KRAS and EGFR Amplifications Mediate Resistance to Rociletinib and Osimertinib in Acquired Afatinib-Resistant NSCLC Harboring Exon 19 Deletion/T790M in EGFR

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Abstract

The critical T790M mutation in EGFR, which mediates resistance to first- and second-generation EGFR tyrosine kinase inhibitors (TKI; gefitinib, erlotinib, and afatinib), has facilitated the development of third-generation mutation-selective EGFR TKIs (rociletinib and osimertinib). We previously reported heterogeneous afatinib-resistant mechanisms, including emergence of T790M-EGFR, and responses to third-generation EGFR TKIs. Here, we used afatinib-resistant lung adenocarcinoma cells [AfaR (formerly AFR3) cells], carrying exon 19 deletion/T790M in EGFR. To identify the novel resistance mechanisms in post-afatinib treatment, RocR1/RocR2 and OsiR1/OsiR2 cells were established using increasing concentrations of rociletinib and osimertinib, respectively. Attenuation of exon 19 deletion and T790M was confirmed in both rociletinib-resistant cells; in addition, EGFR and KRAS amplification was observed in RocR1 and RocR2, respectively. Significant KRAS amplification was observed in the osimertinib-resistant cell lines, indicating a linear and reversible increase with increased osimertinib concentrations in OsiR1 and OsiR2 cells. OsiR1 cells maintained osimertinib resistance with KRAS amplification after osimertinib withdrawal for 2 months. OsiR2 cells exhibited KRAS attenuation, and osimertinib sensitivity was entirely recovered. Phospho-EGFR (Y1068) and growth factor receptor-bound protein 2 (GRB2)/son of sevenless homolog 1 (SOS1) complex was found to mediate osimertinib resistance in OsiR1 cells with sustained KRAS activation. After 2 months of osimertinib withdrawal, this complex was dissociated, and the EGFR signal, but not the GRB2/SOS1 signal, was activated. Concomitant inhibition of MAPK kinase and EGFR could overcome osimertinib resistance. Thus, we identified a heterogeneous acquired resistance mechanism for third-generation EGFR TKIs, providing insights into the development of novel treatment strategies.

Introduction

EGFR tyrosine kinase inhibitors (TKI) of the first (gefitinib and erlotinib) and second (afatinib) generations are administered in patients with non–small cell lung cancer (NSCLC) with activating mutations in EGFR as a first-line therapy. Several clinical trials have shown that the response rates to these therapies are 70% to 80%, and the progression-free survival time is 9 to 14 months (1–3). Mutations in the EGFR gene are present in approximately 32% of Asians and 7% of patients of other ethnicities with NSCLC (4). Somatic mutations in the tyrosine kinase domain of EGFR, including in-frame deletions in exon 19 (exon 19 del) and the L858R point mutation in exon 21, are common mutations accounting for 80% to 90% of EGFR mutations in NSCLC (4). Unfortunately, most patients administered EGFR TKIs develop acquired resistance after 1 to 2 years, accompanied by secondary mutations in EGFR, the emergence of bypass signaling with MET and human EGFR 2 (HER2), and transformation to small-cell lung cancer (SCLC, refs. 2, 3–7). The most common resistance mutation is substitution of methionine for threonine at position 790 (T790M), which is detected in approximately 50% to 60% of patients with NSCLC who exhibit acquired resistance to first- and second-generation EGFR TKIs (8, 9). To overcome acquired resistance due to the emergence of T790M mutations in EGFR, third-generation or T790M-selective EGFR TKIs, including osimertinib (10) and rociletinib (11), have been developed; these drugs inhibit activating mutations and T790M in EGFR in preclinical and clinical settings (12, 13).

Third-generation EGFR TKIs, such as rociletinib and osimertinib, are oral drugs that covalently and irreversible bind to Cys797 in the ATP-binding site of EGFR. Osimertinib has received approval for the treatment of patients with metastatic,
Acquired Third-Generation EGFR-TKI Resistance Mechanisms

EGFR T790M mutation-positive NSCLC who have progressed during or after EGFR-TKI therapy. Although these third-generation EGFR TKIs were active in patients with NSCLC bearing the EGFR T790M mutation in clinical trials (10, 14), development of acquired resistance has still limited the efficacy of this treatment. Improving our understanding of resistance mechanisms to third-generation EGFR TKIs should increase survival rates in patients with NSCLC harboring EGFR mutations and facilitate the development of novel therapeutics to overcome resistance. Recently, the acquisition of two secondary EGFR mutations, C797S and L789I, has been reported in patients with NSCLC with recurrence during osimertinib treatment (15, 16). Although resistance mechanisms for third-generation EGFR TKIs are not fully understood, bypass pathway activation by MET or HER2 (17, 18), MAPK pathway activation by KRAS or MEK mutations (19) has been reported in clinical and preclinical studies.

We previously reported three distinct resistance mechanisms to afatinib in PC-9 lung adenocarcinoma cells, including cells bearing EGFR T790M (designated AFR3 cells; refs. 20). AFR3 cells are sensitive to third-generation EGFR TKIs, including osimertinib and rociletinib. Therefore, in this study, we aimed to elucidate the mechanisms of resistance to the third-generation EGFR TKIs rociletinib and osimertinib using PC-9 afatinib-resistant lung adenocarcinoma cells [designated AfaR (formerly named AFR3)] by stepwise dose escalation. Our findings may lead to the development of new therapeutic options for patients with EGFR-mutant lung cancer.

Materials and Methods

Cell lines, antibodies, and reagents
PC-9 human NSCLC cells were established from a previously untreated patient and donated by K. Hayata (Tokyo Medical College, Tokyo, Japan) during the 1980s. PC-9 cells were cultured in RPMI1640 medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) in a 5% CO2 incubator at 37°C. Cells were passaged for less than 4 months prior to retrieval from frozen stocks. The cell lines used in this study were authenticated by short tandem repeat analysis at the Japanese Collection of Research Bioresources Cell Bank in 2016 and tested for Mycoplasma using a MycoAlert Mycoplasma Detection Kit in 2017 (Lonza). The following antibodies were purchased from Cell Signaling Technology: anti-total EGFR antibodies (#4267), anti–phospho-EGFR (Y1068) antibodies (#3777), anti-EGFR (E746-A750del) antibodies (#2085), anti-total HER2 antibodies (#4290), anti-total ERBB3 antibodies (#4754), anti-total ERBB4 antibodies (#2285), anti-total insulin-like growth factor receptor 1 (IGF1R) antibodies (#3018), anti–phospho-IGF1R antibodies (#3024), anti-total MET antibodies (#8198), anti-total AKT antibodies (#2272), anti–phospho-AKT (S473) antibodies (#2971), anti-total ERK1/2 antibodies (#9102), anti–phospho-ERK1/2 antibodies (#4370), anti–phospho-MAPK kinase (MEK1/2) antibodies (#2338), anti–cleaved PARP antibodies (#5625), and anti–β-actin antibodies (#4970). Antibodies targeting sonic hedgehog 1 (SOS1; sc-17793), growth factor receptor–bound protein 2 (GRB2; sc-8034), KRAS (sc-30), HRAS (sc-29), and NRAS (sc-31) were purchased from Santa Cruz Biotechnology. Cetuximab was purchased from Showa University Hospital (Tokyo, Japan). Afnatinib was provided by Boehringer-Ingelheim, and other inhibitors were obtained from Selleck Chemicals.

Establishment of afatinib-resistant PC-9 (AfaR) cells harboring the EGFR T790M mutation with acquired resistance to rociletinib and osimertinib

To obtain cell lines with acquired resistance, PC-9 cells were exposed to increasing concentrations of afatinib in the growth medium. Starting with a concentration that was one tenth of the half-maximal inhibitory concentration, the concentration was progressively increased over 10 to 12 months to 1 µmol/L afatinib. The resulting PC-9 afatinib-resistant cell line was designated AfaR cells (formerly AFR3 cells) and was maintained continuously in culture medium containing 1 µmol/L afatinib. EGFR exon 19 del and T790M in exon 20 were evaluated in AfaR cells, and the proliferation of these cells was effectively suppressed by rociletinib and osimertinib treatment. Next, to obtain acquired rociletinib- or osimertinib-resistant cell lines from AfaR cells, AfaR cells were exposed to increasing concentrations of rociletinib or osimertinib in the growth medium. Starting with a dose that was approximately 1/10th of the IC50, the dosage was progressively increased over 10 to 12 months to 1 µmol/L rociletinib or osimertinib. Two rociletinib-resistant and two osimertinib-resistant AfaR cells were obtained and designated RocR1 or RocR2 for rociletinib resistance and OsiR1 or OsiR2 for osimertinib resistance; these cells were maintained continuously in a culture medium containing 1 µmol/L rociletinib or osimertinib, respectively (Fig. 1A).

Cell proliferation assay

Cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (Promega), as described previously (21). Briefly, cells (5 × 104/well) were seeded in 96-well plates and incubated overnight, and assays were performed on days 0, 1, 2, 3, 5, and 6. To inhibit cell proliferation, 5 × 104 cells per well were seeded in 96-well plates and incubated overnight, followed by continuous exposure to the indicated concentrations of inhibitor for 72 hours. The optical density at 570 nm (OD570) was then measured with a PowerScan HT microplate reader (BioTek) and expressed as a percentage of the value obtained from the control cells. We prepared 6 to 12 replicates, and the experiments were repeated at least three times. Data were graphically displayed using GraphPad Prism version 7.0 software (GraphPad, Inc.).

Western blot analysis

Treated cells were washed twice with ice-cold PBS and lysed with modified RIPA buffer consisting of 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, and 1.0% protease- and phosphatase-inhibitor cocktails (Sigma-Aldrich). Cell suspensions were centrifuged for 5 minutes at 1,200 rpm at 4°C, and protein concentrations were determined using bicinchoninic acid assays (Sigma-Aldrich). Equal amounts of protein were mixed and boiled in Laemmli buffer, and samples were separated by SDS-PAGE electrophoresis on 8% to 12% gels, followed by transfer to polyvinylidene difluoride membranes. Membranes were probed using the appropriate primary and secondary antibodies (diluted according to the manufacturers’ instructions, 1:1,000–2,000), were treated with enhanced chemiluminescence solution, and were then exposed to film. β-Actin...
and at least one additional protein were used as loading controls. All experiments were performed in triplicate.

**RAS pull-down assay**

Pull-down assays were performed using a glutathione S-transferase fusion protein corresponding to the human RAS-binding domain of RAF-1, which specifically binds to the GTP-bound form of RAS (EMD Millipore). Western blots were developed using anti-KRAS antibodies (F234; Santa Cruz Biotechnology).

**RNA interference**

Nontargeting (N/T) short interfering (si) RNA (controls) and SMARTpool siRNAs targeting EGFR (M003114), KRAS (M005069), SOS1 (M005194), and GRB2 (M019220) were purchased from Dharmacon. Cells were seeded in 6-well plates in

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**Figure 1.** Establishment of third-generation EGFR TKI–resistant cell lines from afatinib-resistant PC-9 (AfaR) cells. 

**A,** Schematic model describing the process of generating rociletinib- and osimertinib-resistant cell lines from afatinib-resistant PC-9 (AfaR) cells. 

**B,** Cells were seeded into 96-well plates at $5 \times 10^3$ cells/50 µL/well, preincubated overnight, followed by treatment with afatinib, rociletinib, and osimertinib at the indicated concentrations for 72 hours. MTT assays were then performed, and OD$_{570}$ values were obtained. Data represent means ± SEMs of data obtained from 6 to 12 replicate wells. 

**C,** Western blot analysis of basal levels of various proteins in PC-9, AfaR, RocR1, RocR2, OsiR1, and OsiR2 cells. β-Actin was included as a loading control. 

**D,** Cells were seeded into 96-well plates at $5 \times 10^3$ cells/100 µL/well. Following MTT assays, OD$_{570}$ values were obtained on days 0, 1, 2, 3, 4, 5, and 6.
Results

Characteristics of AfaR clones with acquired resistance to third-generation EGFR TKIs

We established two rociletinib- and osimertinib-resistant AfaR cell lines over 10 to 12 months (Fig. 1A). Afatinib-resistant PC-9 cells, called AfaR cells, harbored 15-bp deletions in exon 19 (exon 19 del) and the T790M mutation in EGFR, and the third-generation EGFR TKIs rociletinib and osimertinib effectively suppressed proliferation in AfaR cells (Fig. 1B). Notably, RocR1 and RocR2 cells were partially sensitive to afatinib compared with AfaR cells, whereas OsiR1 and OsiR2 cells exhibited resistance to afatinib and rociletinib (Fig. 1B). The EGFR E746-A750 del in exon 19 was decreased in RocR1 and RocR2 cells compared with that in AfaR cells, although similar expression levels were observed in OsiR1 and OsiR2 cells (Fig. 1C) and the other receptors HER2, ERBB3, ERBB4, MET, and IGF1R were not increased in these RocR and OsiR cells. With regard to cell proliferation, RocR1 and RocR2 cells showed significantly slower proliferation than parental AfaR cells. However, the proliferation rates of OsiR1 and OsiR2 cells were similar to that of AfaR cells (Fig. 1D). Thus, these findings suggested that the exon 19 del activating mutation in EGFR was greatly decreased in RocR1 and RocR2 cells but not in OsiR1 and OsiR2 cells (Table 1).

RocR1 and RocR2 cells exhibited KRAS and EGFR gene amplification with loss of EGFR mutations (exon 19 del and T790M)

Direct sequencing of EGFR exons 19 and 20 revealed partial loss of E746-A750del and T790M alleles (Supplementary Fig. S1A). Allelic quantitative distribution analyses using EGFR mutation-specific droplet digital PCR probes detected higher proportions of mutant EGFR alleles for exon 19 del and T790M than WT alleles in parental AfaR cells. Conversely, RocR1 and RocR2 cells exhibited significantly higher proportions of WT EGFR alleles compared with the parental AfaR cells (Table 1). Because the third-generation EGFR TKIs rociletinib and osimertinib were EGFR-mutation-selective inhibitors, RocR1 and RocR2 cells, with increased expression of WT EGFR, could prevent inhibition of EGFR phosphorylation. As shown in Fig. 2A, rociletinib completely suppressed EGFR phosphorylation and downstream signaling through AKT and ERK1/2 at 0.1 μmol/L. These changes led to induction of apoptosis, as observed by increases in cleavable PARP levels in AfaR cells. However, in RocR1 and RocR2 cells, rociletinib did not inhibit EGFR phosphorylation or AKT and ERK1/2 activation; therefore, induction of apoptosis was not observed. Afatinib, RocR1, and RocR2 cells were exposed to increasing concentrations of afatinib, and afatinib did not inhibit EGFR phosphorylation or

Table 1. The proportions of EGFR exon 19 del and T790M alleles in PC-9, AfaR, RocR1, RocR2, OsiR1, and OsiR2 cells, as measured by digital PCR analysis

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<td></td>
<td>Mutation rates (%)</td>
<td>mut/(mut + wt)</td>
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<td>OsiR2</td>
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FISH

KRAS and chromosome 12 FISH analyses were performed by Chromosome Science Lab Inc. using the KRAS/Chr12cen probe.

Statistical analysis

Data are presented as means ± SEMs and were analyzed using GraphPad Prism version 7.0 software (GraphPad, Inc.). Statistical significance was evaluated by two-tailed Student t tests and, unless otherwise noted, differences with P values of less than 0.05 were considered statistically significant.
AKT and ERK1/2 activation in AfaR cells. In RocR1 and RocR2 cells, afatinib effectively inhibited EGFR phosphorylation, and the downstream signaling was inhibited especially in RocR2 cells. Moreover, in RocR2 cells, induction of apoptosis was observed, indicating that afatinib sensitivity was recovered (Fig. 2B). Afatinib inhibited activating mutations and WT EGFR to a greater extent than EGFR T790M, and third-generation EGFR TKIs selectively activated activating EGFR mutations and T790M, but weakly inhibited WT EGFR (12, 13). Afatinib was administered to RocR1 cells at increasing concentrations, leading to concentration-dependent inhibition of EGFR and attenuation of downstream AKT and ERK1/2 phosphorylation. However, this suppression of AKT and ERK1/2 phosphorylation was modest (Fig. 2B) as compared with the levels observed following rociletinib administration to parental AfaR cells (Fig. 2A). Furthermore, elevated KRAS gene copy numbers and KRAS protein overexpression were observed in RocR1 cells, but not in PC-9, AfaR, and RocR2 cells (Supplementary Fig. S2A). In addition, each of the cell lines exhibited similar levels of NRAS and HRAS protein expression (Fig. 2C). Pull-down assays detected elevated KRAS activity in RocR1 cells (Fig. 2D) but not constitutive mutational activation of KRAS, which is indicative of WT KRAS exons 2–4 (Supplementary Fig. S2A; ref. 23).

As reported previously, the relationship between amplification and mutation of KRAS may be mutually exclusive (24). Although RocR1 cells did not show reduced cell proliferation when KRAS levels were downregulated by siRNA, additional treatment with afatinib in KRAS-knockdown cells significantly reduced cell proliferation (Fig. 2E). The attenuation of KRAS by siRNA reduced ERK1/2 activation, but AKT activation was modestly decreased. Although afatinib inhibited EGFR phosphorylation and downstream of AKT and ERK1/2 activation, when KRAS was downregulated by siRNA in RocR1 cells, AKT and ERK1/2 activation was markedly inhibited, and apoptosis was obviously induced (Fig. 2F). This suggested that EGFR signaling may still be activated for survival in RocR1 cells.

MAPK signaling is a well-known KRAS effector pathway, and targeting this pathway using MEK inhibitors has been shown to have limited activity in patients with KRAS mutations (25). In RocR1 cells, selumetinib, a MEK1/2 inhibitor (26), did not suppress cell proliferation, and afatinib alone suppressed cell proliferation only modestly. In combination with selumetinib, afatinib enhanced inhibition of cell proliferation (Supplementary Fig. S2B). Moreover, afatinib suppressed EGFR phosphorylation and AKT activation in a concentration-dependent manner, whereas ERK1/2 was still activated. Therefore, addition of selumetinib, a MEK inhibitor, to block ERK1/2 activation enhanced the induction of apoptosis (Supplementary Fig. S2C). Taken together, these results showed that loss of the rociletinib target (mutated EGFR [exon 19 del/T790M]) and KRAS amplification caused rociletinib resistance in RocR1 cells; combination of a MEK inhibitor and afatinib could overcome this resistance.

EGFR amplification and overexpression were observed in RocR2 cells (Figs. 1C and 2G). When EGFR was knocked down by siRNA in AfaR cells, downstream AKT and ERK1/2 activation was inhibited; furthermore, the induction of apoptosis was observed (Fig. 2H), and cell proliferation was inhibited (Fig. 2I). Moreover, RocR2 cells required EGFR expression and phosphorylation for survival, similar to AfaR cells. Therefore, afatinib administration inhibited cell proliferation, and addition of cetuximab enhanced suppression of cell proliferation, although cetuximab alone did not inhibit cell proliferation (Supplementary Fig. S2D). In RocR2 cells, afatinib inhibited the phosphorylation of EGFR in a concentration-dependent manner and downstream of AKT and ERK1/2, and addition of cetuximab enhanced the inhibition of EGFR/AKT/ERK1/2 and stimulated apoptosis (Supplementary Fig. S2E). Other studies have shown that ligands of EGFR increased, leading to selumetinib resistance; however, in RocR2 cells, EGFR ligands were not increased compared with that in AfaR cells (Supplementary Fig. S2F; ref. 27).

**KRAS amplification and overexpression were observed in OsiR1 and OsiR2 cells.**

In OsiR1 and OsiR2 cells, the distribution of EGFR mutations (exon 19 del and T790M) was similar to that in AfaR cells (Table 1). AfaR cells showed reduced phosphorylation of EGFR and activation of AKT and ERK1/2, and apoptosis occurred in response to osimertinib exposure in a concentration-dependent manner (Fig. 3A). In OsiR1 and OsiR2 cells, EGFR phosphorylation was sustained following osimertinib exposure; downstream AKT and ERK1/2 activation was also sustained (Fig. 3A). Moreover, in a xenograft model, osimertinib reduced the growth of xenograft tumors derived from AfaR cells, but not the growth of tumors derived from OsiR1 and OsiR2 cells (Fig. 3B). In addition, KRAS amplification and overexpression were observed in OsiR1 and OsiR2 cells (Fig. 3C and D). KRAS amplification in OsiR1 and OsiR2 cells was significantly increased by 300- and 30-fold.

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**Figure 2.** RocR1 and RocR2 exhibited KRAS and EGFR gene amplification, respectively, with loss of exon 19 deletion and T790M in EGFR following acquisition of rociletinib resistance. **A** and **B**, AfaR, RocR1, and RocR2 cells were exposed to different concentrations of rociletinib (**A**) or afatinib (**B**) for 24 hours, and cell lysates were subjected to Western blot analysis. β-Actin was included as a loading control. Immunoblot analysis of PARP cleavage was used to screen for the induction of apoptosis. Blots are representative of three independent experiments. **C**, The expression levels of KRAS, NRAS, and HRAS were determined by Western blot analysis. β-Actin was included as a loading control. **D**, Western blot analysis of KRAS expression in PC-9, AfaR, and RocR1 cells and correlations with the results of RAF-RBD pull-down assays. **E**, RocR1 cells were transfected with nontargeting (N/T) siRNA or siRNA directed against KRAS, and transfected cells were then reseeded in the presence or absence of 1 μmol/L afatinib. After a 72-hour incubation, MTT assays were performed, and OD values were obtained. Data represent the means ± SEMs of the data obtained from 6 replicate wells; *, *P* < 0.01. **F**, RocR1 cells were transfected with N/T siRNA or siRNA directed against KRAS for 48 hours and then treated with 1 μmol/L afatinib for 24 hours. KRAS knockdown was determined by Western blot analysis. Lysates were subjected to immunoblot analysis using the indicated primary antibodies. β-Actin was included as a loading control. Immunoblot analysis of PARP cleavage was used to screen for the induction of apoptosis. **G**, Increases in EGFR copy numbers were detected using quantitative PCR analysis of genomic DNA extracted from PC-9, AfaR, RocR1, and RocR2 cells; *, *P* < 0.01. **H**, AfaR, RocR1, and RocR2 cells were transfected with N/T siRNA or siRNA directed against EGFR, and EGFR knockdown was determined by Western blot analysis. Lysates were then subjected to immunoblot analysis using the indicated primary antibodies; β-actin was included as a loading control. Immunoblot analysis for PARP cleavage was used to screen for the induction of apoptosis. Blots are representative of three independent experiments. **I**, AfaR, RocR1, and RocR2 cells were transfected with N/T siRNA or siRNA directed against EGFR, incubated for 72 hours, and subjected to MTT assays. Data (OD values) represent the means ± SEMs of the data obtained from 6 replicate wells; *, *P* < 0.01.
Figure 3.
Acquired increases in KRAS expression in OsiR1 and OsiR2 cells, which were sensitive to cotreatment with a MEK inhibitor and an IGF1R inhibitor. A, AfaR, OsiR1, and OsiR2 cells were exposed to osimertinib at different concentrations for 24 hours, and cell lysates were subjected to Western blot analysis using specific antibodies. β-Actin was included as a loading control. Immunoblot analysis of PARP cleavage was used to screen for the induction of apoptosis. Blots are representative of three independent experiments. B, Female SCID mice were injected with AfaR, OsiR1, or OsiR2 cells. Once tumors reached a volume of approximately 230 to 300 mm³, 5 mice per group were randomized into groups and treated with or without 5 mg/kg osimertinib daily via orogastric gavage for 25 days. Tumors were measured twice weekly with calipers. Data in the figure represent mean tumor volumes ± SEMs; ∗, *P < 0.01. C, The expression levels of KRAS, NRAS, and HRAS were determined by Western blot analysis. β-Actin was included as a loading control. D, KRAS gene copy numbers were detected via quantitative PCR analysis of genomic DNA extracted from PC-9, AfaR, OsiR1, and OsiR2 cells; ∗, *P < 0.01. E, Western blot analysis of KRAS expression in PC-9, AfaR, OsiR1, and OsiR2 cells and correlations with the results of RAF-RBD pull-down assays. OsiR1 (F) and OsiR2 (G) cells were exposed to selumetinib at different concentrations in the presence or absence of 1 µmol/L OSI906 for 24 hours, and cell lysates were subjected to Western blot analysis using specific antibodies. β-Actin was included as a loading control. Immunoblot analysis of PARP cleavage was used to screen for the induction of apoptosis. Blots are representative of three independent experiments.
Acquired Third-Generation EGFR-TKI Resistance Mechanisms

Figure 4.
Aquired increases in KRAS and changes in KRAS levels following removal of osimertinib in OsiR1 and OsiR2 cells. A and C, AfaR cells were cultured in the presence of osimertinib with increasing concentrations from 0.01 to 1 µmol/L for 10 to 12 months to establish OsiR1 (A) and OsiR2 (C) cells. OsiR1 and OsiR2 cell lysates were generated and subjected to Western blot analysis using the indicated primary antibodies. β-Actin was included as a loading control. B and D, Increases in KRAS gene copy numbers were detected via quantitative PCR analysis of genomic DNA extracted from PC-9, AfaR, OsiR1 (B), and OsiR2 (D) cells; *, P < 0.01, compared with AfaR cells. OsiR1 (E) and OsiR2 (G) cells were cultured in the absence of osimertinib for 2 weeks (F2W), 1 month (F1M), or 2 months (F2M), and lysates were generated and subjected to Western blot analysis using the indicated primary antibodies. β-Actin was included as a loading control. KRAS copy numbers were evaluated in PC-9, AfaR, OsiR1 (F), and OsiR2 (H) cells (F2W, F1M, and F2M) by real-time PCR analysis; *, P < 0.01, compared with AfaR cells.
compared with that in AfaR cells. In exons 2–4 of the KRAS gene, no mutations were identified (Supplementary Fig. S1B). Notably, KRAS activation was also increased markedly (Fig. 3E). FISH analysis showed that KRAS amplification was observed as a homogeneously stained region in OsiR1 and OsiR2 cells (Supplementary Fig. S3A).

The combination of a MEK inhibitor and an IGF1R inhibitor overcame KRAS amplification in osimertinib-resistant cells

Using siRNA against EGFR or KRAS, knockdown of EGFR or KRAS reduced AKT and ERK1/2 activation, suggesting that these downstream signals were induced by either EGFR or KRAS (Supplementary Fig. S3B). Interestingly, IGF1R phosphorylation was increased in KRAS-knockdown OsiR1 and OsiR2 cells. Other studies have shown that KRAS attenuation leads to negative feedback activation of PI3K via insulin receptor substrate, and cells are then sensitive to the combination of IGF1R and MEK inhibitors in the presence of activating KRAS mutations (28). When treated with the MEK inhibitor selumetinib, both OsiR1 and OsiR2 cells showed inhibition of ERK1/2 activation in a concentration-dependent manner. Activation of AKT was decreased only modestly but was completely inhibited by addition of the IGF1R inhibitor OSI906, and induction of apoptosis was enhanced (Fig. 3F and G). Cell proliferation was inhibited by selumetinib in both OsiR1 and OsiR2 cells, and further inhibition was observed following addition of OSI906 (Supplementary Fig. S3C and S3D).

Acquired KRAS amplification and changes in KRAS amplification following removal of osimertinib in OsiR1 and OsiR2 cells

While establishing osimertinib-resistant cells, AfaR cells were exposed to osimertinib by increasing the concentration in growth medium; KRAS and EGFR expression levels were then determined. In both OsiR1 and OsiR2 cells, KRAS expression started to change as the concentration of osimertinib increased from 0.06–0.08 to 1 μmol/L. Although the acquired increase in KRAS was maximized at 0.4 to 0.6 μmol/L osimertinib in both OsiR1 and OsiR2 cells, amplified KRAS expression was sustained in OsiR1 cells and showed a 300-fold increase compared with that in AfaR cells (Fig. 4A and B). However, in OsiR2 cells, KRAS expression was decreased from a 120-fold increase to only a 30-fold increase compared with that in AfaR cells (Fig. 4C and D). Conversely, phospho-EGFR expression levels were decreased as the levels of KRAS increased, although basal expression of EGFR was maintained in both OsiR1 and OsiR2 cells (Fig. 4A and C). These findings suggested that KRAS amplification occurred via adaptation to osimertinib, rather than selection.

After establishment of osimertinib-resistant cells, OsiR1 and OsiR2 cells were cultured in the presence of 1 μmol/L osimertinib for at least 2 to 3 months. OsiR1 and OsiR2 cells were then cultured in osimertinib-free medium. Approximately 2 months later, the amplified KRAS expression was sustained in OsiR1 cells (Fig. 4E and F); however, in OsiR2 cells, KRAS expression was decreased to the level of the control parental AfaR cells (Fig. 4G and H). In both OsiR1 and OsiR2 cells, EGFR phosphorylation was increased markedly after osimertinib removal only for 2 weeks; otherwise, basal EGFR expression was maintained (Fig. 4E and G).

Acquired resistance to osimertinib was sustained in OsiR1 cells but reversed in OsiR2 cells, according to KRAS expression, after 2 months of osimertinib withdrawal

After approximately 2 months of culture in osimertinib-free growth medium, OsiR1 and OsiR2 cells exhibited EGFR mutations (exon 19 del/T790M) similar to those of AfaR cells and to OsiR1 and OsiR2 cells without osimertinib withdrawal (Supplementary Table S1). Interestingly, EGFR phosphorylation was inhibited by osimertinib in a concentration-dependent manner in OsiR1 cells at 2 months after osimertinib withdrawal. However, the downstream signals of AKT and ERK1/2 were inhibited modestly, suggesting that overexpression of KRAS prevented complete inhibition (Fig. 5A). Next, for further inhibition of ERK1/2 activation, the MEK inhibitor selumetinib was administered to OsiR1 cells subjected to osimertinib withdrawal for 2 months and then treated with 1 μmol/L osimertinib. The results showed that downstream AKT and ERK1/2 activation was completely inhibited, and apoptosis induction was observed following selumetinib treatment in a concentration-dependent manner (Fig. 5B). The proliferation of OsiR1 cells after 2 months of osimertinib withdrawal was suppressed by the combination of selumetinib and osimertinib (Fig. 5C). In OsiR2 cells after 2 months of osimertinib withdrawal, decreased KRAS expression was observed after removal of osimertinib, and cell proliferation was suppressed by osimertinib but not by afatinib (Fig. 5D). Osimertinib also inhibited EGFR phosphorylation and the activation of AKT and ERK1/2, and induction of apoptosis was clearly observed; however, these effects were not noted when cells were exposed to afatinib.

Figure 5.

Acquired resistance to osimertinib was sustained in OsiR1 cells but reversed in OsiR2 cells, according to KRAS expression, after 2 months of osimertinib withdrawal. A, OsiR1-F2M cells were exposed to graded concentrations of osimertinib for 24 hours, and cell lysates were subjected to immunoblot analysis using specific antibodies. β-Actin was included as a loading control. Immunoblot analysis of PARP cleavage was used to screen for the induction of apoptosis. B, OsiR1-F2M cells were exposed to selumetinib at different concentrations for 24 hours in the presence or absence of 1 μmol/L osimertinib, and cell lysates were subjected to Western blot analysis using the indicated primary antibodies. β-Actin was included as a loading control. Immunoblot analysis of PARP cleavage was used to screen for the induction of apoptosis. Blots are representative of three independent experiments. C, OsiR1-F2M cells were seeded into 96-well plates at 5 × 10^3 cells/50 μL growth medium/well, preincubated overnight, and treated with osimertinib or selumetinib in the presence or absence of 1 μmol/L osimertinib at the indicated concentrations for 72 hours. An MTT assay was performed, and OD absorbance values were measured. Data represent the means ± SEMs for 6 to 12 wells. D, OsiR2-F2M cells were seeded into 96-well plates at 5 × 10^3 cells/50 μL growth medium/well, preincubated overnight, and treated with osimertinib or afatinib at the indicated concentrations for 72 hours. MTT assays were performed, and OD absorbance values were measured. Data represent the means ± SEMs for 6 to 12 wells. E, OsiR2-F2M cells were exposed to different concentrations of osimertinib or afatinib for 24 hours, and cell lysates were subjected to immunoblot analysis using the indicated primary antibodies. β-Actin was included as a loading control. Immunoblot analysis of PARP cleavage was used to screen for the induction of apoptosis. F, Female SCID mice were injected with OsiR2-F2M cells. Once tumors reached a volume of approximately 230 to 300 mm^3, 5 mice per group were treated with or without 5 mg/kg osimertinib daily via orogastric gavage for 25 days. Tumors were measured twice weekly with calipers. Data in the figure represent mean tumor volumes ± SEMs; *, P < 0.01.
Nakatani et al.

**A**

- **P-EGFR**
- **EGFR**
- **Actin**

**B**

- **IB: EGFR**
- **IB: GRB2**
- **IB: Actin**

**C**

- **siRNA N/T SOS1 N/T GRB2**
- **P-EGFR**
- **EGFR**
- **P-AKT**
- **AKT**
- **P-ERK1/2**
- **ERK1/2**
- **SOS1**
- **GRB2**
- **Actin**

**D**

- **siRNA N/T SOS1 N/T GRB2**
- **P-EGFR**
- **EGFR**
- **P-AKT**
- **AKT**
- **P-ERK1/2**
- **ERK1/2**
- **SOS1**
- **Actin**

**E**

- **AfaR OsiR1 OsiR1-F2M**
- **Osimertinib: 1 µmol/L**
- **P-EGFR**
- **EGFR**
- **EGFR**
- **P-AKT**
- **AKT**
- **P-ERK1/2**
- **ERK1/2**
- **Actin**

**F**

- **AfaR OsiR1 OsiR1-F2M**
- **Osimertinib: 1 µmol/L**
- **P-EGFR**
- **EGFR**
- **GRB2**
- **SOS1**
- **KRAS**
- **Actin**
- **KRAS**

**G**

- **siRNA N/T GRB2**
- **Osimertinib: 1 µmol/L**
- **Selumetinib: 1 µmol/L**
- **P-EGFR**
- **EGFR**
- **P-AKT**
- **AKT**
- **P-ERK1/2**
- **ERK1/2**
- **Cle-PARP**
- **GRB2**
- **Actin**

**H**

- **OD570**
- **N/T GRB2**
- **OsiR1**
- **siRNA**
- **P-EGFR**
- **Selumetinib: 1 µmol/L**
- **Osimertinib: 1 µmol/L**
- **Akt**
- **Cle-PARP**
- **GRB2**

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Thus, the distribution of EGFR T790M mutations was still dominant in OsiR2 cells subjected to osimertinib withdrawal for 2 months. Furthermore, xenograft tumors of these cells were sensitive to osimertinib administration (Fig. 5E), and OsiR2 cells were resensitized to osimertinib after 2 months of osimertinib withdrawal. Taken together, these findings supported that EGFR phosphorylation was inhibited by osimertinib in both OsiR1 and OsiR2 cells after 2 months of osimertinib withdrawal.

Association of the phospho-EGFR and GRB2/SOS complex promoted KRAS activity and resistance to osimertinib in OsiR1 cells

In OsiR1 cells, the expression of EGFR was similar to that in AfaR cells and OsiR1 cells after osimertinib withdrawal for 2 months; however, phosphorylation of EGFR was strongly suppressed (Fig. 6A). We next attempted to determine the associations between adapter proteins and EGFR. EGFR and GRB2 were associated strongly in OsiR1 cells, but not in AfaR and OsiR1 cell after osimertinib withdrawal for 2 months (Fig. 6B). Then, using siRNA against GRB2 to disassociate the EGFR/GRB2 complex, EGFR phosphorylation was attenuated in OsiR1 cells, and the activation of downstream AKT and ERK1/2 signaling was also inhibited (Fig. 6C), suggesting that the EGFR/GRB2 association may have a critical role in osimertinib resistance.

The GRB2/SOS1 complex has been shown to activate RAS as a guanine nucleotide exchange factor (GEF; ref. 29). To determine the role of SOS1 in OsiR1 cells, we used siRNA against SOS1 (Fig. 6D). SOS1 downregulation decreased ERK1/2 phosphorylation, but not AKT phosphorylation. Moreover, the GRB2/SOS1 complex bound to phospho-EGFR (Y1068), even following osimertinib exposure, and osimertinib induced the dissociated of the phospho-EGFR and GRB2/SOS1 complex in AfaR cells; no association was observed in OsiR1 cells after osimertinib withdrawal for 2 months (Fig. 6E; Supplementary Fig. S4A). Thus, these findings suggested that the association between the phospho-EGFR (Y1068) and GRB2/SOS1 complex led to sustained EGFR phosphorylation, even following osimertinib treatment, and maintained KRAS activation. KRAS activity was partially inhibited by osimertinib treatment in OsiR1 cells after osimertinib withdrawal for 2 months, although KRAS expression in these cells was similar to that in OsiR1 cells without osimertinib withdrawal (Fig. 6F). Moreover, activated EGFR signals induced Shc, Gab1, and Gab2, except GRB2/SOS1 in OsiR1 cells, after osimertinib withdrawal for 2 months (Supplementary Fig. S4B). Using siRNA against GRB2, we found that downregulation of GRB2 decreased EGFR phosphorylation and that osimertinib inhibited EGFR phosphorylation in this context. Moreover, the combination of osimertinib and selumetinib enhanced the induction of apoptosis in OsiR1 cells (Fig. 6G). The proliferation of OsiR1 cells was significantly inhibited by the combination of osimertinib and selumetinib in GRB2-knockdown OsiR1 cells (Fig. 6H).

Discussion

In this study, we demonstrated that the third-generation EGFR TKIs rociletinib and osimertinib showed novel resistance mechanisms in acquired afatinib-resistant AfaR cells carrying exon 19 del and T790M in EGFR. Gene amplification of KRAS and EGFR was observed in rociletinib-resistant RocR1 and RocR2 cells, with loss of exon 19 del/T790M in EGFR. Moreover, osimertinib resistance was induced by KRAS amplification in OsiR1 and OsiR2 cells carrying exon 19 del/T790M in EGFR, similar to parental AfaR cells. Notably, in OsiR1 cells, withdrawal of osimertinib treatment for 2 months resulted in an increase in EGFR phosphorylation; osimertinib partially inhibited EGFR phosphorylation and, subsequently, KRAS activity. This result could be explained by the dissociation of the phosphorylated EGFR and GRB2/SOS1 complex in OsiR1 cells after osimertinib withdrawal for 2 months and the partial activation of EGFR–KRAS signals. Therefore, the combination of osimertinib plus a MEK inhibitor was able to overcome the resistance in OsiR1 cells after osimertinib withdrawal for 2 months. Furthermore, in OsiR2 cells, KRAS expression was decreased to the level in parental AfaR cells; hence, sensitivity to osimertinib was recovered completely.

The acquired resistance mechanisms to third-generation EGFR TKIs have been reported in both clinical and preclinical settings. Such mechanisms include C797S (15), G796D (30), and L798I (16) mutations in EGFR, suggesting that these mutations may decrease the binding activity to EGFR. Bypass signaling pathways function through activation of HER2, MET, and EGFR or upregulation of ligands of FGFR or EGFR (27, 31–33). Downstream activation by the mutation/amplification of NRAS, KRAS, or Yes1;
histologic transformation to SCLC, and phenotypic epithelial–mesenchymal transition have been shown to act as resistance mechanisms to other generation EGFR TKIs (19, 34–36). Previously, loss of activating mutations in EGFR has been reported to act as a resistance mechanism to first- and second-generation EGFR TKIs (20, 37). Moreover, wild-type EGFR amplification has been observed in rociletinib resistance in PC-9 cells carrying exon 19 del in EGFR and clinical tissue samples post-rociletinib therapy (27, 31, 38). To the best of our knowledge, this is a first report describing partial loss of both exon 19 del and T790M with increased expression of WT EGFR. Furthermore, loss of both exon 19 del and T790M in EGFR is not sufficient for rociletinib resistance. Amplification of KRAS or EGFR was observed in RocR1 and RocR2 cells, respectively; hence, inhibition of the KRAS effector protein MEK, combined with afatinib, was required to overcome RocR1 cell proliferation. For RocR2 cells, further strong inhibition of EGFR activation was necessary, and addition of afatinib to cetuximab was required to suppress cell proliferation.

We have recently shown that WT KRAS amplification and hyperactivation leads to acquired resistance to afatinib in PC-9 cells (i.e., AFR1), which exhibit reversible KRAS amplification following withdrawal of afatinib for 2 months, decreasing to the level of the parental PC-9 cells. Subsequently, afatinib sensitivity was recovered (20). Notably, in OsiR2 cells, KRAS amplification and hyperactivation was reduced after 2 months of osimertinib withdrawal, and KRAS expression then decreased to the level of that in the parental AfaR cells. Subsequently, osimertinib sensitivity was recovered. Similar results were reported by Cepero and colleagues, demonstrating that KRAS and MET gene amplification was involved in the mechanism of MET inhibitor resistance in gastric cancer cells. This KRAS and MET amplification was reversible after drug withdrawal for 1 month, resulting in loss of resistance (39). They postulated that amplified KRAS and MET genes may be induced by ‘replication stress,’ which interferes with DNA replication and disrupts chromatin organization in this region, predisposing it to breakage (40). Furthermore, withdrawal of the inhibitor resulted in excessive signal transduction, which may induce cellular stress and lead to loss of extrachromosomal DNA (41). However, in osimertinib-resistant cells (OsiR1 and OsiR2), FISH analysis showed that the acquired increased copies of KRAS were not located on chromosome 12, where the KRAS gene is located, or on extrachromosomal DNA, but existed on a homogeneously stained region containing an amplified chromosomal segment, which stimulated cancer progression (42). Thus, it is likely that cells gaining KRAS amplification may have a selective advantage under selective pressure with osimertinib. Interestingly, in postprogression plasma samples after osimertinib therapy, amplification of KRAS was identified in circulating tumor DNA, suggesting this is one of the putative mechanisms of resistance to osimertinib (43). Compound EGFR mutations, defined as multiple mutations in the EGFR tyrosine kinase domain, are frequently detected using advanced sequencing technologies. It has been reported that compound EGFR mutations with EGFR exon 19 deletion are rarer than those with L858R mutations (44, 45). Therefore, gene amplification or bypass signals are more likely to act as resistance mechanisms than secondary or tertiary mutations involving EGFR exon 19 deletions. However, this is still a subject of investigation.

There are different patterns of altered KRAS copy numbers observed at various stages of resistance, that is, increased osimertinib concentrations and intermediate stages of osimertinib removal in OsiR1 and OsiR2 cells. In OsiR1 cells, expression of the KRAS gene was increased in parallel with osimertinib concentrations up to 0.6 μmol/L and was then sustained at a high level. In contrast, in OsiR2 cells, amplification of KRAS increased, with a peak at 0.4 μmol/L, and then decreased at 1 μmol/L osimertinib. Interestingly, at osimertinib concentrations of 0.2 to 0.6 μmol/L, activation of AKT and ERK1/2 caused stronger inhibition in OsiR1 and OsiR2 cells, suggesting that strong replication stress may have occurred. At the intermediate stage, KRAS amplification and overexpression were sustained, even after 2 months of osimertinib withdrawal in OsiR1 cells. However, in OsiR2 cells, reduced KRAS amplification and expression were observed. Taken together, these findings clearly indicated that the observed increases in KRAS gene copies were not due to selection of preexisting populations of cells, but rather due to an adaptive process in OsiR1 and OsiR2 cells because KRAS amplification did not show a linear increase. However, the regulatory mechanism of KRAS alterations remains unclear, and requires further investigation.

Notably, we found that association of phospho-EGFR (Y1068) with the GRB2/SOS1 complex sustained KRAS activity and osimertinib resistance in OsiR1 cells. RAS proteins are upregulated by GEFs, such as SOS1, which enhance the exchange of GDP to GTP to promote the activation state, and downregulated by GTPase-activating proteins (GAP), such as p120GAP and neurofibromin 1 (NF1; ref. 46). The reduced NF1 expression was reported to confer resistance to EGFR inhibition in patients with NSCLC harboring EGFR-activating mutations, although the osimertinib-resistant cell lines did not show reduced NF1 expression (Supplementary Fig. S4C; ref. 47). The association of phosphorylated EGFR with the GRB2/SOS1 complex leads to RAS activation. GRB2 is an adaptor protein and is essential for signal transduction. GRB2 binds to phospho-tyrosine residues in receptors via its SH2 domain and binds to proteins containing proline-rich motifs, such as SOS proteins via its SH3 domain (29). In OsiR1 cells, GRB2 downregulation by siRNA decreases the activation of AKT and ERK1/2, resulting in suppression of KRAS activation through disruption of the GRB2/SOS1 complex in OsiR1 cells. Interestingly, GRB2 knockdown leads to decreased phosphorylation of EGFR. Furthermore, osimertinib inhibits EGFR phosphorylation when GRB2 is knocked down by siRNA, suggesting that GRB2 may sustain EGFR phosphorylation. The role of GRB2 in EGFR dynamics, including EGFR internalization, is unclear, and further studies are needed to elucidate these mechanisms. Moreover, the association between phosphorylated EGFR and GRB2/SOS1 was not evaluated in OsiR1 cells after osimertinib withdrawal for 2 months; however, adaptor/scaffold molecules, such as Shc, Gab1, and Gab2, were activated and then inhibited by osimertinib, indicating that phosphorylated EGFR signals were induced downstream to avoid GRB2/SOS1.

Osimertinib treatment after first-line afatinib treatment exhibited a remarkable median time to disease progression of 31.5 months longer than that of other subsequent therapies using chemotherapeutics and first-generation EGFR-TKIs in retrospective analysis of Lux-lung 3, 6, and 7 (48). However, these are premature and retrospective results, the administration of osimertinib in afatinib-relapsed NSCLC patients carrying activating mutations/T790M in EGFR would be promising. Furthermore, combination therapy with EGFR-TKI and MEK-inhibitor has been developed for lung cancers carrying activating EGFR mutations (NCT02143466 and NCT02025114). Consequently, the
combination of third-generation EGFR-TKIs and MEK inhibitors may be effective for treating lung cancers with EGFR mutations and RAS amplification through acquisition of EGFR-TKIs. Therefore, our study indicates that the intermittent treatment with 2 months of osimertinib withdrawal is better than continuous treatment with EGFR-TKIs.

To the best of our knowledge, this is the first report describing the resistance mechanisms to third-generation EGFR TKIs, that is, rociletinib and osimertinib, after the emergence of T790M EGFR following afatinib treatment. Amplification and subsequent overexpression of oncogenes have been observed in several types of neoplasms and are thought to play important roles in the advancement of tumor cells toward increased malignancy (49). In this study, we characterized novel mechanisms of acquired resistance to T790M EGFR-selective third-generation EGFR TKIs. Moreover, KRAS and EGFR gene expression should be evaluated in clinical samples following reacquisition of sensitivity to osimertinib in post-afatinib therapy. We propose a novel concept for the adaptation of T790M-selective inhibitors, that is, KRAS or EGFR amplification with or without loss of EGFR mutations (exon 19 del and T790M). In addition, we describe a preclinical rationale for reversal of acquired KRAS activity; further clinical analyses and clinical trials are needed to evaluate rechallenge with osimertinib at 2 months after osimertinib withdrawal.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

Acquired Third-Generation EGFR-TKI Resistance Mechanisms

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KRAS and EGFR Amplifications Mediate Resistance to Rociletinib and Osimertinib in Acquired Afatinib-Resistant NSCLC Harboring Exon 19 Deletion/T790M in EGFR

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