms and novel therapeutic targetable kinases in cancer.

In this study, Src phosphorylation was suppressed by FAK knockdown, whereas FAK phosphorylation was suppressed on Src knockdown. These results suggested that FAK and Src are mutually phosphorylated in osimertinib-resistant cancer cells. Previous studies have also revealed that the mutual phosphorylation of FAK and Src plays an important role in the survival of malignant tumors.[23–26] The autophosphorylation of FAK creates a high-affinity binding site for the Src homology 2 domain of Src kinase.[27] The binding of Src to FAK leads to the formation of an active FAK-Src complex in which the active Src kinase phosphorylates FAK.[28] Src-mediated phosphorylation of Tyr576 and Tyr577 domains activates FAK.[26,28] Fully activated FAK in turn directly phosphorylates Src.[29] The FAK-Src complex

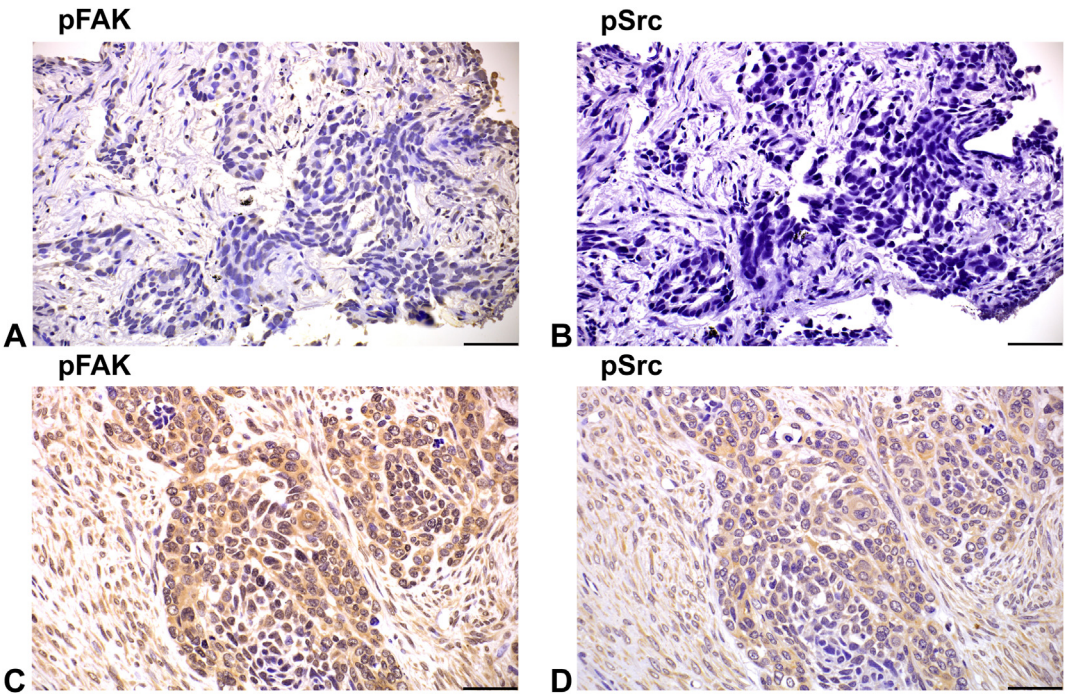

Figure 5. Immunohistochemistry of patients' tumor tissue (patient no. 6). (*A*, *B*) Immunohistochemistry of patients' tumor tissue obtained before osimertinib treatment. (*A*) phosphor FAK (pFAK) and (*B*) phosphor Src (pSrc). (*C*, *D*) Immunohistochemistry of patients' tumor tissue after acquiring resistance to osimertinib. (*C*) Phosphor FAK (pFAK) and (*D*) phosphor Src (pSrc). Scale bars: 50 μm.

leads to mutual protein kinase activation and activates downstream signaling pathways involved in the proliferation, survival, invasion, and migration of cells.[30–32] In this study, we found cases of increased Src and FAK phosphorylation after development of osimertinib resistance in some clinical specimens. Therefore, mutual phosphorylation of FAK and Src is suggested as a mechanism of osimertinib resistance. Mutual phosphorylation of FAK and Src has been suggested as a new mechanism of osimertinib resistance.

This study revealed activation of FAK and SRC in osimertinib-resistant cell lines, whereas previous studies have revealed that activation of FAK and SRC is also associated with survival from initial osimertinib treatment in EGFR TKI-naive *EGFR*-mutant NSCLC cell lines. Increased phosphorylation of Src family kinases and FAK after osimertinib treatment maintains the AKT and MAPK signaling pathways during continuous osimertinib treatment in osimertinib-sensitive cell lines.[33] Osimertinib treatment activates signal transducer and activator of transcription 3 (STAT3) and Src-YES-associated protein 1 (YAP1) signaling in osimertinib-sensitive cell lines.[34] Activation of Src-YAP1 and STAT3 during osimertinib treatment limits therapeutic response and leads to survival past the first EGFR TKI therapy.[34] These previous studies suggest that activation of FAK and SRC occurs as a compensatory response to EGFR TKI treatment and induces osimertinib resistance. Moreover, *EGFR*-mutant NSCLC cells co-express and activate non-RTKs, especially Src, YES, and FAK.[35] In line with these previous findings, the results of this study support the notion that osimertinib treatment induces an increase in the phosphorylation of existing FAK and SRC, leading to cell survival during osimertinib treatment and osimertinib resistance. This is consistent with previous reports revealing that a switch to another kinase dependence is involved in TKI resistance.[36,37]

Both defactinib and saracatinib monotherapies were found to have limited efficacy in vitro and in vivo. A previous study evaluating the effect of FAK inhibitors in *EGFR*-mutant cell lines also revealed the limited efficacy of single-agent defactinib in both TKI-sensitive and TKI-resistant cell lines.[38] Although a phase II study revealed that defactinib was well tolerated in patients with pretreated advanced *KRAS*-mutant NSCLC, defactinib alone had modest clinical efficacy.[39] Dasatinib, another Src inhibitor, also had limited efficacy as a single agent in EGFR TKI-resistant cell lines.[40] In a phase II trial, saracatinib alone had limited efficacy in patients with NSCLC.[41] Nevertheless, this study revealed that the combination of defactinib and saracatinib was highly effective in inhibiting cell proliferation. The inhibitory effect of defactinib on cell proliferation was enhanced after siSrc treatment compared with that after siNC treatment, and that of saracatinib was also enhanced after siFAK treatment compared with that after siNC treatment. Importantly, the synergy scores for the defactinib and saracatinib combination underscored a synergistic interaction. These results suggest that the efficacy of FAK and Src inhibition are synergistic, not additive.

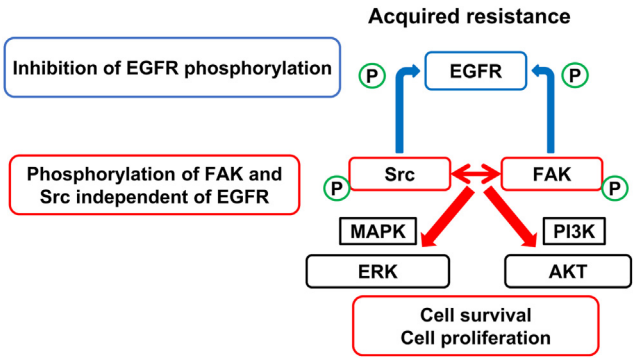

Figure 6. Schematic representation of the resistant mechanism induced by mutual phosphorylation of FAK and Src. Proposed mechanism of osimertinib resistance in which mutual phosphorylation of FAK and Src occurs in an EGFR-independent manner, activating downstream signaling and resulting in cell proliferation and survival.

FAK promotes cancer growth through activation of the PI3K-AKT cascade and up-regulation of cyclin D1 by activation of ERK.[42,43] Src also promotes tumor growth and proliferation through activation of the PI3K-AKT and RAS-MAPK pathways.[44–46] This study revealed that phosphorylation of AKT and ERK was strongly suppressed in osimertinib-resistant strains when treated with a combination of defactinib and saracatinib. In vivo, the defactinib and saracatinib combination was also effective in inhibiting tumor growth. In clinical specimens, patients with tumors without FAK and Src phosphorylation tended to have longer PFS, but FAK and Src phosphorylation were elevated in tumors after osimertinib resistance. These findings further support the idea that mutual phosphorylation of FAK and Src is a potential mechanism of osimertinib resistance.

Figure 6 reveals a schematic diagram of the resistance mechanism induced by the reciprocal phosphorylation of FAK and Src. We hypothesize that the mutual phosphorylation of FAK and Src, which occurs independently of EGFR, leads to cell proliferation and survival. Furthermore, we hypothesize that mutual phosphorylation of FAK and Src suppresses upstream EGFR phosphorylation.

Adding osimertinib to a combination of FAK and SRC inhibitors might be considered. Combination therapy with Src family kinase inhibitors and osimertinib was reported to be more effective than osimertinib alone in EGFR TKI-naive *EGFR*-mutant NSCLC cells.[33–35]

Nevertheless, addition of osimertinib to the defactinib plus saracatinib combination did not enhance the inhibition of cell viability in this study. The osimertinib-resistant cell lines used in this study developed resistance during continued osimertinib treatment. The continuation of osimertinib after the development of osimertinib resistance had limited efficacy. A phase III trial investigating the efficacy of adding platinum-based chemotherapy after gefitinib resistance in patients with *EGFR*-mutant NSCLC revealed that the addition of EGFR TKI to chemotherapy did not have any survival benefit.[47] Nevertheless, osimertinib plus MET-TKI such as tepotinib had meaningful overall response rates and clinical outcomes for patients with *EGFR*-mutant and MET-amplification NSCLC after first-line osimertinib in clinical trials such as INSIGHT 2 (NCT03940703).[48] Moreover, the phase III SACHI and SAFFRON trials investigated the efficacy of combining osimertinib with savolitinib versus platinum-based chemotherapy in patients with NSCLC with MET amplification or overexpression, post-resistance to osimertinib or other EGFR TKIs (NCT05015608, NCT05261399). The efficacy and safety of osimertinib plus MET-TKI after the osimertinib resistance are currently being investigated. In the FLAURA2 trial, first-line treatment with osimertinib–chemotherapy revealed significantly longer PFS compared with that of osimertinib monotherapy in patients with *EGFR*-mutant NSCLC (hazard ratio for disease progression or death, 0.62; 95% confidence interval, 0.49–0.79; $p < 0.001$).[49] Therefore, the addition of osimertinib to a combination of FAK and SRC inhibitors may also be considered in the first-line setting as a therapeutic strategy to overcome innate resistance to osimertinib. A clinical trial (TOTEM study) to evaluate the safety, preliminary efficacy, and pharmacokinetic and pharmacodynamic profiles of repotrectinib and osimertinib is ongoing (NCT047772235). This study has several limitations. The study was based on a small clinical sample size, and the extent to which the mutual phosphorylation of Src and FAK is responsible for osimertinib resistance in real-world clinical practice remains unclear. We were not able to evaluate genetic abnormalities (EGFR C797S or MET amplification) associated with resistance using the clinical samples. More clinical specimens are needed to clarify the involvement of FAK and SRC in the development of osimertinib resistance in clinical practice. Moreover, the efficacy and safety of Src and FAK combination therapy have not been investigated in patients with lung cancer, and further studies using FAK and Src inhibitors are needed to clarify this. For clinical application of our results, comprehensive pharmacodynamic data obtained using the patient-derived xenograft model should be evaluated in future studies.

In conclusion, phosphoproteomic analysis may be useful for elucidating the mechanisms underlying lung cancer resistance to molecular-targeted therapies. Mutual phosphorylation of FAK and Src is involved in osimertinib resistance. Hence, the inhibition of FAK and Src may be a novel treatment for osimertinib-resistant NSCLC.

CRediT Author Contribution Statement

Takehiro Tozuka: Conceptualization, Methodology, Validation, Investigation, Analysis and interpretation of data, Writing—Original Draft, Writing—Review and Editing.

Rintaro Noro: Conceptualization, Methodology, Validation, Investigation, Analysis and interpretation of data, Writing—Original Draft, Writing—Review and Editing, Study supervision.

Keisuke Yoshida: Analysis and interpretation of data, Writing—Review and Editing.

Satoshi Takahashi: Methodology, Writing—Review and Editing.

Mariko Hirao: Methodology, Investigation, Writing—Review and Editing.

Kuniko Matsuda: Methodology, Investigation, Writing—Review and Editing.

Yasuhiro Kato: Writing—Review and Editing.

Shinji Nakamichi: Writing—Review and Editing.

Susumu Takeuchi: Writing—Review and Editing.

Masaru Matsumoto: Writing—Review and Editing.

Akihiko Miyanaga: Writing—Review and Editing.

Shinobu Kunugi: Investigation, Writing—Review and Editing.

Kazufumi Honda: Analysis and interpretation of data, Writing—Review and Editing, Study supervision.

Jun Adachi: Methodology, Investigation, Writing—Review and Editing.

Masahiro Seike: Conceptualization, Methodology, Analysis and interpretation of data, Writing—Review and Editing, Study supervision.

Disclosure

Dr. Tozuka has received honoraria from Chugai Pharmaceutical and AstraZeneca. Dr. Noro has received honoraria from Chugai Pharmaceutical Co., Ltd., AstraZeneca K.K., and Glaxo Smith Klein, and has received 17KK0177 Fund for the Promotion of Joint International Research (Fostering Joint International Research). Dr. Nakamichi has received honoraria from AstraZeneca, Chugai Pharmaceutical, Bristol-Myers Squibb/Ono Pharmaceutical, Taiho Pharmaceutical, Takeda, Merck Sharp & Dohme, and Kyowa Kirin. Dr. Miyanaga has received honoraria from AstraZeneca, Nippon Kayaku, Merck Pharmaceutical, Kyowa Kirin, and Pfizer, Inc. Dr.

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Ethical Statement

All animal experiments were approved by the Laboratory Animal Center of the Nippon Medical School (approval number, 2022-042). The use of patient specimens was approved by the Ethics Committee of Nippon Medical School (approval number B-2022-603). Informed consent from individuals was obtained using the method of opt-out on the website according to instruction by the Ethics Committee of Nippon Medical School.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *JTO Clinical and Research Reports* at www.jtocrr.org and at https://doi.org/10.1016/j.jtocrr.2024.100668.

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