Clinical Cancer Research

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

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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' samples, mainly cell-free DNAs (cfDNAs), and matched 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 inhibitory concentration (IC_{50}) 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-resistance 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. *Clin Cancer Res; 24(13); 3097–107. ©2018 AACR.*

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

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Translational Relevance

Acquired *EGFR* C797S mutation has been reported to mediate osimertinib resistance. However, the remaining resistance mechanisms are largely unknown. Here, we performed next-generation sequencing on the post-progression samples, mainly cell free DNAs (cfDNA), from 93 patients with lung cancer, mostly non–small cell lung cancers (NSCLCs). Secondary mutations of G796/C797, L792, or L718/G719 residues on *EGFR* were collectively found in approximately one-third of the cohort. *In vitro* functional validations further demonstrated that L792 and L718 substitutions significantly decrease their sensitivities to osimertinib causing resistance. In addition, alterations in genes parallel to or downstream of *EGFR* were discovered as potential resistance mechanisms in patients without *EGFR* secondary mutations, such as *MET*, *KRAS*, and *PIK3CA*. Together, we present comprehensive mutation profiles of a large cohort of patients with lung cancer resistant to osimertinib. Moreover, novel *EGFR* secondary mutations identified here are of great clinical relevance, and may shed light on the development of new *EGFR* inhibitors.

(16). The acquired *EGFR* C797S mutation has been identified as an osimertinib-resistant mechanism in six of 15 patients (17, 18); however, the remaining resistance mechanisms to this TKI are largely unknown (19).

We previously reported three clinical cases of osimertinib-resistant lung adenocarcinoma with *EGFR* secondary mutations at the L792 site, which is located in the hinge pocket of the ATP-binding domain in close proximity to both C797S and T790M (20). Structural prediction suggested that L792 mutations could interrupt osimertinib binding to the receptor and potentially introduce drug resistance. To better understand the prevalence of L792 variants, and to identify a new resistance mechanism in patients with lung cancer treated with osimertinib, we analyzed the mutation profiles of 93 relapsed Chinese patients, mainly NSCLCs, using hybridization-based targeted next-generation sequencing (NGS) with a comprehensive pan-cancer gene panel, among which 12 patients have paired pretreatment samples for analysis. Because of the unavailability of tumor biopsy specimen, cell-free DNA (cfDNA) isolated from blood plasma or pleural effusion as liquid biopsy were used for sequencing analysis in most cases. We identified *EGFR* secondary mutations in approximately one-third of this patient cohort. *In vitro* functional studies demonstrated that these mutations can render EGFR resistance to osimertinib at different levels. Further analysis of the 12 patients with paired pretreatment samples revealed that these *EGFR* secondary mutations were acquired during the treatment. In addition, other potential resistant mechanisms were also identified. In conclusion, our data strongly suggest that *EGFR* L792 and L718Q mutations serve as clinically relevant, alternative resistance mechanisms to osimertinib, which may shed light on the development of new targeted drugs.

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. 5 to 10 mL peripheral blood was 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 de-paraffinized 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 (median: 70.5 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) supplemented with 10% FBS. All the cell lines were cultured in a 5% CO_2-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 *Sal*I site of the pBABE-puro retroviral vector (Addgene). Retroviral production was followed by a modified protocol described previously (26). In brief, a pCL-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 BaF3 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 10^4 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 cfDNA 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 cfDNAs with the rest being tumor tissues, whereas all their corresponding post-osimertinib samples are cfDNAs (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

Table 1. Clinical characteristics of patients

Characteristics	Cohort ($n = 93$)
Age at diagnosis	Years
Median	60
25th–75th percentiles	52–67
Range	34–86
Gender	*n*
Female	54
Male	39
Smoking history	*n*
Nonsmoker	70
Smoker	10
ND[a]	13
Histology type at initial diagnosis	*n*
Adenocarcinoma	86
Squamous	2
Small cell	3
ND[a]	2
Clinical stage at initial diagnosis	*n*
II–III	6
IV	77
ND[a]	10
Treatment history before osimertinib	*n*
Gefitinib	42
Erlotinib	15
Icotinib	22
Afatinib	1
Gefitinib + afatinib	3
Gefitinib + erlotinib	2
Gefitinib + bevacizumab	1
Erlotinib + afatinib	1
Icotinib + erlotinib	1
Icotinib + afatinib	1
TKI (ND[a])	1
Bevacizumab	2
Treatment-naive	1
Post-osimertinib specimens	*n*
Tumor tissue	
FFPE	5
Fresh tumor	5
cfDNA	
Plasma	81
Pleural effusion	2

[a]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 IC_{50} of 3.48 and 4.77 nmol/L, respectively. Positive control cells with additional C797S mutation were markedly resistant to osimertinib (IC_{50} > 1 μmol/L), consistent with the previous reports that C797S significantly decreases the sensitivity to osimertinib (17). Cells with additional L792-mutant variants generally exhibited an intermediate resistance to osimertinib (IC_{50} range, 10–100 nmol/L),

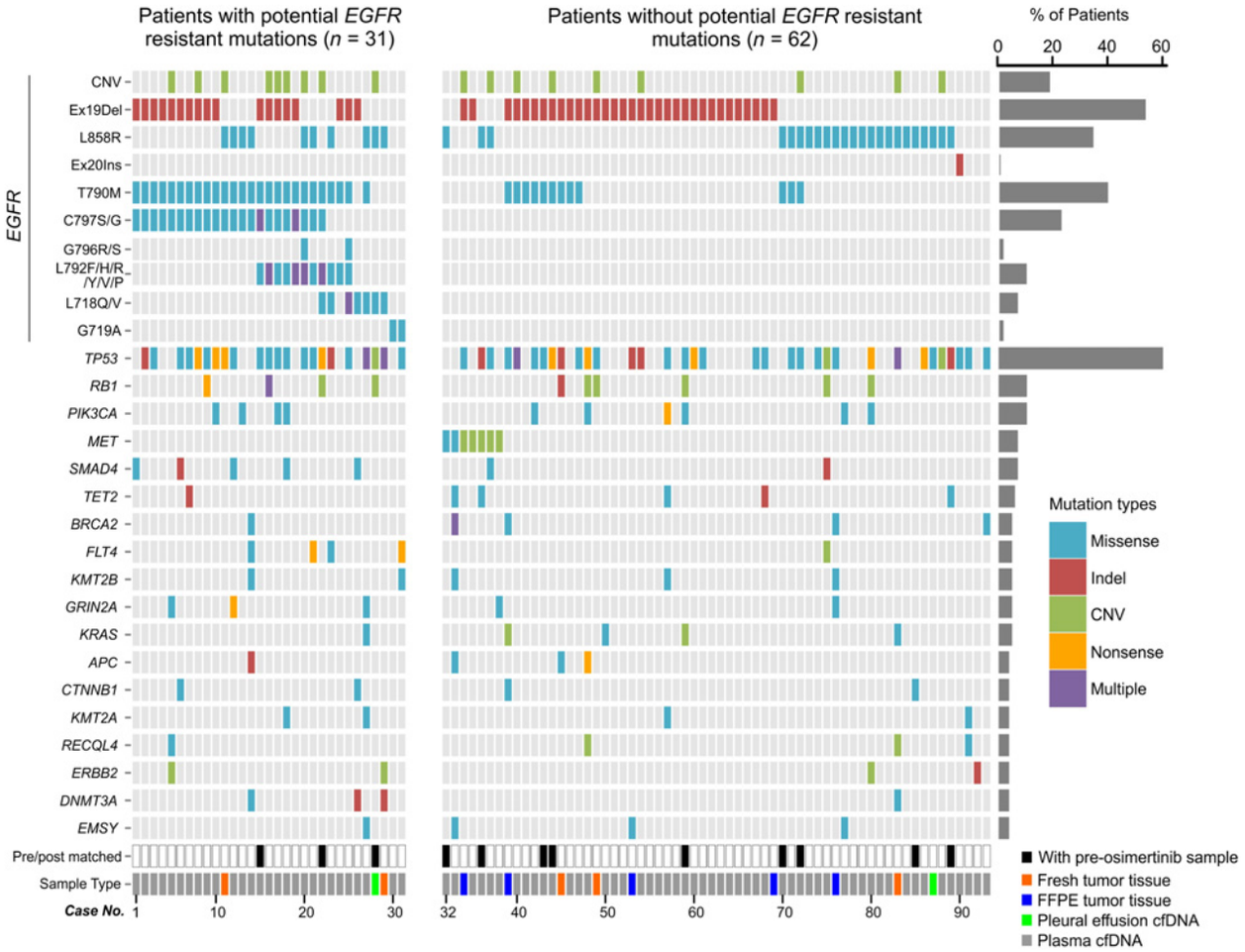

Figure 1.

Comutation plot of post-osimertinib treatment patients. Patients were grouped into two cohorts (left and right panels) by the presence of major *EGFR* tertiary mutations at the positions of L718, G719, L792, G796, and C797. *EGFR* mutations identified in post-osimertinib treatment samples are clustered at the top. Mutations of other cancer-relevant genes are indicated below. Only genes with more than 4 occurrences of alterations are shown. The percentages of patients identified with the indicated alterations are shown on the right panel. Alteration types are represented by indicated colors. Whether or not there is a matched pretreatment sample for this case, and the sample type of each post-treatment specimen used for mutation profiling is indicated at the bottom.

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 confliction 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 IC_{50} of >1 μmol/L, but showed slightly less resistance (IC_{50} 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-line osimertinib treatment (30), we then asked whether acquiring these secondary mutations on top of the *EGFR* activating mutations without preexisting first-generation TKI-resistant T790M mutation can confer osimertinib resistance. To investigate this, we generated Ba/F3 cell lines stably expressing all the above *EGFR* mutants except for T790M mutation (Fig. 4C and D; Fig. 5; Supplementary Fig. S3C and S3D). Our results showed that C797S still confers remarkably osimertinib-resistance, which is consistent with the findings in AURA study (30). Surprisingly, cell lines 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 L858R. 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 IC_{50} 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,

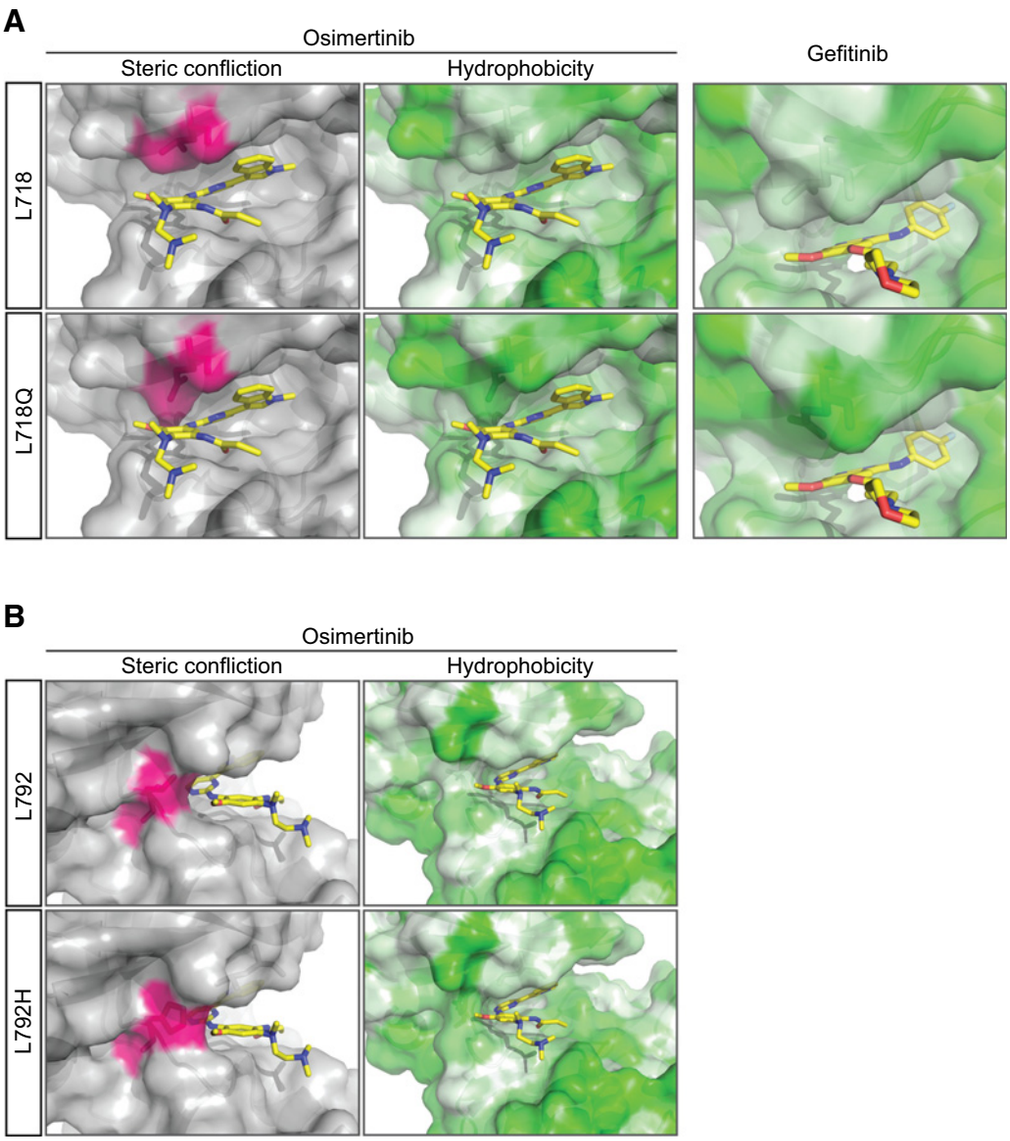

Figure 2.

In silico protein structure modeling of *EGFR* L718Q and L792H mutants for TKI binding. L718Q (**A**) and L792H (**B**) substitutions could prevent osimertinib (PDB id: 4ZAU) binding by introducing spatial confliction (hot pink) and decreasing the local hydrophobicity (green). EGFR are shown in surface. Decreased hydrophobicity is represented by the color change from white to green. Right, the EGFR/gefitinib (PDB id: 4WKQ) structure is disturbed by L718Q due to a remarkable reduction of local hydrophobicity.

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

Figure 3.
Acquired mutations during treatment in 12 patients who had matched pre- and post-osimertinib treatment samples. Alteration types are represented by indicated colors. The number of acquired mutations identified in each patient is shown on the top. Acquired *EGFR* alterations identified are shown individually and clustered at the top. The sample type of each pre- and posttreatment specimen used for mutation profiling is indicated at the bottom.

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 *MET* P97Q 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

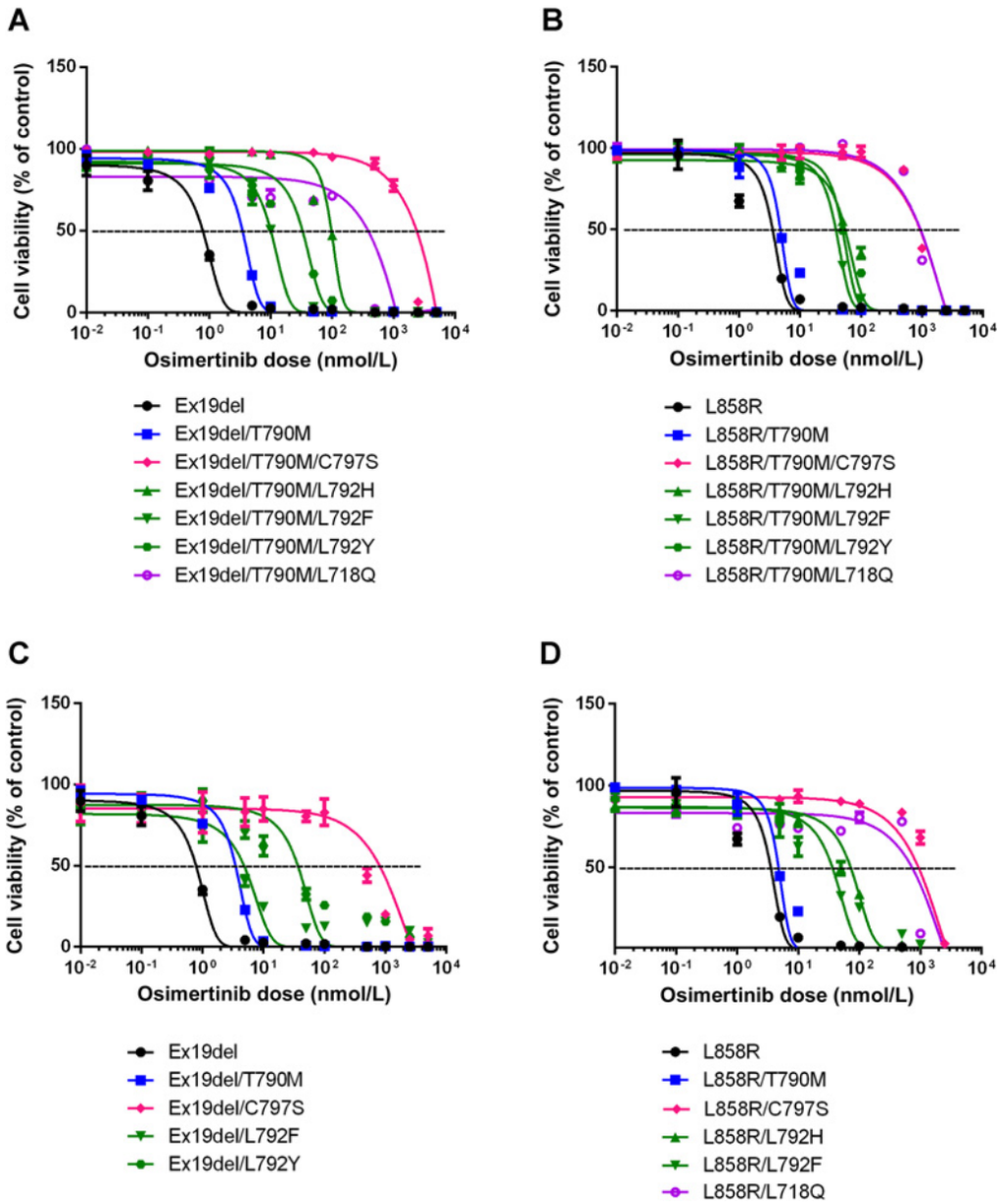

Figure 4.

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.

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. L718/G719 mutants were identified in another nine cases (10%). 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

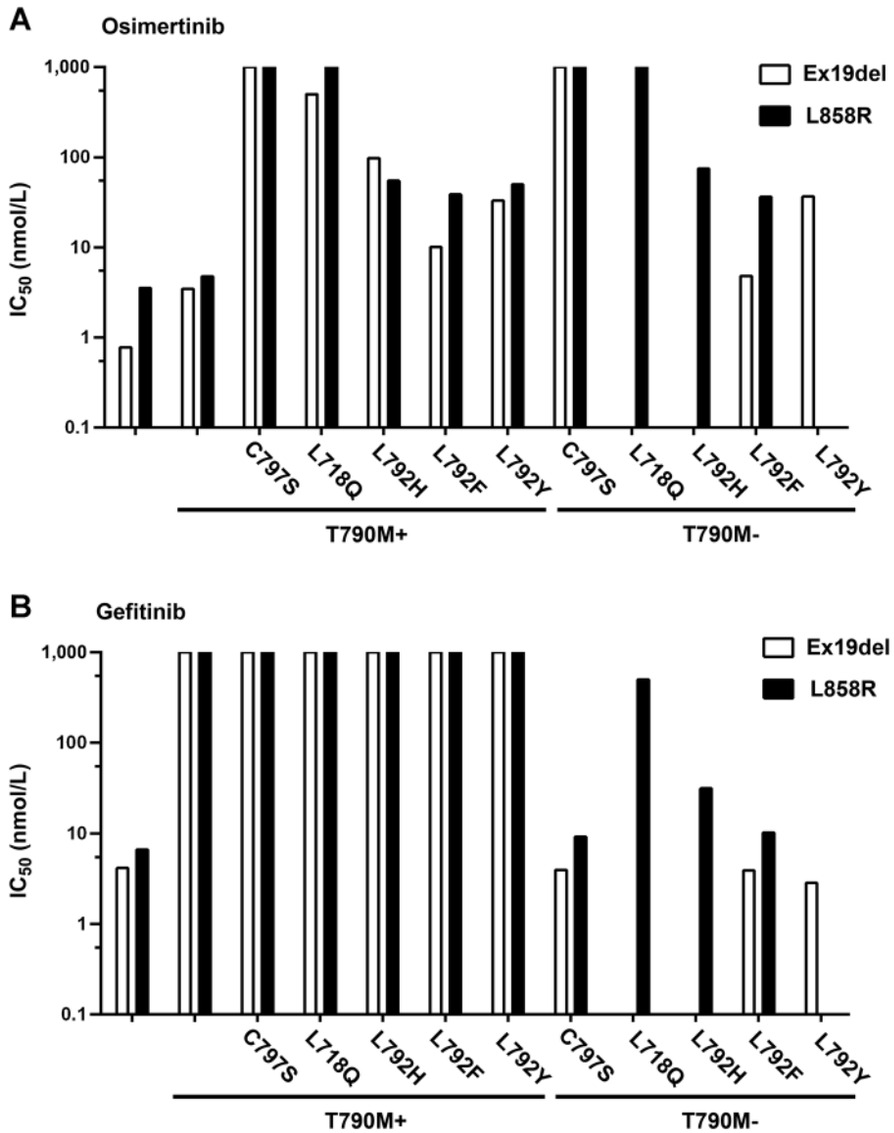

Figure 5.
A summary of IC_{50} values of different *EGFR* mutants to osimertinib and gefitinib. IC_{50} of each cell line to different drugs were calculated by Graphpad Prism 6 software (Supplementary Table S6), and plotted in bar graphs for comparison. IC_{50} over 1,000 nmol/L was presented as 1,000 nmol/L. Ex19del/L718Q, Ex19del/L792H, and L858R/L792Y expressing Ba/F3 cells failed to proliferate in the absence of IL3.

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 can still confer remarkably osimertinib resistance. L792 variants exhibited mild resistance to osimertinib, whereas L718Q when *in cis* with L858R displayed strong osimertinib resistance. We further determined the sensitivity of these *EGFR* mutants to the first-generation TKI gefitinib, and the results showed that C797S or L792-mutant variants remained sensitive to gefitinib, but *EGFR* L858R/L718Q exhibited strong gefitinib resistance. All these results may be valuable and of clinical relevance when we observed such mutants in clinical practice.

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 harbor any secondary *EGFR* mutations. Along with previous report (35), these data indicate that *MET* alterations 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* primary 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.

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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

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