Investigating Novel Resistance Mechanisms to Third-Generation EGFR Tyrosine Kinase Inhibitor Osimertinib in Non-Small Cell Lung Cancer Patients

Zhe Yang1, Nong Yang2, Qiuxiang Ou3, Yi Xiang4, Tao Jiang5, Xue Wu3, Hua Bao2, Xiaoling Tong3, Xiaonan Wang6, Yang W. Shao7,8, Yunpeng Liu8, Yan Wang9, and Caicun Zhou5

Abstract

Purpose: The third-generation EGFR tyrosine kinase inhibitor osimertinib is approved to treat patients with EGFR T790M-positive non–small cell lung cancer (NSCLC) who have developed resistance to earlier-generation drugs. Acquired EGFR C797S mutation has been reported to mediate osimertinib resistance in some patients. However, the remaining resistance mechanisms are largely unknown.

Experimental Design: We performed mutation profiling using targeted next-generation sequencing (NGS) for 416 cancer-relevant genes on 93 osimertinib-resistant lung cancer patients’ pretreatment samples of 12 patients. In vitro experiments were conducted to functionally study the secondary EGFR mutations identified.

Results: EGFR G796/C797, L792, and L718/G719 mutations were identified in 24.7%, 10.8%, and 9.7% of the cases, respectively, with certain mutations coexisting in one patient with different prevalence. L792 and L718 mutants markedly increased the half inhibition concentration (IC50) of osimertinib in vitro, among which the L718Q mutation conferred the greatest resistance to osimertinib, as well as gefitinib resistance when not coexisting with T790M. Further analysis of the 12 matched pretreatment samples confirmed that these EGFR mutations were acquired during osimertinib treatment. Alterations in parallel or downstream oncogenes such as MET, KRAS, and PIK3CA were also discovered, potentially contributing to the osimertinib-resistance in patients without EGFR secondary mutations.

Conclusions: We present comprehensive mutation profiles of a large cohort of osimertinib-resistant lung cancer patients using mainly cfDNA. Besides C797 mutations, novel secondary mutations of EGFR L718 and L792 residues confer osimertinib resistance, both in vitro and in vivo, and are of great clinical and pharmaceutical relevance.

Introduction

Lung cancer is the second leading cause of cancer-related deaths globally in 2015 (1). A distinct subtype of lung cancer is epidermal growth factor receptor (EGFR) mutation positive non–small cell lung cancer (NSCLC). Approximately 10% to 50% of patients with NSCLC harbor EGFR activating mutations, such as in-frame deletions in exon 19 (Ex19del) or missense mutation in exon 21 (L858R), although the frequencies are significantly associated with ethnicity, gender, and smoking history (2–4). EGFR mutated lung cancer exhibits sensitivity to EGFR tyrosine kinase inhibitors (TKI) such as gefitinib and erlotinib (5–7). However, acquired resistance inevitably develops. The occurrence of an EGFR secondary mutation (T790M) is the most frequent resistance mechanism to the first and second generations of TKIs, and is detected in more than 50% of the patients after disease progression (8, 9). Osimertinib (AZD9291), a third-generation EGFR TKI, selectively blocks the activated EGFR mutant with T790M-resistant mutation (10). This pyrimidine-based compound binds to the EGFR kinase domain irreversibly by targeting the C797 residue in the ATP binding pocket via covalent bond formation (11–13). Osimertinib has proven effective in patients with NSCLC who have developed the T790M mutation (11, 14, 15), but the majority of patients will develop resistance and undergo progressed disease.
Materials and Methods

**Patients and sample collection**

A total of 93 patients with lung cancer were enrolled from multiple hospitals across China, including Shanghai Pulmonary Hospital (Shanghai), Shandong Provincial Hospital (Jinan), Hunan Cancer Hospital (Changsha), and National Cancer Center/Cancer Hospital (Beijing). Written consent was collected according to ethical regulations of each participating hospital. The NGS tests were performed in a centralized clinical testing center (Nanjing Geneseeq Technology Inc.) according to protocols reviewed and approved by the ethical committee of each participating hospital. NGS was performed on 5 to 10 mL peripheral blood collected from each patient in EDTA-coated tubes (BD Biosciences). Plasma was extracted within 2 hours of blood collection and shipped to the central testing laboratory within 48 hours. Formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks/sections or fresh tumor tissues were obtained from the hospitals, with confirmation by the pathologists for diagnosis and tumor purity.

**Targeted NGS and data processing**

DNA extraction, sequencing library preparation, and targeted capture enrichment were carried out following the methods as previously described with modifications (3). In brief, genomic DNA from fresh tumor tissue and whole blood were extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer’s protocols. FFPE samples were deparaffinized with xylene followed by genomic DNA extraction using QIAamp DNA FFPE Tissue Kit (Qiagen) following the manufacturer’s instruction. Plasma sample was first centrifuged at high speed to remove any cell debris, followed by cfDNA extraction from the supernatant using QIAamp Circulating Nucleic Acid Kit (Qiagen). Since our previous study showed that cfDNA of pleural effusion has a better detection rate for tumor-specific mutations compared to the cells in pleural effusion sediments (21), cfDNA from pleural effusion was prepared for NGS testing in this study. Similar to plasma cfDNA, we first removed cells from the pleural effusion by low-speed centrifugation, followed by high-speed centrifugation to remove any debris. The resultant supernatant was then subjected to cfDNA extraction using Qiagen QIAamp Circulating Nucleic Acid Kit. The total amount of cfDNA extracted and the input for library preparation of each case are listed in Supplementary Table S1. Sequencing libraries were prepared using the KAPA Hyper Prep Kit (KAPA Biosystems) according to manufacturer’s suggestions for different sample types. In brief, 6.08 to 200 ng of cfDNA or 1 μg of fragmented genomic DNA underwent end-repairing, A-tailing and ligation with indexed adapters sequentially, followed by size selection using Agencourt AMPure XP beads (Beckman Coulter). Hybridization-based target enrichment was carried out with GeneseeqOne pan-cancer gene panel (416 cancer-relevant genes), and xGen Lockdown Hybridization and Wash Reagents Kit (Integrated DNA Technologies). Captured libraries by Dynabeads M-270 (Life Technologies) were amplified in KAPA HiFi HotStart ReadyMix (KAPA Biosystems) and quantified by qPCR using the KAPA Library Quantification Kit (KAPA Biosystems) for sequencing. The libraries were paired-end sequenced on Illumina HiSeq4000 NGS platforms (Illumina) according to the manufacturer’s instructions. The mean coverage depth was >100× for the whole blood control samples, and >300× for tumor tissues after removing PCR duplicates. For cfDNA samples, the original targeted sequencing depth was >3,000×, however, the de-duplicated sequencing depths are variable due to different cfDNA inputs (see Supplementary Table S1 for details). Trimmomatic (22) was used for FASTQ file quality control (below 15 or N bases were removed). Reads were then mapped to the reference Human
Genome (hg19) using Burrows-Wheeler Aligner (BWA-mem, v0.7.12; https://github.com/lh3/bwa/tree/master/bwakit). Local realignment around the indels and base quality score recalibration was applied with the Genome Analysis Toolkit (GATK 3.4.0; https://software.broadinstitute.org/gatk/), which was also applied to detect germline mutations. VarScan2 (23) was used for somatic mutation detection. Somatic variant calls with at least 0.2% mutant allele frequency (MAF) and with at least three supporting-reads from both directions were retained. Common SNPs were filtered out using dbSNP (v137) and the 1,000 Genomes database, followed by annotation using ANNOVAR (24). Genomic fusions were identified by FACTERA (25) with default parameters. Copy-number variations (CNVs) were detected using ADTEx (http://adtex.sourceforge.net) with default parameters. Somatic CNVs were identified using paired normal/tumor samples for each exon with the cut-off of 0.65 for copy-number loss and 1.50 for copy-number gain.

Cell culture and reagents
The Ba/F3 cell line was maintained in RPMI1640 medium ( Gibco) supplemented with 10 ng/µL of mouse IL3 (Cell Signaling Technology). 293T cells were maintained in DMEM ( Gibco) with 10% FBS. All the cell lines were cultured in a 5% CO₂-humidified atmosphere at 37°C. Gefitinib and osimertinib used in functional validation experiments were purchased from Selleck Chemicals.

Plasmid construction, retroviral production, and transduction
Full-length cDNAs of EGFR containing specific mutations were generated by mutagenesis using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) following the manufacturer’s instructions with mutagenesis primers listed in Supplementary Table S2. The mutated full-length EGFR cDNAs were confirmed by Sanger sequencing, and then subsequently cloned into the SaI site of the pBABE-puro retroviral vector (Addgene). Retroviral production was followed by a modified protocol described previously (26). In brief, a pC-L-Eco retroviral packaging vector was co-transfected with pBABE-puro plasmid at a 1:1 ratio into 293T cells using FuGENE6 transfection reagent (Promega). The medium was replaced with fresh DMEM plus 30% FBS 12 hours after transfection. The culture medium was collected and the viral particles were harvested by centrifugation at 200 × g for 5 minutes at RT in 48 hours. The viral supernatant was used directly to infect Ba/F3 cells in addition to an equal volume of fresh culture medium for Ba/F3 cells. Stable transfectant pools were selected by culturing cells in the presence of 2 µg/mL of puromycin for 2 × 48 hours, and further subject to Western blot analysis for the confirmation of EGFR expression.

Western blot analysis
Total cell lysates were prepared using RIPA buffer supplemented with protease inhibitors (Halt Protease inhibitor cocktail, Thermo Scientific) and 40 µg/mL PMSF (Sigma). Protein concentration was determined by a standard Bradford assay (BioRad) and measured by a microplate reader (BioTek). Twenty-five microgram of total protein was resolved on SDS-PAGE gel, transferred to PVDF membrane (Bio-Rad), and analyzed by Western blot analysis with specific antibodies. EGFR antibody (1 in 1,000 dilution) was purchased from Cell Signaling Technology. ERK2 antibody (1 in 1,000 dilution; Santa Cruz Biotechnology) was used as loading control. HRP-conjugated secondary antibodies (LI-COR Biosciences) were used at a final dilution of 1:2,000. Blots were developed in the LI-COR Western Sure ECL substrate and were imaged by LI-COR C-Digit Blot Scanner. The results were analyzed using LI-COR Image Studio Digits software (version5.2).

Proliferation assay for IL3 independency
A total of 10^6 Ba/F3 cells were plated in each well of a 96-well plate and grown in RPMI1640 medium with 10% FBS in the absence of IL3 for the indicated period of time. The cell proliferation rate was examined at indicated time points using AlamarBlue cell viability assay (Thermo Scientific) according to the manufacturer’s protocol. The experiment was performed three times in triplicates each time.

In vitro growth inhibition assay
A total of 10^4 Ba/F3 cells expressing different EGFR-mutant variants were plated in each well of a 96-well plate with IL3-free culture medium and each drug at indicated doses for 72 hours. The inhibitory effects of gefitinib and osimertinib on cell growth were examined using AlamarBlue cell viability assays (same as above). The experiment was performed twice in quadruplicates.

Results

Overview of patient cohort
The clinical information of all 93 Chinese patients with lung cancer are summarized in Table 1 with details in Supplementary Tables S3 and S4. Demographic characteristics reflect that 77 patients (83%) were at stage IV disease, and the majority of the patients (95%) had NSCLCs, among which 98% are adenocarcinoma confirmed at the initial histological diagnosis (Table 1; Supplementary Tables S3 and S4). There are slightly more female patients in this cohort, and at least 75% of the patients were nonsmokers. Ninety of 93 patients received first- and/or second-generation TKIs. Two patients were initially treated with VEGF inhibitor Bevacizumab, and one patient received osimertinib as first-line treatment (Supplementary Tables S3 and S4).

To characterize the potential resistance mechanisms to osimertinib, we performed mutation profiling using targeted NGS for 416 cancer-relevant genes on the posttreatment samples of these 93 patients who progressed on osimertinib, among which 12 patients have matched pre-osimertinib treatment samples. Because of the unavailability of tumor surgical or biopsy specimens, 89% of the post-osimertinib samples (83/93) were cDNA from plasma (n = 81) or pleural effusion (n = 2), with the rest of the cases being FFPE or fresh tumor tissues (Supplementary Tables S3 and S5). About half of the pretreatment samples (7 out of 12) are cDNAs with the rest being tumor tissues, whereas all their corresponding post-osimertinib samples are cDNAs (Supplementary Table S3). In summary, one to 27 somatic mutations (median: 5) were identified in each sample (Supplementary Tables S3 and S5). Beside EGFR and TP53 genes, RB1, PIK3CA, MET, and SMAD4 are the top mutated genes (Fig. 1).

EGFR mutational status
The EGFR mutation status was summarized in Fig. 1. EGFR-activating mutations, Ex19del, L858R, or Ex20ins (exon 20 insertion), were detected in a total of 91% of the patients. Nineteen percent of the patients also harbor copy-number gain of the mutated EGFR. EGFR T790M was detected in 38 patients...

Published OnlineFirst March 5, 2018; DOI: 10.1158/1078-0432.CCR-17-2310

www.aacrjournals.org
Clin Cancer Res; 24(13) July 1, 2018
3099

Downloaded from clincancerres.aacrjournals.org on June 30, 2021. © 2018 American Association for Cancer Research.
Table 1. Clinical characteristics of patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cohort (n = 93)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td>Years</td>
</tr>
<tr>
<td>Median</td>
<td>60</td>
</tr>
<tr>
<td>25th–75th percentiles</td>
<td>52–67</td>
</tr>
<tr>
<td>Range</td>
<td>34–86</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>54</td>
</tr>
<tr>
<td>Male</td>
<td>39</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>70</td>
</tr>
<tr>
<td>Smoker</td>
<td>10</td>
</tr>
<tr>
<td>NDa</td>
<td>13</td>
</tr>
<tr>
<td>History type at initial diagnosis</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>86</td>
</tr>
<tr>
<td>Squamous</td>
<td>2</td>
</tr>
<tr>
<td>Small cell</td>
<td>3</td>
</tr>
<tr>
<td>NDa</td>
<td>2</td>
</tr>
<tr>
<td>Clinical stage at initial diagnosis</td>
<td></td>
</tr>
<tr>
<td>II–III</td>
<td>6</td>
</tr>
<tr>
<td>IV</td>
<td>77</td>
</tr>
<tr>
<td>NDa</td>
<td>10</td>
</tr>
<tr>
<td>Treatment history before osimertinib</td>
<td></td>
</tr>
<tr>
<td>Gefitinib</td>
<td>42</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>15</td>
</tr>
<tr>
<td>Icotinib</td>
<td>22</td>
</tr>
<tr>
<td>Afatinib</td>
<td>1</td>
</tr>
<tr>
<td>Gefitinib + afatinib</td>
<td>3</td>
</tr>
<tr>
<td>Gefitinib + erlotinib</td>
<td>2</td>
</tr>
<tr>
<td>Gefitinib + bevacizumab</td>
<td>1</td>
</tr>
<tr>
<td>Erlotinib + afatinib</td>
<td>1</td>
</tr>
<tr>
<td>Icotinib + erlotinib</td>
<td>1</td>
</tr>
<tr>
<td>TKI (NDa)</td>
<td>1</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>2</td>
</tr>
<tr>
<td>Treatment-naive</td>
<td>1</td>
</tr>
<tr>
<td>Post-osimertinib specimens</td>
<td></td>
</tr>
<tr>
<td>FFPE</td>
<td>5</td>
</tr>
<tr>
<td>Fresh tumor</td>
<td>5</td>
</tr>
<tr>
<td>cfDNA</td>
<td>81</td>
</tr>
<tr>
<td>Plasma</td>
<td>2</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td></td>
</tr>
</tbody>
</table>

*Not determined.

(41%). However, five patients do not have any detectable EGFR mutations in the plasma cfDNAs. We identified 22 patients (24%) that had the EGFR C797 mutations. The C797S substitution prevents covalent binding of several irreversible EGFR TKIs including osimertinib (17, 18, 27, 28), and therefore can account for the drug resistance observed in those patients. A C797G mutation was also observed in two patients with coexisting C797S mutation although at relatively low MAF of around 1%. When simultaneously detected, C797S/G mutations were always in cis to T790M. Mutations at G796 site were detected in two patients. Although it cannot change the covalent binding to osimertinib, in silico protein structure modeling predicts that G796R has a dramatic impact on EGFR/osimertinib complex by introducing strong spatial conflict, while G796S may have a milder effect on osimertinib binding to EGFR (Supplementary Fig. S1).

Mutations at our previously reported L792 residue (20) were detected in 11 patients (12%; Fig. 1). A majority of the L792 mutations (10 of 11) coexist with other secondary EGFR mutations, and they were always in cis with T790M, but in trans to G796/C797 alterations when present in the same patient, suggesting that L792 mutations may independently lead to osimertinib resistance. In addition, the variety of L792 substitutions is very diverse, and multiple types of substitutions at L792 within the same patient with different prevalence were also observed, among which L792H ranks the highest.

Seven cases (8%) carried L718 substitutions with L718Q clones to be fairly dominant (Fig. 1; Supplementary Tables S3 and S5). Notably, a majority of the L718 mutation–positive patients (6 of 7) do not have coexisting C797 mutations, suggesting that L718 mutations could be another strong candidate accounting for osimertinib-resistance. L718 residue is located in the ATP binding site of the EGFR kinase domain (29), and in silico protein structure modeling predicted that the alteration from leucine to glutamine at this position causes spatial restriction and reduced hydrophobic interaction with osimertinib (Fig. 2A). Finally, G719A mutations were identified in two patients. It is plausible that G719 alteration may also confer resistance to osimertinib through the same mechanism due to its close proximity to the L718 site. Indeed, in silico protein structure analysis demonstrated that G719A may cause a decrease in EGFR binding to osimertinib due to an increase in steric restriction (Supplementary Fig. S1). Interestingly, no other detectable EGFR mutations were identified in these two patients when progressed on osimertinib, although L858R or T790M mutation was initially identified in each patient, respectively, before osimertinib treatment (Supplementary Table S4). It raises the possibility that cells harboring this mutation may represent the expanded primary drug-resistant clones during disease progression.

We further analyzed the mutation profiles of the 12 patients before and after osimertinib treatment (Supplementary Table S3). Three patients (25%) carried secondary EGFR mutations on residues of C797, L792, and L718, and they were confirmed to be newly acquired mutations during treatment (Figs. 1 and 3). Taken together, secondary EGFR mutations with the potential of causing resistance to osimertinib have been identified in one third of the patients in our cohort, 30% of which further showed multiple coexisting secondary EGFR mutations suggesting the high degree of clonal heterogeneity during disease progression and osimertinib resistance.

**EGFR L792 and L718 mutations confer resistance to osimertinib in vitro**

We next functionally investigated whether EGFR L792 and L718 mutations contribute to the osimertinib resistance. We generated mouse Ba/F3 cell lines stably expressing EGFR activating mutation (either Ex19del or L858R) and T790M (in cis) with or without L797, L792, or L718 mutations individually. All EGFR mutants were expressed at comparable levels to the wild-type EGFR control (Supplementary Fig. S2). All Ba/F3 cells expressing these EGFR mutants, but not wild-type EGFR, achieved IL3-independent proliferation (Supplementary Fig. S3). We then exposed these cell lines to increased doses of osimertinib (Fig. 4A and B; Fig. 5A; Supplementary Table S6). As expected, cells expressing the double mutations (Ex19del/T790M or L858R/T790M) were very sensitive to osimertinib with an IC50 of 3.48 and 4.77 nmol/L, respectively. Positive control cells with additional C797S mutation were markedly resistant to osimertinib (IC50 range, 10–100 nmol/L).
among which, the L792H substitution is the strongest inducer of osimertinib resistance. Our in silico protein structure modeling further shows that L792H likely introduces a spatial conflict and decreased local hydrophobicity that prevents osimertinib binding to EGFR (Fig. 2B). L718Q brought greater resistance to osimertinib than all L792-mutant variants. Remarkably, when in cis with L858R/T790M mutations, L718Q displayed similar resistance to osimertinib as C797S with IC₅₀ of >1 μmol/L, but showed slightly less resistance (IC₅₀ of ~500 nmol/L) when in cis with Ex19del/T790M mutations (Fig. 4B).

Considering that osimertinib has been actively tested in clinical trials as first-line treatment for EGFR-mutated lung cancer patients with promising results (30, 31), and T790M has not been identified so far in patients progressed on first-generation TKI-resistant T790M mutation (30). Our results showed that C797S still confers remarkably osimertinib-resistance, which is consistent with the findings in AURA study (30). Surprisingly, cells expressing L718Q and L792H could not grow upon IL3 withdraw when in cis with Ex19del, as well as L792Y expressing cells when in cis with T790M. In the transformed cells, L792 variants exhibited different levels of, but mild, resistance to osimertinib (Fig. 5A). Notably, cells expressing L718Q in cis with L858R displayed strong osimertinib-resistance with IC₅₀ of >1 μmol/L.

We also determined the sensitivity of these EGFR mutants to the first-generation TKI gefitinib (Fig. 5B; Supplementary Fig. S4). As expected, all cells containing EGFR T790M were resistant to gefitinib. Strikingly, although T790M-negative cells expressing C797S or L792-mutant variants remained sensitive to gefitinib,
those expressing EGFR L858R/L718Q exhibited strong resistance to the drug. The EGFR/gefitinib structure revealed that L718Q remarkably decreases the local hydrophobicity, leading to an impaired interaction between EGFR and gefitinib (Fig. 2A, right). In addition, it is worthy to note that cells expressing EGFR activating mutations, such as Ex19del or L858R, are more sensitive to osimertinib than gefitinib (Fig. 5; Supplementary Table S6). Overall, these data show that the EGFR L718Q mutation with or without T790M can confer similar resistance to osimertinib as the previously identified C797S mutation, and that the EGFR L858R/L718Q variant is also resistant to gefitinib.

**Other potential resistance mechanism in patients without EGFR secondary mutations**

In the patient cohort without secondary EGFR mutations at G796/C797, L792, or L718/G719 sites (n = 62, 67%), other genetic alteration might be responsible for inducing the resistance to osimertinib (Supplementary Tables S3 and S5). TP53 is significantly mutated in the whole patient cohort, which is consistent with the previous report that more TP53 mutations were observed in advanced disease (32). Five out of the 12 patients (42%) with paired pre- and posttreatment samples acquired TP53 mutations (Fig. 3). Somatic alterations in MET gene were observed in seven
of these patients, but not the patients with secondary EGFR mutations (Fig. 1), among which two cases were confirmed to be acquired upon drug-resistance when comparing to the available pretreatment samples (Fig. 3). Specifically, five of these seven cases (5%) were MET amplification, which may serve as a bypass osimertinib-resistance mechanism. The other two cases contained rare mutations of METP97Q and I865F (Fig. 1; Supplementary Tables S3 and S5). However, little is known about the function of these point mutations. Although not significant, more mutations were identified in TET2 and KRAS genes in this cohort. Comparison of the pre- and posttreatment samples further revealed other genes with acquired somatic alterations when progressed on osimertinib (Fig. 3). However, further studies in a larger patient cohort and functional analysis need to be carried out in order to confirm their roles in causing resistance to osimertinib.

Discussion

Understanding and identifying the resistance mechanisms to targeted therapies is critical, as it allows treatment strategies to be adjusted and provides patients with the best possible clinical care. Here, we established the mutation profiles of 93 Chinese patients with lung cancer developing resistance to osimertinib treatment, and observed a number of resistance mechanisms to this drug. We
identified the well-documented EGFR C797S variant (24%), as well as coexisting C797G mutations in two cases. Mutations at adjacent G796 sites were also discovered with strong implication as potential resistant mutations based on protein structure prediction. We previously reported that mutations at the EGFR L792 site could be the potential resistant mechanisms in three NSCLC patients (20). In this larger patient cohort, alterations at the L792 site account for 12% of the cases, and the subsequent in vitro functional studies demonstrated that L792 substitutions could confer intermediate resistance to osimertinib compared to C797S. Specifically, L718Q variant, which was previously reported in one case report (33) exhibited similar resistance to osimertinib as C797S when combined with L858R, which was further confirmed in our in silico structure modeling analysis and in vitro experiments. We also found that the majority of L718/G719 mutations were observed in patients without coexisting C797S mutations, suggesting that these mutations represent another key mediator of

Figure 4. L718 and L792 substitutions induce osimertinib resistance in vitro. Ba/F3 cells harboring Ex19del (A, C) or L858R (B, D) plus indicated mutations, with or without T790M, were treated with osimertinib at the indicated concentrations in the absence of IL3. Cell viability was evaluated after 72 hours of treatment and plotted relative to the untreated control cells. Experiments were repeated twice in triplicate each time with mean ± standard deviation plotted at each concentration. The curves were fitted using a nonlinear regression model with a sigmoidal dose response in the Graphpad Prism 6.
osimertinib resistance. It is worthy to be noted that 11 patients harbor multiple coexisting secondary EGFR mutations with different prevalence and distinct resistant potential, suggesting the high degree of clonal heterogeneity during disease progression and osimertinib resistance.

Approximately 97% of the patients in this cohort received prior first- and/or second-generation of TKI treatments. Only one patient (Case 91) with small cell lung cancer received osimertinib as the first-line treatment, but the EGFR status information at initial diagnosis is not available (Supplementary Table S4). Interestingly, this patient achieved a progression-free survival (PFS) of 25 months, and no EGFR mutations were detected after disease progression. This is substantially improved from the reported median PFS (11 months) of the first-generation TKI such as gefitinib and erlotinib (34), and from the median PFS (10–14 months) of osimertinib as second-line treatment (14). In line with this, Soria and colleagues 2017 reported that osimertinib improves PFS by 54% and extended the median PFS to 18.9 months comparing to the standard first-line treatment (gefitinib or erlotinib) in patients with advanced EGFR mutated NSCLC (31). The putative resistance mechanisms to osimertinib as the first-line treatment have been reported by Ramalingam and colleagues 2017 in 9 out of 19 patients with detectable circulating tumor DNA, which include one case of EGFR G719S/MEK1 G128V, two cases of kinase domain-activating variants (HER2 exon 20 insertion and JAK2 V617F), along with those previously reported resistant mechanisms of second-line treatment, such as EGFR C797S mutation, MET, EGFR, KRAS amplification and activating mutations of PIK3CA and KRAS (30). As expected, acquired EGFR T790M mutation was not detected in this patient cohort. In Case 91 of this study, we identified TP53, KMT2A and RECQL4 mutations in the posttreatment cfDNA of the patient. However, without pre-treatment sample for comparison, we cannot determine which mutation was associated with acquired resistance to osimertinib. To further explore the potential resistance mechanisms of first-line osimertinib treatment, we tested...
whether acquiring those secondary EGFR mutations identified on top of the EGFR activating mutations without preexisting first generation TKI-resistant T790M mutation can confer osimertinib resistance, and found that C797S or L792-mutant variants to the first-generation TKI gefitinib. In addition, we detected five cases with MET copy-number gains (approximately 5.4% of all patients) and two patients with MET mutations in the post-osimertinib samples, which do not harboring any secondary EGFR mutations. Among 19 patients report (32), these data indicate that MET amplification may represent an EGFR-independent bypass mechanism of osimertinib resistance. However, considering that the majority of our samples are cfDNAs from liquid biopsy, we may underestimate the overall frequency of MET amplification due to the detection sensitivity of copy-number variation by NGS method in cfDNA samples with lower circulating tumor DNA content (3). Consistently, Ramalingam and colleagues also reported that MET amplification was only detected in one out of the 19 patients (about 5%) who received osimertinib as first-line TKI by liquid biopsy (30). As a result, patients with NSCLC harboring both MET alteration and EGFR mutations may benefit from combined therapy with EGFR TKI and MET kinase inhibitors (36–38).

Taken together, we have identified secondary EGFR mutations conferring osimertinib resistance in one third of the patients. The novel EGFR secondary mutations identified here are of great clinical relevance, and may shed light on the development of new EGFR inhibitors. We also discovered that 7.5% of the cases harbor MET alterations, which may represent the EGFR-independent resistance mechanism. Further studies of larger patient cohorts with matched pretreatment samples may be of interest to study the osimertinib resistance mechanisms in the remaining patients.

**Disclosure of Potential Conflicts of Interest**

Q. Ou, X. Wu, H. Bao, X. Tong, and Y.W. Shao are the shareholder or employees of Geneseeq Technology Inc. X. Wang is the shareholder and employee of Nanjing Geneseeq Technology Inc. The remaining authors declare that they have no competing interests.

**Authors’ Contributions**

Conception and design: Z. Yang, N. Yang, Q. Ou, X. Wang, Y. Wang, C. Zhou

Development of methodology: Z. Yang, N. Yang, Q. Ou, Y. Wang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Yang, N. Yang, Q. Ou, X. Wang, Y.W. Shao, Y. Wang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Q. Ou, Y. Xiang, T. Jiang, X. Wu, H. Bao, X. Tong

Writing, review, and/or revision of the manuscript: Z. Yang, N. Yang, Q. Ou, Y. Xiang, T. Jiang, X. Wu, H. Bao, X. Wang, Y.W. Shao, Y. Liu, Y. Wang, C. Zhou

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Wang

Study supervision: Y.W. Shao, C. Zhou

Acknowledgments

We would like to thank the patients and family members who gave their consent on presenting the data in this study, as well as the investigators and research staff at all hospitals and research sites involved.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 8, 2017; revised December 1, 2017; accepted February 26, 2018; published first March 5, 2018.

**References**


Novel Resistance Mechanisms to Osimertinib in NSCLC Patients


Clinical Cancer Research

Investigating Novel Resistance Mechanisms to Third-Generation EGFR Tyrosine Kinase Inhibitor Osimertinib in Non–Small Cell Lung Cancer Patients

Zhe Yang, Nong Yang, Qiuxiang Ou, et al.

Clin Cancer Res 2018;24:3097-3107. Published OnlineFirst March 5, 2018.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-17-2310

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2018/03/03/1078-0432.CCR-17-2310.DC1

Cited articles
This article cites 36 articles, 6 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/24/13/3097.full#ref-list-1

Citing articles
This article has been cited by 13 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/24/13/3097.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/24/13/3097.
Click on "Request Permissions" which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.