

On-target Resistance to the Mutant-Selective EGFR Inhibitor Osimertinib Can Develop in an Allele-Specific Manner Dependent on the Original EGFR-Activating Mutation



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Abstract

Purpose: The third-generation EGFR inhibitor, osimertinib, is the first mutant-selective inhibitor that has received regulatory approval for the treatment of patients with *EGFR*-mutant lung cancer. Despite the development of highly selective third-generation inhibitors, acquired resistance remains a significant clinical challenge. Recently, we and others have identified a novel osimertinib resistance mutation, G724S, which was not predicted in *in vitro* screens. Here, we investigate how G724S confers resistance to osimertinib.

Experimental Design: We combine structure-based predictive modeling of G724S in combination with the 2 most common EGFR-activating mutations, exon 19 deletion (Ex19Del) and L858R, with *in vitro* drug-response models and patient genomic profiling.

Results: Our simulations suggest that the G724S mutation selectively reduces osimertinib-binding affinity in the

context of Ex19Del. Consistent with our simulations, cell lines transduced with Ex19Del/G724S demonstrate resistance to osimertinib, whereas cells transduced with L858R/G724S are sensitive to osimertinib. Subsequent clinical genomic profiling data further suggest G724S occurs with Ex19Del but not L858R. Furthermore, we demonstrate that Ex19Del/G724S retains sensitivity to afatinib, but not to erlotinib, suggesting a possible therapy for patients at the time of disease relapse.

Conclusions: Altogether, these data suggest that G724S is an allele-specific resistance mutation emerging in the context of Ex19Del but not L858R. Our results fundamentally reframe the problem of targeted therapy resistance from one focused on the "drug-resistance mutation" pair to one focused on the "activating mutation–drug-resistance mutation" trio. This has broad implications across clinical oncology.

Introduction

Oncogenic mutations in the EGFR tyrosine kinase domain are found in approximately 15% to 30% of non-small cell lung carcinomas (NSCLCs; refs. 1, 2). Of these cases, approximately 90% can be attributed to in-frame deletions within exon 19

(Ex19Del) or missense mutations in exon 21 (L858R), which occur with approximately equal prevalence (1, 2). Multiple phase III clinical trials have shown that patients with *EGFR*-mutant tumors experience >70% radiographic response rates and a statistically significant improvement in progression-free survival when treated with first-generation (erlotinib, gefitinib) or second-generation (afatinib) EGFR tyrosine kinase inhibitors (TKI) as compared with platinum-based chemotherapy (3–6). However, response to these targeted agents is transient, and acquired therapeutic resistance typically develops within 8 to 10 months. In approximately 60% of cases, resistance is acquired through acquisition of a secondary EGFR mutation, EGFR T790M (7–9). Osimertinib, a mutant-selective third-generation covalent inhibitor, was developed specifically to target T790M. For these reasons, the clinical standard of care for *EGFR*-mutant NSCLC has been treatment with first- or second-generation TKIs followed by treatment with osimertinib postprogression on first-line therapy (10). Recently, osimertinib became approved as first-line therapy (11).

Unfortunately, resistance mutations may also emerge against osimertinib therapy (12, 13). The most well described to date is C797S, which is detected in approximately 10% to 19% of patients with first-line and second-line osimertinib resistance (14, 15). Mutation of C797 to serine prevents covalent adduct formation between osimertinib and the EGFR kinase domain (16, 17). We (18) and others (14, 19, 20) have also identified G724S as a mutation that is selected for in osimertinib-resistant tumors.

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Translational Relevance

Osimertinib is the first mutant-selective EGFR tyrosine kinase inhibitor to receive regulatory approval. Despite impressive clinical benefit, acquired resistance to osimertinib presents a significant challenge. Herein, we describe the emergence of G724S as a new mutation within the EGFR kinase domain that is detected at the time of osimertinib resistance. Notably, our *in silico*, *in vitro*, and patient-level data show that G724S is an allele-specific resistance mutation emerging in the context of EGFR exon 19 deletion (Ex19Del) but not EGFR L858R. The findings are immediately translatable for (1) surveillance—patients with Ex19Del mutations are susceptible to G724S-mediated resistance and (2) treatment—our results provide structural and *in vitro* evidence for the efficacy of afatinib in treating patients with osimertinib resistance mediated by the emergence of G724S mutation.

Unlike C797S, G724S was not predicted based on *in vitro* studies (21, 22), and the precise mechanism whereby G724S mutation confers osimertinib resistance is unknown.

The most fundamental principle of structural biology is that sequence determines structure and structure determines function. To determine the relationship between classical EGFR kinase-activating mutations (Ex19Del and L858R), acquired G724S mutation, and osimertinib resistance, we employed an integrated computational/experimental approach. Our results suggest that G724S is a resistance mutation that develops with Ex19Del but not L858R and provide mechanistic insight into this process at the structural level.

Materials and Methods

Inhibitor source and preparation

EGFR TKIs were purchased from Selleck Chemicals. All drugs were prepared and stored as a stock solution at 10 mmol/L in DMSO (Sigma-Aldrich).

Cell culture

293FT cells were purchased from Invitrogen. NR-6 cells were a gift from Dr. William Pao (Vanderbilt University) (23). 293FT and NR-6 cells were cultured in DMEM with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Mediatech) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals) and penicillin (100 U/mL)/streptomycin (100 µg/mL; Mediatech). Ba/F3 cells were purchased from DSMZ and were cultured in RPMI 1640 with L-glutamine (Mediatech) supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL)/streptomycin (100 µg/mL), and 1 ng/mL IL3 (Thermo Fisher Scientific) until retroviral transduction and subsequent IL3 withdrawal. Cells were grown in a humidified incubator with 5% CO₂ at 37°C and were routinely evaluated for mycoplasma using a Venor GeM Mycoplasma Detection Kit (Sigma-Aldrich).

Immunoblot analysis

Cells were washed with PBS and lysed in radioimmunoprecipitation analysis buffer [50 mmol/L Tris HCl, pH 8.0, 150 mmol/L sodium chloride, 5 mmol/L magnesium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 40 mmol/L sodium

fluoride, 1 mmol/L sodium orthovanadate, and complete Protease Inhibitor Cocktail (Roche Diagnostics)]. Western Lightning ECL reagent (PerkinElmer) was used for signal detection. β-Actin antibody (A2066) was purchased from Sigma-Aldrich. EGFR (#2232), pEGFR Y1068 (#2234), pEGFR Y1173 (#2244), ERK (#9102), pERK T202/Y204 (#9101), horseradish peroxidase (HRP)-conjugated anti-mouse (#7076), and HRP-conjugated anti-rabbit (#7074) antibodies were purchased from Cell Signaling Technology. Each experiment was performed twice.

CellTiter Blue cell viability assay

Ba/F3 cells were seeded in 96-well plates at a density of 20,000 cells/well and treated with varying concentrations of indicated compounds, with 6 technical replicates per concentration. After 72 hours, CellTiter Blue Reagent (Promega) was added to wells according to the manufacturer's instructions, and cells were incubated at 37°C with 5% CO₂ for 2 to 4 hours. Absorbance was detected at 590 nm with a Synergy HTX microplate reader (BioTek Instruments). Each experiment was performed 3 times.

Statistical analysis

All experiments were performed at least 3 times, and the differences were determined by 1-way ANOVA. Differences were considered significant when $P < 0.05$.

Molecular modeling

Structural models of the EGFR kinase exon 19 deletion mutants (Ex19Del) were generated through complementary use of the structure-prediction software package Rosetta utilizing the REF2015 score function (24–26) and molecular dynamics (MD) simulation with AMBER16 (27). Comparative models of Ex19Del kinase domain were created with RosettaCM (24, 25) by modeling the kinase domain sequence sans β3-αC residues E746–A750 for the canonical variant model, or a valine substituted for the range E746–S752 for the rare variant model, and applying PDB IDs 2GS6 and 2GS7 as templates for the active and inactive state models, respectively (28). Active and inactive state Rosetta models of EGFR were minimized and allowed to equilibrate in a rectangular box of TIP4PEW explicit solvent neutralized with monovalent chlorine anions (29, 30). Solute was buffered on all sides with 12 Å solvent. Afterward, dual-boost Gaussian-accelerated MD (GaMD) simulations were performed to enhance conformational sampling (31–34). Protein-ligand-binding-free energy calculations were performed with MM/GBSA implemented in the AMBER suite in combination with the quasi-harmonic approximation of entropy (35). For a detailed description of model building, MD simulations, and binding-free energy calculations, please see the Supplementary Methods section.

Genomic profiling of patient samples

Hybrid capture-based next-generation sequencing was performed on formalin-fixed paraffin-embedded tissue sections or circulating tumor DNA isolated from blood samples in a Clinical Laboratory Improvement Amendments–certified, College of American Pathologists–accredited laboratory (Foundation Medicine) as described previously (36, 37). Approval for this study, including a waiver of informed consent and a Health Insurance Portability and Accountability Act (HIPAA) waiver of authorization, was obtained from the Western Institutional Review Board (Protocol No. 20152817).

Results

A G724S-mediated conformational change in the glycine-rich P-loop reduces binding affinity of osimertinib to Ex19Del/G724S but not to L858R/G724S

To determine the structural effects of G724S mutation on osimertinib binding, we performed a series of GaMD simulations (32, 34) of wild-type EGFR (WT), Ex19Del (unless otherwise stated, the canonical variant E746_A750del), Ex19Del/G724S, L858R, and L858R/G724S in the drug-unbound (apo) state (Supplementary Table S1). Analysis of our initial simulations suggests G724S may increase P-loop backbone conformation fluctuations (Supplementary Fig. S1). These data are intriguing because EGFR has previously been shown to bind osimertinib with a characteristic "bent" P-loop conformation (17), and we hypothesized that G724S could reduce osimertinib binding through disruption of the bent P-loop conformation. Previous literature on protein conformational dynamics has cautioned against inferring functional mechanisms from root mean square fluctuation (RMSF) statistics alone (38). Therefore, to test our hypothesis, we performed GaMD simulations of Ex19Del, Ex19Del/G724S, L858R, and L858R/G724S reversibly bound with osimertinib. We similarly examined these 4 mutants with the second-generation, wild-type selective EGFR TKI, afatinib, as a control. Afatinib was selected as a control in our study for multiple reasons. Afatinib has previously been reported to be a potential therapeutic agent in the setting of Ex19Del/G724S-mediated NSCLC based on a patient case report (18). Similar to osimertinib, afatinib is an irreversible EGFR inhibitor that has received regulatory approval for treatment of EGFR-mutant lung cancer.

Osimertinib and afatinib both irreversibly bind EGFR through covalent adduct formation. In order to form an irreversible complex, they must first form a reversible, noncovalent complex (Fig. 1A). Disruption of the reversible complex formation is expected to reduce formation of adduct. A previously determined crystallographic structure of EGFR kinase reversibly bound to osimertinib demonstrates that osimertinib binding is accommodated through a well-defined "bent" P-loop conformation (Fig. 1B; ref. 17). This bent P-loop conformation allows the F723 phenyl ring to make an energetically favorable contact with the indole ring of osimertinib, contributing to its affinity (17).

Our GaMD simulations illustrate that G724S rigidifies the tip of the P-loop by stabilizing a β -bend conformation (Fig. 1C and D; Supplementary Fig. S2). As a result, Ex19Del/G724S and L858R/G724S cannot form a stable bent P-loop conformation when bound to osimertinib. The rigidified P-loop displaces F723 from contact with osimertinib (Fig. 1C–F). Interestingly, however, we found evidence of reduced stability of the osimertinib-bound Ex19Del/G724S complex but not the osimertinib-bound L858R/G724S complex. In our simulations, osimertinib maintains an RMSD of 1 to 2 Å from its native binding pose in Ex19Del and L858R. Displacement of F723 from contact with osimertinib is associated with an increase in osimertinib RMSD to 3–4 Å in Ex19Del/G724S but not in L858R/G724S (Fig. 1E and F). In contrast, afatinib forms a stable reversible complex in all 4 cases (Ex19del, Ex19del/G724S, L858R, and L858R/G724S; Supplementary Fig. S3). These models suggest that structural perturbations from G724S, which disrupt binding of osimertinib, fail to notably effect binding of afatinib. These data support a potential role for afatinib in treating patients with G724S.

To further investigate these differences, we applied the molecular mechanics-generalized Born surface area method (MM/GBSA) to compute the binding-free energies of osimertinib with Ex19Del, Ex19Del/G724S, L858R, and L858R/G724S. Our calculations predict a 2.3 kcal/mol reduction in osimertinib binding-free energy ($\Delta\Delta G_{\text{bind}}$) with Ex19Del/G724S (Fig. 1G). Our binding-free energy calculations also suggest that osimertinib reversibly binds L858R more tightly than Ex19Del by 1.9 kcal/mol (Fig. 1G). The osimertinib binding-free energies computed for Ex19Del and L858R/G724S are indistinguishable, within error, suggesting that the reduction in binding affinity accompanying the addition of the G724S mutation in L858R should not confer osimertinib resistance (Supplementary Table S2).

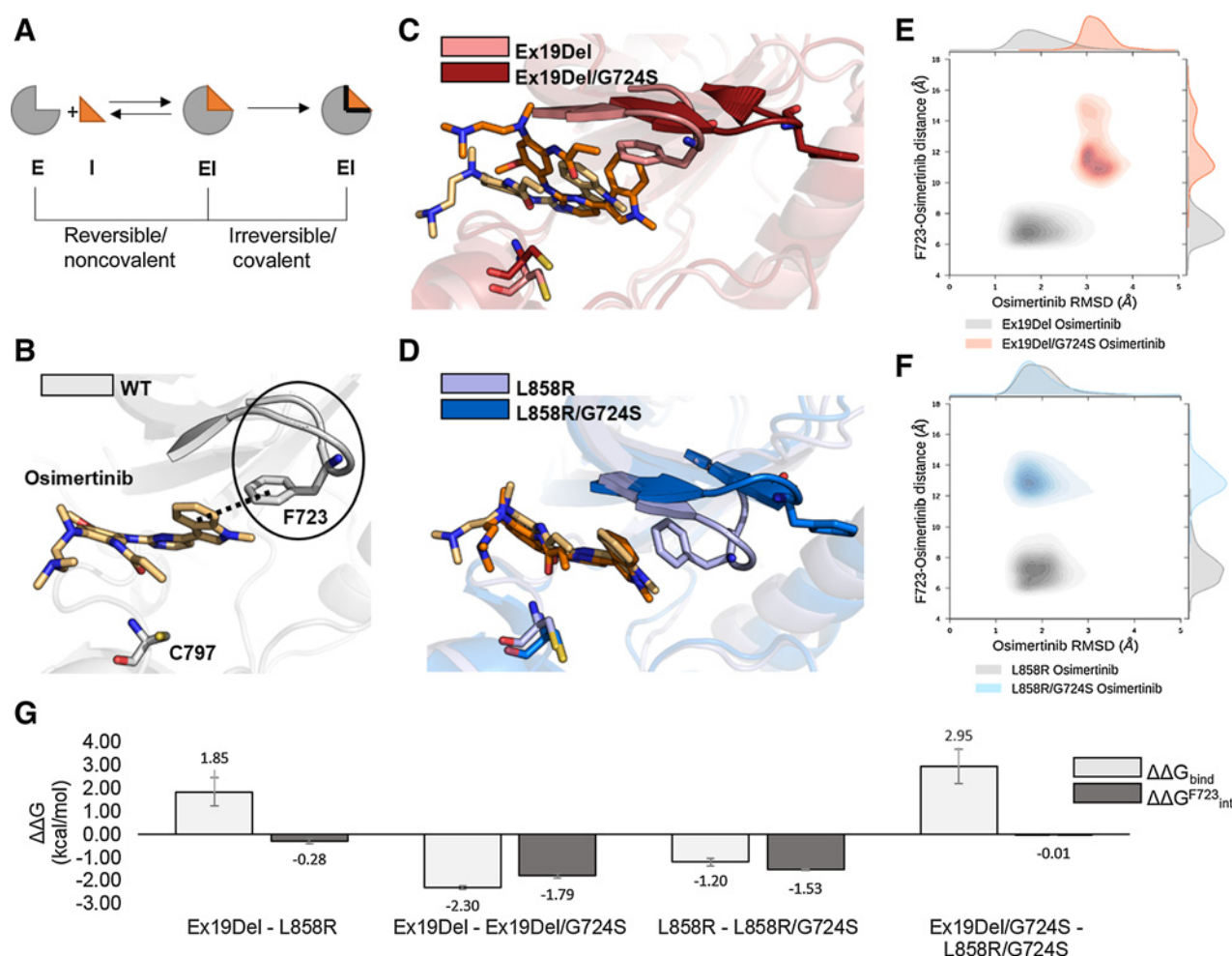
In addition, energy decomposition analysis supports our qualitative observation that F723 contributes favorably to osimertinib binding in both Ex19Del and L858R (the interaction energy of F723, $\Delta G_{\text{int}}^{\text{F723}}$ defined $\Delta G_{\text{int}}^{\text{F723}} = \Delta E_{\text{MM}} + \Delta G_{\text{solv}}$ is approximately -1.8 and -1.5 kcal/mol, respectively), and that addition of G724S prevents this interaction (Fig. 1G). As expected based on crystallographic evidence, our simulations show that F723 contributes considerably less to the interaction of EGFR with afatinib (ref. 39; Supplementary Table S2). Consistent with Fassunke and colleagues (20), our afatinib relative binding-free energies are less affected by G724S versus osimertinib (Supplementary Table S2). Altogether, these data suggest G724S may function as a resistance mutation to osimertinib in Ex19Del/G724S, but not in L858R/G724S.

In vitro expression of Ex19Del/G724S, but not L858R/G724S, is associated with osimertinib resistance

To test our simulation predictions, we first examined the ability of osimertinib to inhibit EGFR autophosphorylation of various EGFR single, double, and triple mutants. Of note, to date, G724S has been detected in both the absence and presence of T790M (18, 19). Therefore, we modeled all possibilities in our experimental studies. Osimertinib was effective at inhibiting EGFR autophosphorylation in 293FT cells expressing Ex19Del and Ex19Del/T790M, but not in 293FT cells expressing Ex19Del/C797S or Ex19Del/T790M/C797S, as C797S mutation has previously been associated with osimertinib resistance (refs. 16, 40; Fig. 2A). Likewise, osimertinib was ineffective at blocking autophosphorylation of EGFR Ex19Del/G724S and Ex19Del/T790M/G724S mutants.

We also tested the efficacy of osimertinib against L858R variant combinations. Analogous to the Ex19Del data above, phosphorylation of L858R and L858R/T790M was inhibited by osimertinib, whereas C797S-containing variants (L858R/C797S and L858R/T790M/C797S) were insensitive to this agent (Fig. 2B). In contrast to the Ex19Del variant data, phosphorylation of L858R/G724S and L858R/T790M/G724S was potently inhibited by osimertinib (Fig. 2B). These data are consistent with our simulations, which suggested a difference in the drug-binding properties between Ex19Del and L858R when combined with G724S mutation. Altogether, these data suggest that G724S functions as a resistance mutation in the context of Ex19Del but not L858R.

Next, we attempted to define strategies to overcome osimertinib resistance mediated by G724S mutation. In particular, we focused on the efficacy of earlier generations of wild-type selective EGFR TKIs (Supplementary Table S3). Previous studies have demonstrated that C797S-containing EGFR variants, which are resistant to osimertinib, retain sensitivity to the

**Figure 1.**

Stability of osimertinib in reversible complexes with EGFR mutants. EGFR mutants reversibly bound to osimertinib were simulated with GaMD. A schematic representation of a simplified binding equilibrium for a covalently binding inhibitor is depicted such that E = Enzyme target, I = Inhibitor, and EI = Enzyme-Inhibitor complex (A). Each simulation was performed in triplicate for a total of 12 independent 250 ns GaMD simulations. Representative images of osimertinib reversibly bound to WT [PDB ID 4ZAU; the solid black line indicates the bent P-loop; the dashed black line indicates the contact between the F723 phenyl and osimertinib indole ring (B), Ex19Del and Ex19Del/G724S (C), and L858R and L858R/G724S (D)] are displayed. Trajectory frames were extracted every 10 ps and plotted as osimertinib RMSD from the equilibrated start structure (*x* axis) and distance between the phenyl ring of F723 and the indole ring of osimertinib (*y* axis; E–F). RMSD vs. distance plots include data from 3 independent trajectories for each mutant–inhibitor pair (E–F). Select relative osimertinib binding-free energies are plotted as averages across 3 independent trajectories; error bars indicate SEM (G). $\Delta G_{\text{bind}} = \Delta E_{\text{MM}} + \Delta G_{\text{solv}} - T\Delta S$; $\Delta G_{\text{int}}^{\text{F723}} = \Delta E_{\text{MM}} + \Delta G_{\text{solv}}$; $\Delta\Delta G = \Delta G_1 - \Delta G_2$.

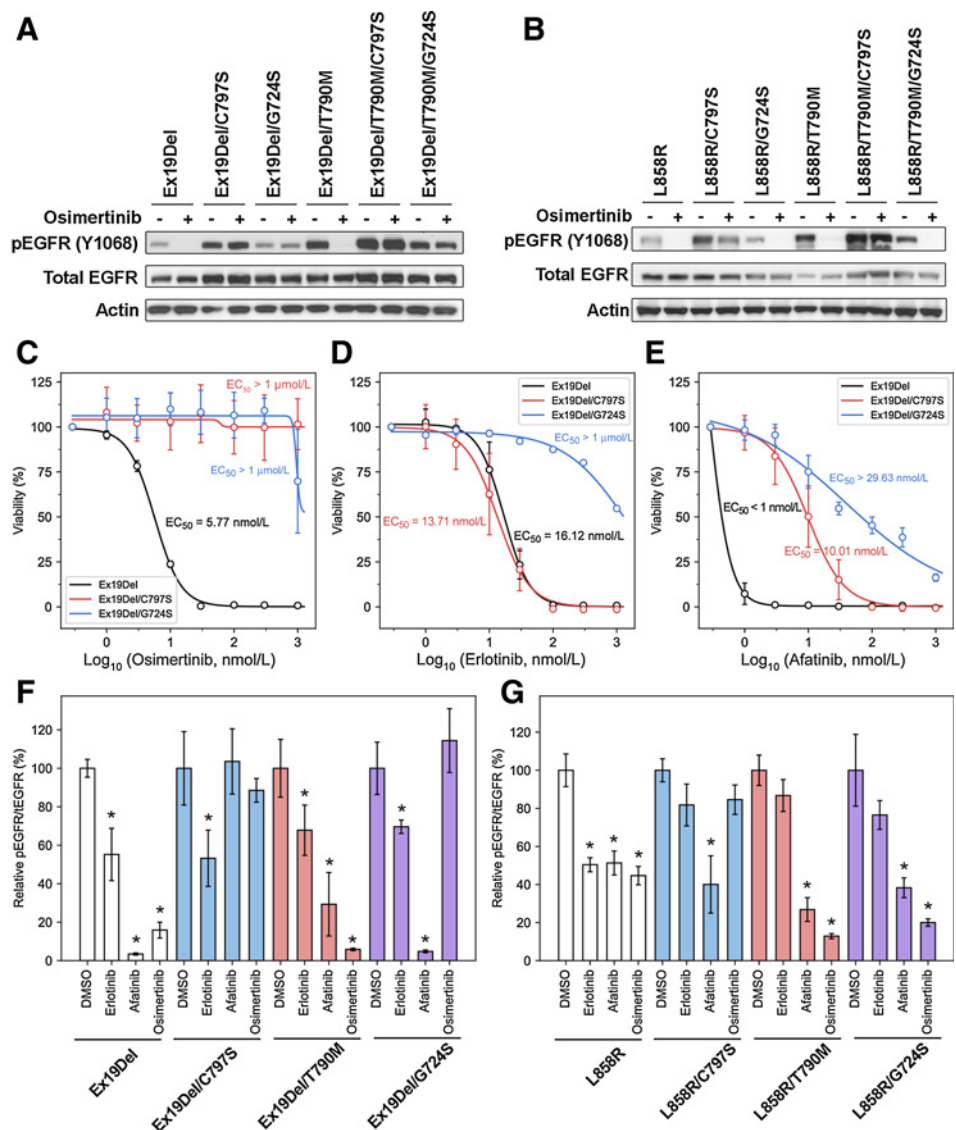
first-generation EGFR TKIs (erlotinib, gefitinib; ref. 16). We sought similar strategies for G724S-containing EGFR variants. We quantitatively evaluated several TKIs on Ex19Del-series mutants by stably transducing Ex19Del EGFR variants into Ba/F3 cells and measuring IL3-independent growth at multiple inhibitor concentrations (Fig. 2C–E). As expected, growth of cells expressing EGFR Ex19del/C797S and EGFR Ex19del/G724S was insensitive to osimertinib. Cell lines expressing Ex19del/C797S and Ex19del/G724S were also cross-resistant to another mutant-selective EGFR-TKI, rociletinib (Supplementary Fig. S4). In accord with previous data (16), cells expressing Ex19Del/C797S were sensitive to the effects of the first-generation EGFR TKI, erlotinib (Supplementary Table S3), with an EC_{50} paralleling that of the original Ex19Del single mutant (16.12 nmol/L vs. 13.71 nmol/L, respectively, Fig. 2D). However, the Ex19Del/G724S mutant was

insensitive to the effects of erlotinib ($EC_{50} > 1 \mu\text{mol/L}$). Our structural data suggested that afatinib may retain efficacy against the Ex19Del/G724S double mutant (Supplementary Fig. S3). In accord with these data, the growth of cells expressing this double mutant was inhibited with an EC_{50} of 29.63 nmol/L afatinib (Fig. 2E). Likewise, autophosphorylation of the Ex19Del/G724S in stably transduced NR6 cells was potently inhibited by afatinib, but not erlotinib or osimertinib (Fig. 2F; Supplementary Fig. S5), whereas the autophosphorylation of the L858R/G724S was potently inhibited by both afatinib and osimertinib (Fig. 2G; Supplementary Fig. S5).

Importantly, previous *in vitro* screens failed to identify G724S as a resistance mutation (21, 22). Our data suggest that this may be because these screens generated missense mutants beginning with WT, L858R, or L858R/T790M. Our data suggest that G724S

Figure 2.

EGFR G724S mediates osimertinib resistance in EGFR Ex19Del but not EGFR L858R mutants. **A**, 293FT cells transduced with different EGFR del19 variants were treated with 100 nmol/L osimertinib for 4 hours. Cellular lysates were probed with the indicated antibodies. **B**, 293FT cells transduced with different EGFR L858R variants were treated with 100 nmol/L osimertinib for 4 hours. Cellular lysates were probed with the indicated antibodies. **C**, Ex19Del, Ex19Del/C797S, and Ex19Del/G724S were treated with increasing amount of (C) osimertinib, (D) erlotinib, or (E) afatinib for 72 hours. CellTiter Blue assays were performed to assess cell viability. Each point represents 3 replicates. Data are presented as the mean percentage of viable cells compared with control \pm SD. NR6 cells transduced with (F) different EGFR del19 variants or (G) different EGFR L858R variants were treated with either DMSO, 100 nmol/L erlotinib, 100 nmol/L afatinib, or 100 nmol/L osimertinib for 4 hours. Relative pEGFR/TEGFR values are calculated by the density of pEGFR signal divided by the density of TEGFR signal, then normalized by the DMSO-treated group in each cell line. Density of Western blots was analyzed by ImageJ. *, $P < 0.05$ as compared with DMSO-treated group in each cell line. Representative Western blots are shown in Supplementary Fig. S6.



functions as a resistance mutation in the context of Ex19Del but not L858R. Moreover, our results provide additional evidence that afatinib, but not osimertinib or erlotinib, can function effectively as an inhibitor of Ex19Del/G724S.

G724S emerges as a resistance mutation in Ex19Del but not L858R-mediated NSCLC

To date, 4 independent reports (14, 18–20) have identified G724S as an emergent mutation in patients who have developed acquired resistance to osimertinib, with the frequency of G724S being 13% (higher than the frequency of C797S) in a recent article by Fassunke and colleagues (20). Interestingly, all of these patients harbored Ex19Del as the original activating mutation (14, 18–20). Our computational and experimental data suggest that G724S confers resistance to osimertinib in Ex19Del but not L858R; nevertheless, it is possible that L858R/G724S exists in a subset of EGFR-mutant NSCLC patients. To investigate the prevalence of EGFR G724S mutation, we analyzed data from tissue and plasma DNA samples within the Foundation Medicine data-

base (Supplementary Table S4). Consistent with our computational and experimental evidence, G724S co-occurred with an Ex19Del variant in 15 of 19 cases, and L858R/G724S was not identified (Fig. 3A). Given that the likelihood of observing Ex19Del versus L858R in EGFR-mutant NSCLC is approximately equal (41), it is exceedingly unlikely that L858R-activating mutation would not be found in any of our patient samples without an additional bias.

In 4 cases (all Ex19Del variants), we were able to obtain tissue genomic profiling data at 2 unique time points. In 3 of these cases (Fig. 3C–E), G724S allelic frequency is positively correlated with Ex19Del allelic frequency over time and decline of the T790M allele. Moreover, G724S is not present in the tumor biopsy from any of these 4 patients prior to Ex19Del; that is, the mutant allele frequency of G724S starts at zero in all of these matched cases (Fig. 3B–E). These data suggest that G724S emerges in a fraction of Ex19Del patients to promote disease progression.

To highlight one particular case (patient No. 15, Fig. 3E), a 54-year-old Caucasian gentleman never smoker was diagnosed

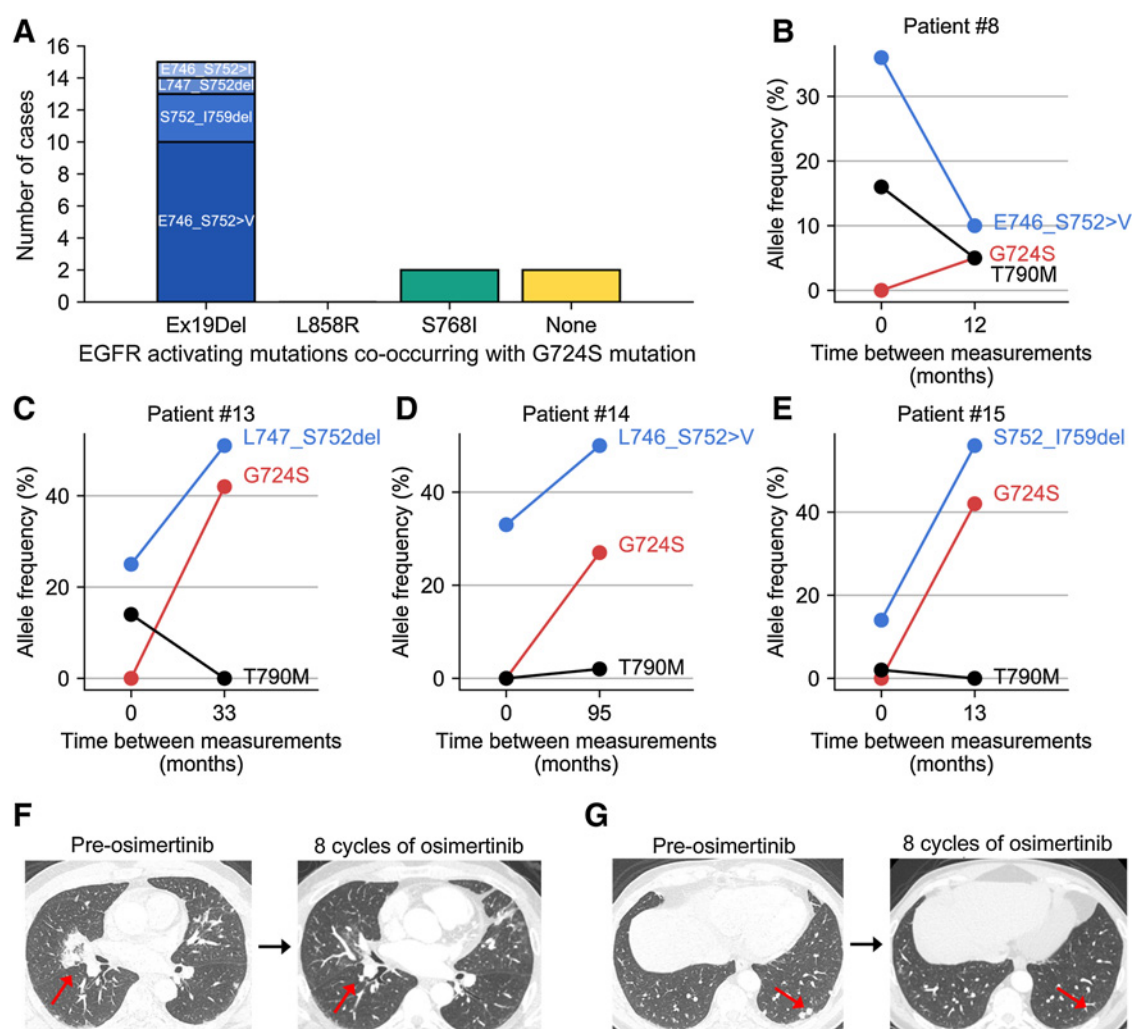


Figure 3.

Prevalence of oncogenic EGFR mutations in NSCLC patient samples with G724S. **A**, Bar chart depicting the number of cases of each oncogenic EGFR mutation associated with G724S in NSCLC patient samples with genomic profiling obtained through Foundation Medicine (total $n = 19$). **B–E**, Allelic frequencies for the specific Ex19Del variant, T790M, and G724S are plotted versus time between measurements for 4 cases for which tissue genomic profiling results were available at 2 independent time points. **F** and **G**, Radiographic images for patient No. 15 taken prior to osimertinib therapy (left) and after 8 cycles of osimertinib (right). The red arrows in the CT scan images show sites of disease that responded to osimertinib.

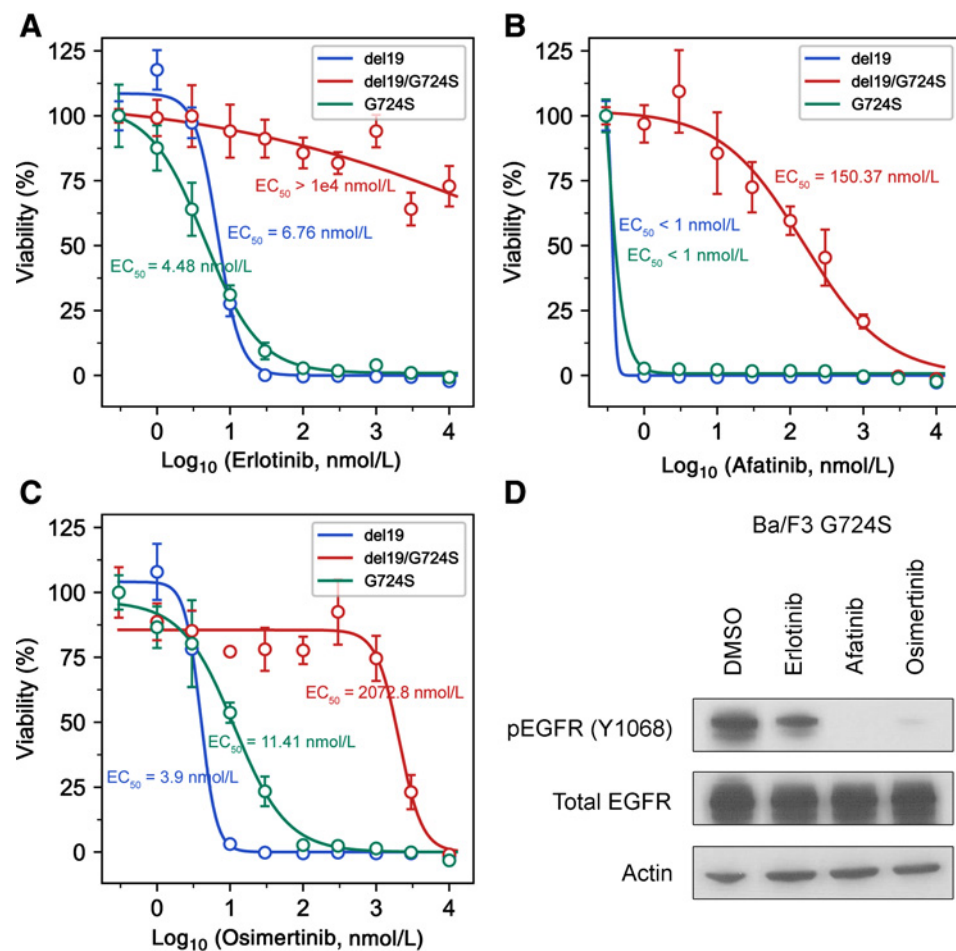
with stage IV lung adenocarcinoma after presenting with abdominal pain. Tumor mutational testing was positive for an *EGFR* Ex19Del mutation. He was treated with first-line erlotinib plus bevacizumab with partial response. Fifteen months after starting this combination therapy, he experienced progression of disease with enlargement of bilateral pulmonary nodules and a ground glass opacity in the left upper lobe. Repeat biopsy confirmed metastatic lung adenocarcinoma, and tumor genetic testing at that time revealed the presence of *EGFR* Ex19Del and T790M mutations. He was thereafter treated with osimertinib and had a partial response lasting 30 months (Fig. 3F). He experienced progression of disease with new metastases to the skull, liver, and bone. Tumor genetic testing of a repeat biopsy revealed the presence of *EGFR* Ex19Del, loss of T790M mutation, and gain of *EGFR* G724S mutation. He was treated with radiotherapy to the skull followed by systemic therapy with carboplatin and pemetrexed. Approximately 4 months after starting cytotoxic chemotherapy, he devel-

oped symptomatic pleural and pericardial effusions, which ultimately resulted in his demise.

Of note, G724S was also detected with the oncogenic missense mutant S768I in 2 of 19 cases. Shan and colleagues previously demonstrated that S768I stabilizes the active conformation by improving hydrophobic packing between the α C-helix and the β 9-strand. G724S also occurred as an individual missense mutation in 2 of 19 cases (Fig. 3A). The latter suggests that G724S could potentially be independently oncogenic. Indeed, G724S could support oncogenic growth of Ba/F3 cells (Supplementary Fig. S6A). Of note, the G724S single mutant exhibits a TKI sensitivity profile very similar to Ex19Del in that this mutant can be effectively inhibited by erlotinib, afatinib, and osimertinib (Fig. 4; Supplementary Fig. S6B). In addition, we identified 9 cases of *EGFR* G724S as an isolated mutation in patients with small-cell lung carcinoma, bladder urothelial carcinoma, glioblastoma, breast cancer, and colorectal cancer (Supplementary Table S5).

Figure 4.

The EGFR G724S single mutant can be effectively inhibited by EGFR TKIs. Ba/F3 cells stably expressing EGFR Ex19Del, G724S, and Ex19Del/G724S were treated with increasing amounts of (A) erlotinib, (B) afatinib, or (C) osimertinib for 72 hours. CellTiter Blue assays were performed to assess cell viability. Each point represents 4 replicates. Data are presented as the mean percentage of viable cells compared with control \pm SD. D, Ba/F3 cells transduced with EGFR G724S were treated with either DMSO, 100 nmol/L erlotinib, 100 nmol/L afatinib, or 100 nmol/L osimertinib for 4 hours. Cellular lysates were probed with the indicated antibodies.



These data are consistent with recent evidence implicating G724S as an oncogenic driver in colorectal cancer (42) and suggest that patients with tumors harboring an isolated G724S mutation could be treated with FDA-approved EGFR TKIs, such as afatinib.

The catalytically active conformation of EGFR is better stabilized by E746_S752>V/G724S than by E746_A750del/G724S

Unexpectedly, all of the Ex19Del alterations co-occurring with G724S in patient tumor samples were rare variants (Supplementary Table S4). The Ex19Del variant occurring most frequently with G724S in this cohort was E746_S752>V (10/19), followed by S752_I759del (3/19), E746_S752>I (1/19), and L747_S752del (1/19). For context, approximately 67% of Ex19Del cases are attributed to the canonical variant, E746_A750del, whereas less than 2% are attributed E746_S752>V (43). To better understand this enrichment in Ex19Del rare variants, we performed GaMD simulations for E746_S752>V and E746_S752>V/G724S in the apo-state and in reversible complex with osimertinib.

We utilized MM/GBSA to compute the relative binding-free energies between the 2 sets of Ex19Del variants. The results displayed large statistical uncertainty in the calculation of the binding-free energies (Supplementary Table S2), that we attribute to increased P-loop fluctuations in E746_S752>V and E746_S752>V/G724S relative to WT and the other variants (Supplementary Fig. S1; Supplementary Movies S1–S2). The

majority of this difference is attributable to increased fluctuations in E746_S752>V, and just as in the cases of WT and E746_A750del, the additional fluctuations associated with G724S in E746_S752>V occur primarily at the tip of the P-loop (Supplementary Fig. S1). Nevertheless, E746_S752>V, but not E746_S752>V/G724S, is able to stabilize a favorable contact between F723 and the indole ring of osimertinib, consistent with results obtained in the previous E746_A750del and E746_A750del/G724S osimertinib-binding simulations (Supplementary Table S2).

EGFR kinase activation is achieved through asymmetric dimerization of an acceptor EGFR kinase α C-helix with a donor kinase α H-helix. The acceptor kinase is the catalytically active dimer subunit (28). In a seminal article on EGFR dynamics, Shan and colleagues demonstrated that common oncogenic mutations increase activity by stabilizing the α C-helix inward conformation to promote asymmetric dimerization (44). We hypothesized that the unexpected enrichment of the E746_S752>V/G724S double mutant in clinical samples may result from increased stabilization of the α C-helix inward conformation in E746_S752>V/G724S relative to E746_S752>V. To test this hypothesis, we performed a detailed analysis of the conformational free-energy landscape profiles of each EGFR variant in the apo-state.

Consistent with Shan and colleagues, results from our GaMD simulations of WT, E746_A750del, and L858R demonstrate increased stabilization of the α C-helix inward conformation

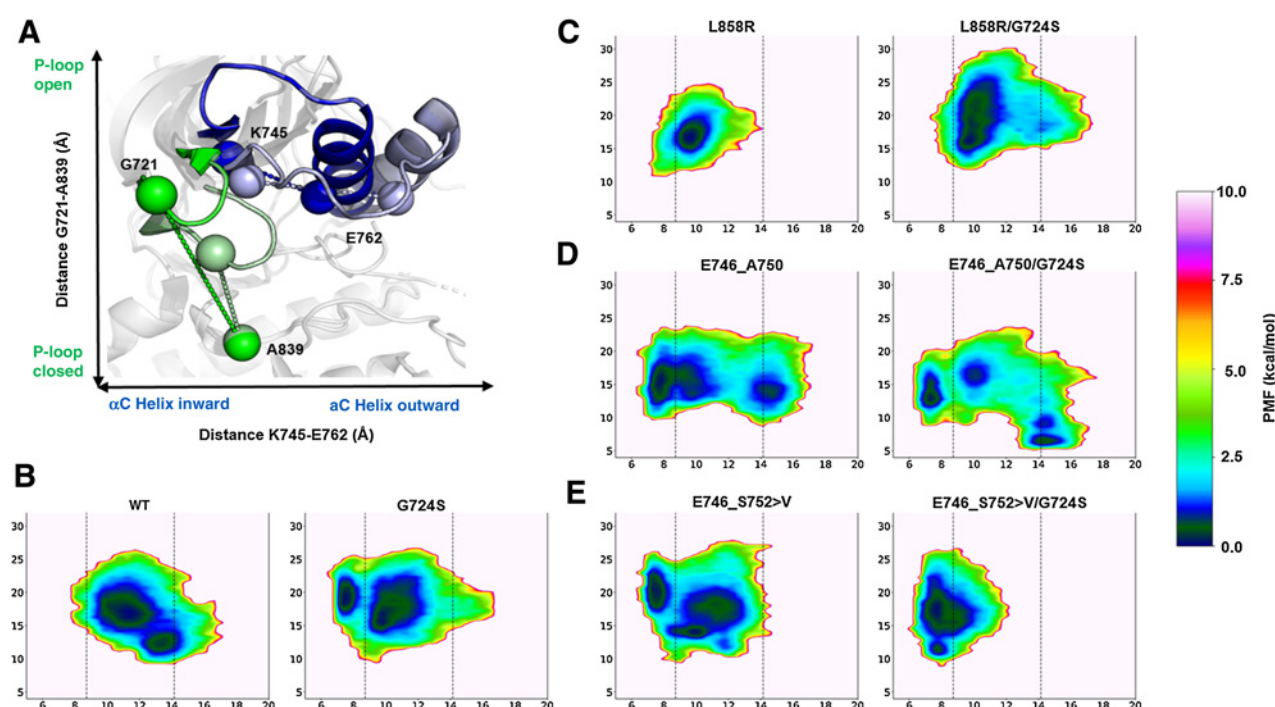


Figure 5.

Conformational free-energy landscape of EGFR kinase domain mutants. The reaction coordinate reference for the conformational free-energy landscape of EGFR kinase mutants is indicated on a model of WT in the active (PDB ID 2ITX; bold colors) and inactive (PDB ID 3GT8; faded colors) conformations (**A**). Green spheres represent the distance (Å) between Ha1 of G721 and CB of A839. Blue spheres represent the distance between CB of K745 and CB of E762. The potential of mean force with respect to the positions of the α C helix (x axis) and P-loop (y axis) is plotted for WT and G724S, L858R and L858R/G724S, E746_A750 and E746_A750/G724S, and E746_S752>V and E746_S752>V/G724S (**B**). The left and right vertical dashed lines on the free-energy plots (**C–E**) indicate center-of-mass distances between K745 and E762 in active (PDB ID 2GS6) and inactive (PDB ID 2GS7) EGFR kinase, respectively. The left vertical dashed line therefore represents the canonical EGFR kinase α C-helix inward conformation, whereas the right vertical dashed line represents the canonical EGFR kinase α C-helix outward conformation. All depicted simulations start from the active (α C-helix inward, activation loop outward) conformation. The energetic reweighting factor was approximated with cumulant expansion to the second order. Free-energy landscapes from the 500 ns GaMD simulations are depicted here.

compared with WT (44). In addition, our simulations show that E746_S752>V stabilizes the α C-helix inward conformation relative to WT. Critically, our computational analyses suggest that E746_S752>V/G724S stabilizes the active α C-helix inward conformation even more than E746_S752>V (Fig. 5E). In contrast, E746_A750del/G724S visits α C-in conformations less frequently than E746_A750del (Fig. 5D). These results suggest that E746_S752>V/G724S could lead to enhanced dimerization-dependent activation compared with E746_S752>V, whereas E746_A750del/G724S could lead to reduced dimerization-dependent activation compared with E746_A750del.

Collectively, these data support G724S as a resistance mutation in Ex19Del over L858R, and that specific Ex19Del mutants may preferentially co-occur with G724S, potentially driven by differences in active conformation stability in the presence of G724S. In addition, our results suggest that G724S (as a single point mutation) also stabilizes the α C-helix inward conformation, consistent with reports that G724S may function as an oncogenic variant in colorectal cancer (ref. 42; Fig. 5A–D; Supplementary Fig. S7). Our data more broadly suggest that the underlying activating mutation profile of EGFR influences the development of drug resistance mutations. This has important implications for clinical management of patients with EGFR-mutant NSCLC.

Discussion

Notable advancements have been observed through the development of increasingly selective inhibitors of mutant oncoproteins (11). The design and clinical implementation of mutant-selective third-generation EGFR TKIs, such as osimertinib, are an excellent example. Unfortunately, despite these advances, the development of resistance mutations to TKI therapy remains a significant barrier in attaining the best outcomes for patients with EGFR-mutant NSCLC. In addition to the previously identified C797S resistance mutation, our results demonstrate osimertinib resistance may emerge in the form of G724S mutations within the P-loop of the EGFR kinase domain. However, unlike C797S, our results also suggest that G724S-mediated resistance preferentially occurs in Ex19Del but not L858R. Indeed, extensive atomic-detail simulations at the structural level, multiple independent *in vitro* models, and patient genomic profiling all demonstrate G724S to be an Ex19Del-specific resistance mechanism to osimertinib. Retrospectively, we identified multiple patient cases now observed in the literature where patients with EGFR Ex19Del-mutant NSCLC displayed tumor progression post-osimertinib treatment in the presence of G724S (14, 18–20). Together with the data we have presented here, these case studies suggest G724S functions as a resistance mutation in an allele-specific manner. To our knowledge, ours is the first evidence directly demonstrating

that the underlying activating mutation (e.g., Ex19Del vs. L858R) influences the emergence of resistance mutations under selective pressure from a specific TKI.

Enhanced α C-helix stabilization in L858R results from polar interactions between the substituted arginine and neighboring negatively charged amino acids. In contrast, enhanced α C-helix stabilization in Ex19Del mutations likely results from alterations at the β 3- α C interface. Structural superimposition of our active state deletion models onto EGFR WT shows that the position of L747 in WT is occupied by S752 (WT numbering) in E746_A750del and by the inserted valine in E746_S752>V (Supplementary Fig. S8). Our data suggest that the P-loop conformational changes induced by G724S lead to destabilization of the α C-helix inward conformation in the presence of polar β 3- α C interface substitutions.

Interestingly, Ex19Del/G724S displays phospho-EGFR levels similar to Ex19Del, but reduced phospho-EGFR compared with Ex19Del/C797S (Fig. 2A). Our modeling suggests that stabilization of the α C-helix can vary between mutants upon introduction of G724S (Fig. 5). Similarly, C797S may preferentially stabilize the α C-helix inward conformation of specific Ex19Del variants. C797 is a critical member of the structurally distinct catalytic spine (C-spine). The C-spine does not contribute to the interface formed by the glycine-rich loop and β 3- α C linker region. Nevertheless, previous network analysis by McClendon and colleagues (45) suggests that the dynamics of the glycine-rich loop and the C-spine may be highly correlated. We therefore suspect C797S may influence interdomain correlations.

Fundamentally, our observations are similar to a concept familiar to clinical oncologists—that sequence variations in mutant proteins can affect drug binding. Osimertinib was developed to bind T790M with higher affinity than non-T790M EGFR mutants (46). Here, we show that sequence variations corresponding to the original activating mutation should also be taken into account when considering mechanisms of TKI resistance. Our findings have several important and immediate clinical implications. First, we further knowledge on a novel osimertinib resistance mutation that was not predicted by *in vitro* studies (21, 22). Recent studies have shown that G724S may be as prevalent as C797S in osimertinib-resistant tumors (20). However, there are critical differences. Although C797S-containing EGFR mutants (e.g., Ex19Del/C797S) regain sensitivity to first-generation EGFR TKIs, erlotinib and gefitinib, the same G724S-containing EGFR variants are cross-resistant to these inhibitors. In fact, there is an ongoing phase I clinical trial (NCT03122717) of osimertinib plus gefitinib combination therapy in patients with treatment-naïve advanced EGFR-mutant NSCLC. This trial aims to test the hypothesis that circumventing C797S-mediated osimertinib resistance with gefitinib will prolong response. This concept will clearly not apply for patients with G724S-mediated osimertinib resistance. However, our results support a role for afatinib therapy in treating Ex19Del patients with disease progression on osimertinib via C797S or G724S in the absence of T790M (Fig. 2). Furthermore, in cases where G724S is potentially an independent oncogenic driver of other cancers (Supplementary Table S5), our results suggest possible treatment strategies with existing FDA-approved inhibitors. This level of evidence is critical to nominate variants of uncertain clinical significance, such as isolated G724S mutation, for eligibility into clinical trials such as NCI MATCH (NCT02465060).

These clinical consequences are rooted in structural perturbations to EGFR kinase. Detailed mechanistic understanding of these perturbations can provide critical insight to guide therapeutic intervention. Just prior to submission of this article, Fassunke and colleagues published investigations into the structural basis of EGFR G724S-mediated osimertinib (20). The authors coupled structure-based alignment of EGFR WT to EGFR D770_N771insNPG (exon 20 mutation) with P-loop RMSF calculations derived from short, single-trajectory cMD simulations. Specifically, Fassunke and colleagues demonstrated an elevated RMSF in both WT and E746_A750del when G724S is introduced. From that result, the authors postulated 2 potential, opposing mechanisms of G724S-mediated third-generation TKI resistance: (1) steric repulsion of the inhibitor, or (2) loss of important interactions with the inhibitor. However, RMSF calculations alone are rarely sufficient to provide detailed mechanistic insights (38). Moreover, osimertinib resistance occurs in Ex19Del/G724S variants (Fig. 2) but not G724S single mutants (Fig. 4). The broad mechanisms previously posited do not provide adequate detail to address these data.

Here, we performed multiple independent GaMD-enhanced sampling simulations in the presence and absence of osimertinib or afatinib totaling over 23 μ s. For each EGFR mutant, we computed the relative binding-free energies of osimertinib and afatinib as well as the conformational free-energy landscape profiles of the apo-state structures. Although our RMSF calculations are consistent with Fassunke and colleagues, our results further suggest that G724S hyperstabilizes a β -bend conformation of the glycine-rich P-loop. This prevents contact of the F723 phenyl ring with the osimertinib indole ring. Our calculations suggest that L858R reversibly binds osimertinib with higher affinity than Ex19Del, and consequently loss of the F723-osimertinib contact fails to disrupt binding in L858R. In Ex19Del, the addition of G724S destabilizes the reversible complex necessary for covalent adduct formation (Fig. 1; Supplementary Table S2).

Moreover, we identified differences in P-loop conformational preferences between Ex19Del/G724S and L858R/G724S (Fig. 4A–D; Supplementary Fig. S7). In addition to our findings in Fig. 1, it is possible that L858R/G724S is less poised to accommodate substrate binding versus Ex19Del/G724S, resulting in L858R/G724S functioning as a catalytically inefficient receiver kinase in an asymmetric dimer (Supplementary Fig. S7G and S7H); however, additional experiments would be required to test this hypothesis. It is also possible that L858R/G724S conformations may be less primed to support dimerization compared with L858R. The α C-helix of L858R/G724S bows outward over the course of the simulation, suggesting increased local instability. Despite still favoring the active state relative to WT, it is possible that with longer simulation times the α C-helix of L858R/G724S would more rapidly transition to a state incapable of supporting asymmetric dimerization than L858R (Fig. 4C; Supplementary Fig. S7G and S7H).

Importantly, our simulations also suggest that G724S increases the stability of the EGFR-active conformation in the E746_S752 variant of Ex19Del, but reduces stability of the E746_A750del variant. Greater stability of the active α C-inward conformation in E746_S752>V/G724S offers a possible explanation for the enrichment of the rare variant Ex19Del in the Foundation Medicine cohort of NSCLC patients with G724S. Interestingly, of the 4 patients with genomic profiling data presented in Fassunke and

colleagues, all of them saw an increase in molecular fraction of G724S post-osimertinib therapy, and all of them had uncommon variants of Ex19Del (20).

These findings have implications in other, non-EGFR-mutant cancers as well. For example, ALK (anaplastic lymphoma kinase) rearrangements can be found in approximately 5% of NSCLC cancers (47). Over a dozen fusion partners have been identified across ALK⁺ cancers (47). Even the most frequently occurring fusion partner in ALK⁺ NSCLC, echinoderm microtubule-associated protein-like 4 (EML4), has >10 identified unique fusion variants (48). In addition, on-target acquired resistance to first- and second-generation ALK TKIs occurs in the form of approximately a dozen unique missense mutations (49). Recent data suggest that a particularly recalcitrant ALK solvent front mutation, G1202R, is more likely to cause resistance in the context of EML4-ALK E6;A20 (V3) fusion rather than the more common EML4-ALK E13;A20 (V1) fusion (50). A structural basis for this observation was not presented; however, analogous to our current study, it could be that the unique structural and biochemical properties of the original activating mutation foreshadowed the development of a specific resistance mutation.

In summary, we have employed an interdisciplinary computational and experimental approach which provides evidence that on-target osimertinib resistance in EGFR-mutant NSCLC occurs in an allele-specific manner dependent on the underlying activating mutation. Our data support a potential structural mechanism for Ex19Del/G724S osimertinib resistance and open the door for further studies on TKI-EGFR interactions. We hope these mechanistic studies will be exploited to develop novel EGFR TKIs that circumvent multiple drug resistance mutations. Finally, we hope that insights from our investigations will be applied to develop increasingly effective targeted therapies for additional genetically defined cancers.

Disclosure of Potential Conflicts of Interest

J.S. Ross has ownership interests (including patents) at Foundation Medicine. V.A. Miller is an employee of and has ownership interests (including patents) at Revolution Medicines and Foundation Medicine/ROCHE. S. Ali has ownership interests (including patents) at Foundation Medicine, is a consultant/advisory board member for Incysus Therapeutics, Revolution Medicines, Azitra, and Princetpx. L. Bazhenova is a consultant/advisory board member for Takeda, AstraZeneca, Genentech, Abbvie, Loxo, Eli Lilly and Novartis, and reports receiving commercial research grants from Beyondspring Pharma. A. B. Schrock is an employee of Foundation Medicine, Inc. C.M. Lovly is an employee of Foundation Medicine, is a consultant/advisory board member for Pfizer, Novartis, AstraZeneca, Genoptix, Sequenom, Ariad, Takeda, Blue-

prints Medicine, Cepheid, reports receiving commercial research grants from Xcovery and Novartis. No potential conflicts of interest were disclosed by the other authors.

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