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Structural, biochemical and clinical characterization of epidermal growth factor receptor (EGFR) exon 20 insertion mutations in lung cancer

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Materials and Data Availability: The following protein structures were deposited into the protein data bank (PDB): PDB ID 4LRM (EGFR kinase of D770_N771insNPG + PD168393) and PDB ID 4LQM (EGFR kinase of L858R + PD168393).

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Abstract

Epidermal growth factor receptor (*EGFR*) gene mutations (G719X, exon 19 deletions/insertions, L858R and L861Q) predict favorable responses to EGFR tyrosine kinase inhibitors (TKIs) in advanced non-small-cell lung cancer (NSCLC). However, *EGFR* exon 20 insertion mutations (∼10% of all *EGFR* mutations) are generally associated with insensitivity to available TKIs (gefitinib, erlotinib and afatinib). The basis of this primary resistance is poorly understood. We study a broad subset of exon 20 insertion mutations, comparing *in vitro* TKI sensitivity with responses to gefitinib and erlotinib in NSCLC patients; and find that most are resistant to EGFR TKIs. The crystal structure of a representative TKI-insensitive mutant (D770_N771insNPG) reveals an unaltered ATP-binding pocket and the inserted residues form a wedge at the end of the C-helix that promotes the active kinase conformation. Unlike EGFR-L858R, D770_N771insNPG activates EGFR without increasing its affinity for EGFR TKIs. Unexpectedly, we find that EGFR-A763_Y764insFQEA is highly sensitive to EGFR TKIs *in vitro*; and patients whose NSCLCs harbor this mutation respond to erlotinib. Analysis of the A763_Y764insFQEA mutant indicates that the inserted residues shift the register of the C-helix in the N-terminal direction, altering the structure in the region that is also affected by the TKI-sensitive EGFR-L858R. Our studies reveal intricate differences between EGFR mutations, their biology and their response to EGFR TKIs.

Keywords

lung cancer; non-small-cell lung cancer; epidermal growth factor receptor; EGFR; erlotinib; gefitinib; afatinib; exon 20 insertion; x-ray crystallography

INTRODUCTION

Non-small-cell lung cancers (NSCLCs) continue to be the leading cause of cancer-related deaths [1]. Epidermal growth factor receptor (*EGFR*) gene mutations were initially reported in 2004 and defined a prevalent molecularly-classified subgroup of NSCLC [2–7]. The most common *EGFR* mutations are in-frame deletions around the LREA motif (amino-acid residues 747 to 750) of exon 19 (45% of *EGFR* mutations) and the exon 21 L858R point mutation (40% of *EGFR* mutations) [5,6,8]. These *EGFR* mutations are oncogenic in both cell lines and mouse models [9,10]. They activate the EGFR signaling pathway in the absence of ligand, promote downstream pro-survival and anti-apoptotic signals such as phosphatidylinositol-3-kinases (PI3K)/protein kinase B (AKT) and extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), and render *EGFR* mutated cells dependent on constitutively-active EGFR for their survival [11,12]. The inhibition of EGFR upregulates pro-apoptotic molecules (such as BIM) in models driven by EGFR-delE746_A750 or L858R, activates the intrinsic mitochondrial apoptotic pathway and ultimately leads to cell death [13–16]. Most patients whose tumors harbor exon 19 deletions or L858R *EGFR* activating mutations have radiographic responses to monotherapy with the reversible adenosine triphosphate (ATP)-competitive EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib [17–23] and the irreversible EGFR TKI afatinib [24]. Other *EGFR* mutations have also been associated with some sensitivity to gefitinib and erlotinib. These include exon 18 point mutations in position G719 (G719A, C or S - ∼3% of *EGFR* mutations), rare inframe exon 19 insertions [25], and the exon 21 L861Q mutant (∼2% of *EGFR* mutations) [26–28].

The other main group of *EGFR* mutations in NSCLC is composed of inframe insertions within exon 20 of *EGFR* (Figure 1A). Exon 20 insertion mutations comprise 4–10% of all *EGFR* mutations [27,29–32]. Most of these mutations lie near the end of the C-helix within the N-lobe of the kinase, after residue M766, but a small subset map to the middle of the C-helix (affecting amino-acids E762 to Y764) [5,33,34]. Unlike *EGFR* exon 19 deletions and L858R–bearing tumors, most NSCLCs with *EGFR* exon 20 insertion mutations don't respond radiographically or clinically to gefitinib or erlotinib. The reported response rate (RR) is below 5% and most patients have short intervals of disease control [35]. The precise mechanisms that determine the primary insensitivity to EGFR TKIs in the most prevalent exon 20 insertion mutations and the response of less prevalent exon 20 insertion mutations to gefitinib or erlotinib remain elusive. We herein elucidate the molecular and structural mechanisms that underlie the patterns of response or resistance of *EGFR* exon 20 insertion mutations to EGFR TKIs.

RESULTS

Sensitivity of *EGFR* exon 20 insertion mutations to EGFR TKIs *in vitro*

To better understand the patterns of resistance or response to EGFR TKIs of exon 20 insertion mutations, we studied representative EGFR exon 20 mutations using *in vitro* systems. We selected two mutations that lie within the C-helix (A763_Y764insFQEA [structurally identical to D761_E762insEAFQ] and Y764_V765insHH) and five mutations that lie at the end of the helix or within the loop following it (M766_A767insAI, A767_V769dupASV [identical to V769_D770insASV], D770_N771insNPG, D770_N771insSVD [identical to S768_D770dupSVD] and H773_V774insH [identical to P772_H773insH]) (Fig. 1A, Sup. Table 1). In aggregate, these mutations represent more than half of reported *EGFR* exon 20 insertion mutations [31,32,35]. In addition, we used gefitinib/erlotinib-sensitive (L858R and exon 19 deletion mutations [delL747_S752 and delL747_P753insS]) and gefitinib/erlotinib-resistant (L858R+T790M and exon 19 deletion mutations+T790M) mutations as assay controls [36,37].

We created Ba/F3 cells stably expressing the aforementioned EGFR mutations. All were able to proliferate in the absence of IL3 (Sup. Fig. 1), indicating transforming ability of these mutations. We next measured proliferation in the presence of erlotinib and showed that only cells with EGFR-delL747_P753insS, L858R and the atypical A763_Y764insFQEA - among all EGFR exon 20 insertions - were inhibited by erlotinib concentrations below 0.1 μM (Fig. 1B, 1C and Sup. Table 2). All other EGFR exon 20 insertions and T790M–bearing mutations had 50% inhibitory concentrations (IC_{50}s) that exceeded 2 μM erlotinib (Fig. 1B, 1C and Sup. Table 2). Nearly identical results were obtained with gefitinib (Sup. Table 2), and a similar pattern of insensitivity was noted with the more potent irreversible EGFR TKI afatinib (Sup. Table 2). Transient transfection and overexpression of these EGFR constructs in Cos-7 cells led to comparable results (Sup. Fig. 2). Examination of EGFR signaling revealed that phosphorylation of EGFR, AKT and ERK were not fully inhibited by 1 μM erlotinib in cells with the inhibitor-insensitive EGFR exon 20 insertions mutations (Fig. 1D). A dose-response experiment disclosed that an inhibitor-insensitive EGFR exon 20 insertion (A767_V769dupASV) lacked deactivation of phosphorylated EGFR, downstream targets (PI3K/AKT and MAPK/ERK) and upregulation of the apoptotic cascade, as measured by levels of BIM (a marker of EGFR TKI-induced cell killing [13]), with 1 μM or submicromolar concentrations of erlotinib (Fig. 1E). In stark contrast, the inhibition of these downstream phosphorylation events and upregulation of BIM were seen with EGFR-A763_Y764insFQEA in a similar fashion as with inhibitor-sensitive L858R (Fig. 1D, 1E)

Enzyme kinetic analysis of representative EGFR exon 20 insertion mutants

Based on our cell line studies, we selected a representative inhibitor-resistant exon 20 insertion mutant, D770_N771insNPG, and the inhibitor-sensitive A763_Y764insFQEA for detailed enzyme kinetic studies. We expressed and purified the kinase domain of these mutants (residues 696–1022 of EGFR), and of the L858R mutant and wild-type (WT) EGFR. Consistent with their transforming activity (Sup. Fig. 1), both insertion mutants exhibited higher catalytic activity than WT EGFR; although they were not as active as EGFR-L858R (Table 1).

The exquisite sensitivity of EGFR-L858R and exon 19 deletions to EGFR TKIs stems from two effects: their enhanced intrinsic affinity for these TKIs and their markedly decreased affinity for substrate ATP [38,39]. Thus we measured the Michaelis constant for ATP ($K_{m[ATP]}$) for D770_N771insNPG and A763_Y764insFQEA (Table 1, Sup. Fig. 3), as well as the inhibition constant (K_i) for gefitinib for these mutants (Table 1, Sup. Fig. 4). Notably, the inhibitor-sensitive A763_Y764insFQEA mutant bound gefitinib ~10-fold more tightly than the insensitive D770_N771insNPG mutant (Table 1). The D770_N771insNPG mutant bound gefitinib with a K_i similar to that of WT EGFR (K_i=25.7 nM for D770_N771insNPG and K_i=16.4 nM for WT EGFR). The K_i value for A763_Y764insFQEA (2.5 nM) was comparable to that we measured for the L858R mutant (6.4 nM). Because inhibitors must compete with substrate ATP for binding, the ratio of $K_i/K_{m[ATP]}$ provides a better estimate of potency that the K_i alone. Based on this measure, A763_Y764insFQEA is predicted to be almost as sensitive to gefitinib as the L858R mutant, whereas D770_N771insNPG is expected to be many fold less sensitive (Table 1). The practical effect of these alterations in kinetic parameters can be seen in inhibition curves measured at a range of ATP concentrations; like L858R, the A763_Y764insFQEA mutant is effectively inhibited by gefitinib in the presence of 1 mM ATP (which approximates the concentration of ATP in cells) while D770_N771insNPG is not (Sup. Fig. 4).

EGFR exon 20 insertion mutations and response to reversible EGFR inhibitors in patients with NSCLC

To determine whether clinical responses matched the pattern of sensitivity to EGFR TKIs that we observed *in vitro*, we assembled data from 19 NSCLC patients with diverse *EGFR* exon 20 insertion mutations who were treated with reversible EGFR TKIs (Fig. 2A, 2B, Sup. Table 3).

Consistent with previous reports [35,40–44], almost all 19 NSCLC patients from our centers displayed progressive disease (PD) in the course of treatment with gefitinib or erlotinib (Fig. 2B, Sup. Table 3, and Sup. Fig. 5). However, three patients with A763_Y764insFQEA *EGFR* mutated NSCLCs either achieved stable disease (SD) or partial response (PR) when treated with erlotinib (Fig. 2A, 2B, Sup. Fig. 5, Sup. Table 3). Even in this small case series, the RR of gefitinib or erlotinib was significantly higher for A763_Y764insFQEA (2/3; 66.6%) when compared to all other mutations within or following the C-helix (0/16, 0%; p=0.0175); furthermore the progression-free survival of these patients exceed that of patients with other exon 20 mutations (Sup. Fig. 6). Thus, as predicted by our preclinical studies, NSCLCs harboring *EGFR* A763_Y764insFQEA appear to respond to gefitinib and erlotinib.

Characterization of an A763_Y764insFQEA mutated lung cancer cell line

To further study the properties and inhibitor sensitivity of the EGFR-A763_Y764insFQEA insertion, we derived a lung cell line from the malignant pleural effusion of a patient with a NSCLC containing *EGFR* A763_Y764insFQEA (Fig. 3A; Sup. Table 3, and Sup. Fig. 5). The original biopsy disclosed an adenocarcinoma (Sup. Fig. 7) and the derived cell line (named BID007) was adherent to tissue culture plate (Sup. Fig. 8). Sequence of exons 18–21

of *EGFR* from DNA isolated from BID007 confirmed the presence of the 12 nucleotides corresponding to the A763_Y764insFQEA insertion (Fig. 3A). DNA was also analyzed for additional hotspot mutations in 113 known oncogenes and tumor suppressor genes (Sup. Table 4), and no additional mutation was identified. A detailed genome-wide SNP analysis to determine copy number variations in BID007's DNA showed that many regions of the cells' chromosomes had broad and focal copy gains or losses (Sup. Fig. 9A). The region around chromosome 7, corresponding to *EGFR*, demonstrated slight amplification (Sup. Fig. 9B). In addition, BID007 had a focal area of deletion encompassing the *CDKN2A* tumor suppressor gene (Sup. Fig. 9C); an area commonly deleted in cancers [45].

To determine if BID007 requires mutant EGFR for its viability, we used siRNAs against *EGFR* in BID007, as well as in lung cancer cell lines H3255 (*EGFR*-L858R and known to undergo cell proliferation arrest and cell death upon inactivation of EGFR) and A549 (*KRAS* mutated). In both BID007 and H3255, EGFR-specific siRNAs decreased expression of EGFR and suppressed cell growth (Fig. 3B). As expected, EGFR siRNAs had no effect on the growth of *KRAS* mutated A549 cells (Fig. 3B). These results demonstrate that BID007 cells, similar to other *EGFR* mutated cell lines, are dependent on aberrant EGFR signaling.

The sensitivity of BID007 cells to EGFR TKIs was compared to that of a group of three EGFR TKI-sensitive *EGFR* mutated NSCLC lines (PC9 [EGFR-delE746_A750], HCC827 [delE746_A750] and H3255) and one erlotinib-resistant line (H1975 [L858R+T790M]). Proliferation assays with increasing concentrations of EGFR TKIs disclosed that growth of BID007, PC9, HCC827 and H3255 cells was inhibited by submicromolar concentrations of erlotinib (Fig. 3C, Sup. Table 2), gefitinib and afatinib (Sup. Table 2), indicating augmented sensitivity relative to the inhibitor-resistant H1975 cell line.

The levels of phosphorylated EGFR, AKT and ERK in BID007, PC9, HCC827 and H3255 were diminished by 1 μM of erlotinib (Fig. 3D). A dose-response experiment highlighted that only BID007 and H3255 had deactivation of phosphorylated EGFR, AKT, ERK and upregulation of BIM with submicromolar concentrations of erlotinib (Fig. 3E), and underwent apoptosis with submicromolar doses of erlotinib, as shown by PARP cleavage (Fig. 3E). These results supported our siRNA experiments, and further detailed that BID007 cells were not only dependent on EGFR signaling but were also sensitive to clinically achievable submicromolar concentrations of gefitinib and erlotinib.

Structural analysis of EGFR TKI-resistant and TKI-sensitive EGFR exon 20 mutants

To better understand the mechanism of activation of TKI-resistant EGFR exon 20 insertion mutations, we determined the structure of D770_N771insNPG in complex with inhibitor PD168393 at 3.5Å resolution (Sup. Table 5). For comparison, we also determined the structure of EGFR-L858R in complex with the same compound (Sup. Table 5). PD168393 is a pre-clinical irreversible anilinoquinazoline inhibitor that is structurally related to afatinib and dacomitinib [46,47]. Preparation of a covalent complex with PD168393 stabilized the protein during final stages of purification and was required for crystallization. The D770_N771insNPG mutant adopts an active conformation with the C-helix in its inward position (Fig. 4A). The structure contains five molecules in the crystallographic asymmetric unit arranged in a continuous chain of "asymmetric dimer" interactions in which the C-lobe of one molecule interacts with the N-lobe of the next (Sup. Fig. 10A). This mode of interaction mediates EGFR activation by ligand-induced dimerization [48], and is typically recapitulated in crystals of the EGFR kinase in its active conformation. The D770_N771insNPG insertion lies at the C-terminal end of the C-helix, immediately following D770 (Fig. 4A). Together with D770, the three inserted residues form a tight turn, with a hydrogen bond formed between the main chain carbonyl of D770 and the amide of the inserted glycine (Fig. 4B, Sup. Fig. 10B, 10C). Beyond this glycine, the conformation of

the polypeptide chain is quite similar to that of the WT and L858R kinases [38], and overall the D770_N771insNPG structure superimposes well with the L858R structure in complex with the same inhibitor (Fig. 4B, 4C). Differences, which likely arise from the divergent crystal packing in D770_N771insNPG, include a small change in the relative orientation of the N- and C-lobes, and the conformation of the activation loop, which is fully ordered in the D770_N771insNPG structure but not in the present L858R structure. Within the ATP site, the conformation of the covalently bound inhibitor is essentially the same as in the L858R structure, and while the resolution of this study is relatively low (3.5Å), there is no evidence that the D770_N771insNPG mutation alters interactions with the inhibitor (Fig. 4C). Likewise, we expect that binding of gefitinib and erlotinib would be unperturbed in this mutant, consistent with the closely similar K_i values for gefitinib we measure for D770_N771insNPG and WT EGFR (Table 1).

D770_N771insNPG may activate EGFR by blocking the conformational rearrangements required for the inactive conformation of the kinase. In the inactive state, the C-helix pivots outward, and shifts by ∼3.5Å toward the insertion site. The insertion is at the "pivot point" of the C-helix, thus the inserted residues may sterically inhibit the reorientation of the C-helix that is characteristic of the inactive state. This mutation may also interfere with an adjacent "electrostatic switch" interaction that is proposed to stabilize an inactive EGFR dimer [49]. Irrespective of the detailed mechanism of activation, our structure and kinetic data show that D770_N771insNPG promotes the active conformation, as reflected by its enhanced catalytic activity (Table 1) and a propensity to form the asymmetric dimer as seen in the crystal structure (Sup. Fig. 10A) and in solution (Sup. Fig. 11).

Because we have been unable to crystallize the A763_Y764insFQEA mutant EGFR, we studied it further using homology modeling. The four residue FQEA insertion occurs just C-terminal to A763, in the middle of the C-helix. From a structural perspective, the four inserted residues are expected to form approximately one turn of helix and shift the register of adjacent residues in the helix, either toward the N-terminus or toward the C-terminus (Fig. 5A). The C-helix contains a key active site residue, E762, just N-terminal to the site of the insertion [50]. A shift in register toward the C-terminus would leave this catalytically important residue unaltered. Alternatively, a shift toward the N-terminus would displace this residue by one turn, but effectively replace it with a glutamic acid residue introduced by the insertion (FQEA, Fig. 5A). To distinguish between these two possibilities, we prepared two altered versions of this mutant: one in which the endogenous E762 is mutated to glutamine (E762Q_insFQEA) and a second in which the inserted glutamic acid residue is mutated to glutamine (A763_Y764insFQQA). We found that E762Q_insFQEA, like A763_Y764insFQEA, was highly active in a ligand independent manner but A763_Y764insFQQA was inactive (Fig. 5B). Thus, we conclude that the insertion shifts the register of the helix in the N-terminal direction and that the glutamic acid residue in the FQEA insertion assumes the active site role of E762 in the WT kinase. Based on these findings, we prepared homology models of the A763_Y764insFQEA mutant using both the active and inactive conformations of the kinase (Fig. 5C, 5D). In both models, the insertion was modeled in a helical conformation, shifting the register of the C-helix toward its N-terminus, and resulting in a longer loop at its N-terminal end (the β3-αC loop). Examination of the homology model of the inactive state suggests a mechanism of activation related to that of L858R [38]. The shift in register in the C-helix effectively replaces I759 with alanine (Fig. 5D). Isoleucine at position 759, together with L747, L858 and L861, is part of a cluster of interacting hydrophobic residues that helps to stabilize the inactive conformation. The L858R and L861Q mutations within this cluster are well-characterized activating mutations [28], and mutation of L747 in this cluster to proline or serine is believed to underlie the activity of EGFR-exon 19 insertions [25]. Thus we hypothesize that the de facto I759A replacement caused by the FQEA insertion may activate EGFR via a similar mechanism

(destabilization of the inactive conformation). Furthermore, we note that like both exon 19 deletion and insertion mutants [25], A763_Y764insFQEA must alter the structure of the β3-αC loop. The model based on the active state supports the hypothesis that the glutamic acid residue in the A763_Y764insFQEA insertion can effectively replace E762 in the active site (Fig. 4A, 4C). Finally, inspection of the model indicates that the FQEA insertion is compatible with formation of the asymmetric dimer and, consistent with this, analysis of the A763_Y764insFQEA mutant using size-exclusion chromatography revealed a monomer-dimer equilibrium in solution (Sup. Fig. 11).

DISCUSSION

EGFR exon 20 insertion mutations comprise a unique set of EGFR activating mutations. These have been previously described as insensitive to clinically-available reversible EGFR TKIs, gefitinib and erlotinib [31,32,35,51]. In the present report, we provide detailed characterization of a broad spectrum of these mutations and their correlation with patient response to EGFR TKIs. Our study was limited by a number of factors, including a paucity of available NSCLC cell lines with *EGFR* exon 20 insertion mutations, a lack of in vivo models and the small number of exon 20 insertion mutated NSCLC cases that were treated with reversible EGFR TKIs in our centers. Within these constraints, our data confirm that most exon 20 insertions are not inhibited by submicromolar concentrations of reversible EGFR TKIs. The most common EGFR exon 20 insertions that have been reported in NSCLC samples are post C-helix insertions of one to four amino-acids, which in aggregate account for 80–90% of all exon 20 insertions [31,32,35]. We characterized Y764_V765insHH, M766_A767insAI, A767_V769dupASV, D770_N771insNPG, D770_N771insSVD and H773_V774insH using *in vitro* models and found that cells bearing these mutant EGFR proteins are not inhibited by clinically-achievable doses of gefitinib, erlotinib [12,35,52–54] or the irreversible EGFR inhibitor afatinib [35,55]. These results are consistent with our observations that tumors harboring these mutations did not achieve clinical or radiographic responses to usual doses of gefitinib (250 mg daily) or erlotinib (150 mg daily) in NSCLC patients. Therefore, based on our data and previously published reports [31,32,35], the aforementioned mutations affecting amino-acids Y764 to V774 should be classified as non-sensitizing to the reversible EGFR TKIs gefitinib and erlotinib.

Unexpectedly, we identified an EGFR exon 20 insertion mutation that was inhibited by gefitinib and erlotinib at submicromolar concentrations: EGFR A763_Y764insFQEA. This sequence alteration accounts for 10–20% of all reported *EGFR* exon 20 insertions [31,32,35]. Our kinetic characterization of this mutant revealed that it is activated, relative to WT EGFR, and that it is much more potently inhibited by gefitinib than is WT EGFR. *In vitro* characterization of A763_Y764insFQEA demonstrated that it had an IC_{50} of < 0.05μM to erlotinib or gefitinib, which is more than 10-fold lower than the clinically achievable serum concentrations of these EGFR TKIs (gefitinib 250 mg/day has serum troughs of ∼0.5μM and erlotinib 100–150 mg/day has serum troughs of ∼1–2μM) at their clinical dosing schemes [12,52]. In addition, submicromolar concentrations of erlotinib inhibited phosphorylation of EGFR in A763_Y764insFQEA mutant cells, led to deactivation of the PI3K/AKT and MAPK/ERK pathways, and subsequently induced apoptosis in our models. These results were also confirmed in BID007, a NSCLC-derived cell line bearing the *EGFR* A763_Y764insFQEA mutation. Three patients with NSCLCs harboring *EGFR* A763_Y764insFQEA had clinical and radiographic regressions or stable disease on erlotinib 150 mg daily. Consequently, we conclude that *EGFR* A763_Y764insFQEA is an EGFR TKI-sensitizing mutation. Structurally, EGFR-A763_Y764insFQEA is very different from other exon 20 insertions. Notwithstanding the limitations inherent in homology modeling, our structural analysis and supporting mutagenesis indicate that the inserted FQEA sequence shifts the register of the C-helix toward its N-terminus, altering the length of the β3-αC loop

leading into the helix and effectively leading to an I759A replacement. The β3-αC loop is the site of exon 19 deletion mutations, and the I759A alteration is immediately adjacent to L858R and L861Q, and we hypothesize that it leads to catalytic activation in a related manner. Thus at a structural and enzyme kinetic level, A763_Y764insFQEA may more closely resemble the L858R and exon 19 deletion mutants than it does other exon 20 insertion mutations. EGFR-A763_Y764insFQEA and the identical D761_E762insEAFQ amino acid sequence appear to be unique among reported EGFR exon 20 insertions in having the ability to induce an N-terminal shift in the C-helix while maintaining or replacing active site residue E762.

The structure of D770_N771insNPG, which we studied as a representative EGFR TKI-insensitive exon 20 mutation, shows how the three amino acid insertion forms a “wedge” at the end of the C-helix that may effectively lock the helix in its inward, active position. While each will differ in detail, we expect that other EGFR exon 20 insertion mutants that insert one to three residues at or near the end of the C-helix will have a similar structural effect. The D770_N771insNPG structure, together with our enzyme kinetic studies, shows that this insertion mutant binds EGFR TKIs with a binding mode and apparent affinity similar to that of WT EGFR. Thus the clinical resistance of this mutant does not stem from steric interference with inhibitor binding. Rather, D770_N771insNPG simply does not sensitize to gefitinib and erlotinib inhibition. L858R and exon 19 deletion mutants exhibit increased $K_{m[ATP]}$ and higher affinity (5–10 fold) for gefitinib or erlotinib than the WT receptor [39,50,56]. Together with the oncogene-addicted state of tumors bearing these mutations [39,50,56], these factors explain their sensitivity to EGFR TKIs [50]. Because the dose-limiting toxicities of gefitinib and erlotinib arise from inhibition of WT EGFR in normal tissues, the enhanced sensitivity of these mutants relative to WT EGFR effectively creates the “therapeutic window” that makes gefitinib and erlotinib useful drugs [39,50,56]. In these sensitive EGFR mutants, serum concentrations can be achieved that functionally shut down the oncogenic mutant EGFR, but relatively spare the WT receptor in the skin and gastrointestinal tract. Unlike EGFR-L858R and exon 19 deletion mutants, the D770_N771insNPG mutation lacks this “Achilles’ heel”, since it activates EGFR without markedly diminishing its affinity for ATP or increasing its affinity for EGFR TKIs.

Our findings have important implications for efforts to develop EGFR TKIs effective against D770_N771insNPG and other “insensitive” exon 20 insertion mutants. The EGFR-T790M mutation confers resistance by reversing the sensitization conferred by primary activating mutations, but it does so by altering the gatekeeper residue in the ATP-site that is known to be an important determinant of inhibitor specificity [50,57]. Thus, the T790M structural alteration in essence “enabled” discovery of inhibitors that selectively inhibit T790M–bearing mutants, when in association with L858R or exon 19 deletions, relative to WT EGFR [58,59]. By contrast, there are no structural alterations proximate to the ATP binding cleft in D770_N771insNPG. Additionally, the similar ATP and TKI binding properties of this mutant, as compared with WT EGFR, suggest that it will be difficult to achieve the requisite selectivity versus the WT receptor [39,50,56,58,59]. Indeed, it seems that EGFR monoclonal antibodies [60] and EGFR WT kinase sparing covalent T790M selective TKIs [61] are ineffective in typical EGFR exon 20 insertion mutants. Biochemical screening of large compound libraries to identify potent inhibitors of the D770_N771insNPG mutant, with counterscreening against WT EGFR to identify mutant-selective compounds or scaffolds, may be the most rational approach to what appears to be a very challenging drug discovery problem. Interestingly, the close homology of EGFR and ERBB2 insertion mutations [46,62] may indicate that future therapeutic options for EGFR exon 20 insertion mutations may also be applicable to other cohorts of NSCLC.

In summary, our results not only explain the intricate interplay between different EGFR mutations and their response to EGFR TKIs, but also provide guidance for the selection (in the case of A763_Y764insFQEA) or omission (in the case of other exon 20 insertion mutations) of clinically-available TKIs for the treatment of *EGFR* exon 20 insertion mutated NSCLCs.

MATERIALS AND METHODS

See Supplementary Materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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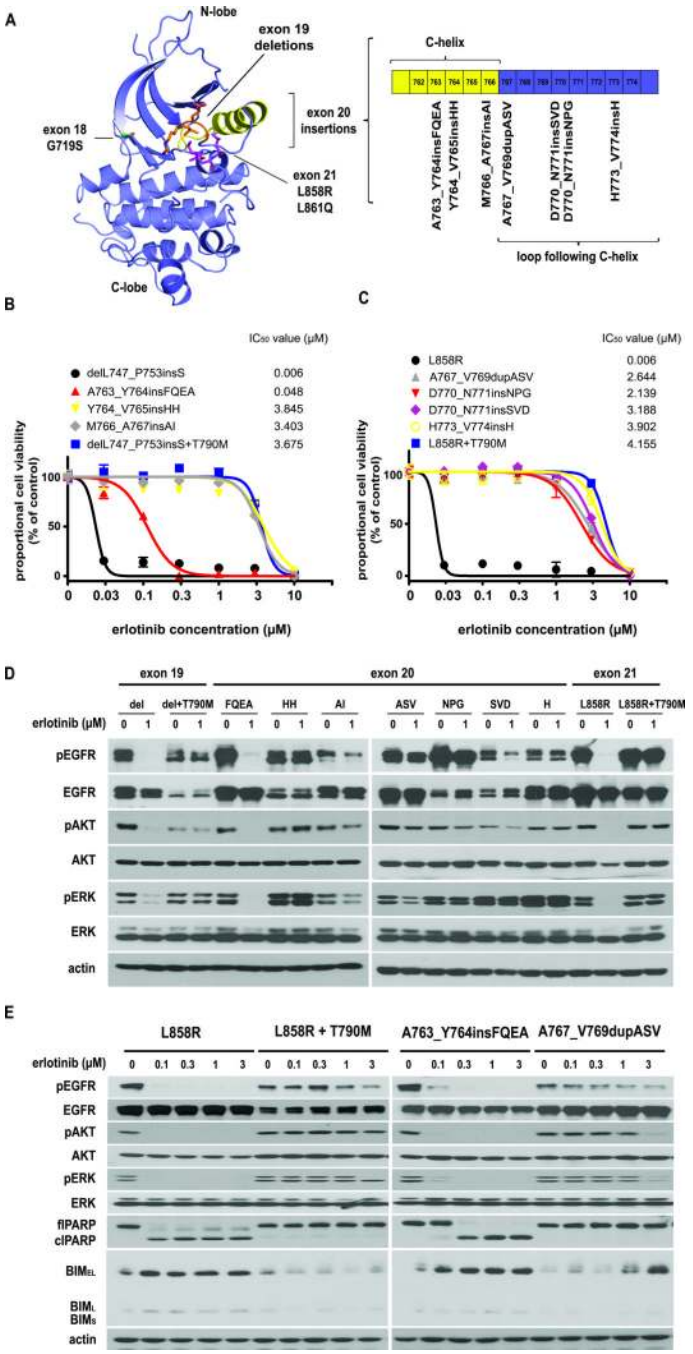
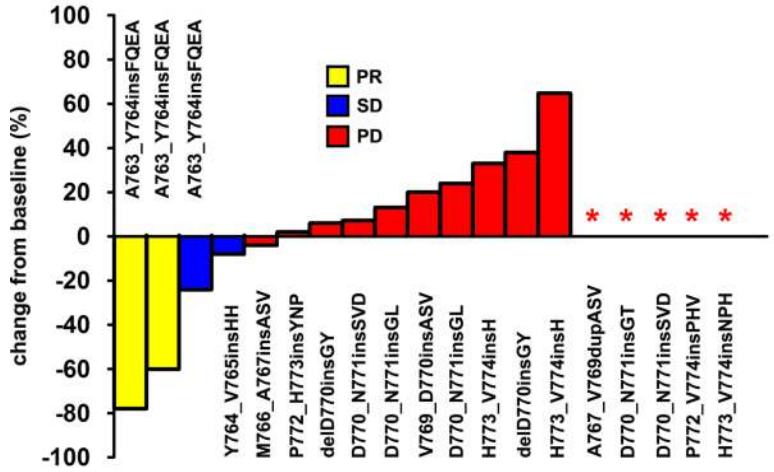


Figure 1.

EGFR exon 20 insertion mutations and their response to EGFR TKIs. A. Structure of the EGFR kinase in the inactive conformation, highlighting the locations of diverse EGFR mutations (drawn from PDB ID 1XKK). The schematic on the right depicts the site of EGFR exon 20 insertion mutations studied here. B. and C. Dose-dependent cell growth inhibition of Ba/F3 cells expressing EGFR-delL747_P753insS, delL747_P753insS+T790M, A763_Y764insFQEA, Y764_V765insHH, M766_A767insAI, A767_V769dupASV, D770_N771insNPG, D770_N771insSVD, H773_V774insH, L858R and L858R+T790M. Ba/F3 cells expressing aforementioned EGFR mutations were treated with the indicated doses of erlotinib for 72 hours. Cell survival was measured using a CellTiter Aqueous One

Solution Cell Proliferation Assay. Error bars indicate standard deviation (n=3). Calculated average IC_{50} values of eleven EGFR mutation types are shown (n=3). D. Inhibition of EGFR signaling by erlotinib. Ba/F3 cells expressing all generated EGFR mutations were treated with 1 μM erlotinib for 6 hours. Phosphorylation of EGFR, AKT, and ERK proteins were detected by immunoblotting. E. Dose-response of erlotinib in Ba/F3 cells expressing EGFR L858R, L858R–T790M, A763_Y764insFQEA, and V769_D770dupASV. The cells were treated with indicated doses of erlotinib for 24 hours. Immunoblotting was done against the indicated proteins (EGFR, AKT, and ERK, as well as full length [flPARP] or cleaved PARP [clPARP] and isoforms of BIM [extra long, BIM_{EL}; long, BIM_L; and short, BIM_S]).

A



B

	Best response to reversible EGFR TKI				
EGFR mutation	drug	PR	SD	PD	RR [%]
A763_Y764insFQEA	erlotinib	2	1	-	66.6%
Y764_V765insHH	gefitinib	-	1	-	0%
M766_A767insASV	erlotinib	-	-	1	0%
A767_V769dupASV	gefitinib	-	-	1	0%
V769_D770insASV	erlotinib	-	-	1	0%
D770_N771insGL	erlotinib	-	-	2	0%
D770_N771insGT	erlotinib	-	-	1	0%
D770_N771insSVD	erlotinib	-	1	1	0%
delD770insGY	erlotinib	-	-	2	0%
P772_H773insYNP	gefitinib	-	-	1	0%
P772_V774insPHV	erlotinib	-	-	1	0%
H773_V774insH	gefitinib/ erlotinib	-	-	2	0%
H773_V774insNPH	erlotinib	-	-	1	0%

Figure 2.

Response to EGFR TKIs of NSCLCs harboring *EGFR* exon 20 insertion mutations. A. Waterfall plot of best responses of target tumor lesions after exposure to gefitinib or erlotinib in relation to baseline measurements for each patient. The plot highlights that all A763_Y764insFQEA bearing tumors decreased in size after exposure to erlotinib, while other mutations had either minimal changes or increase in target lesions. Yellow bars indicate partial response (PR), blue bars stable disease (SD) and red bars progressive disease (PD). * indicates patients who displayed non-measurable PD. B. Detailed response of each individual mutation type analyzed.

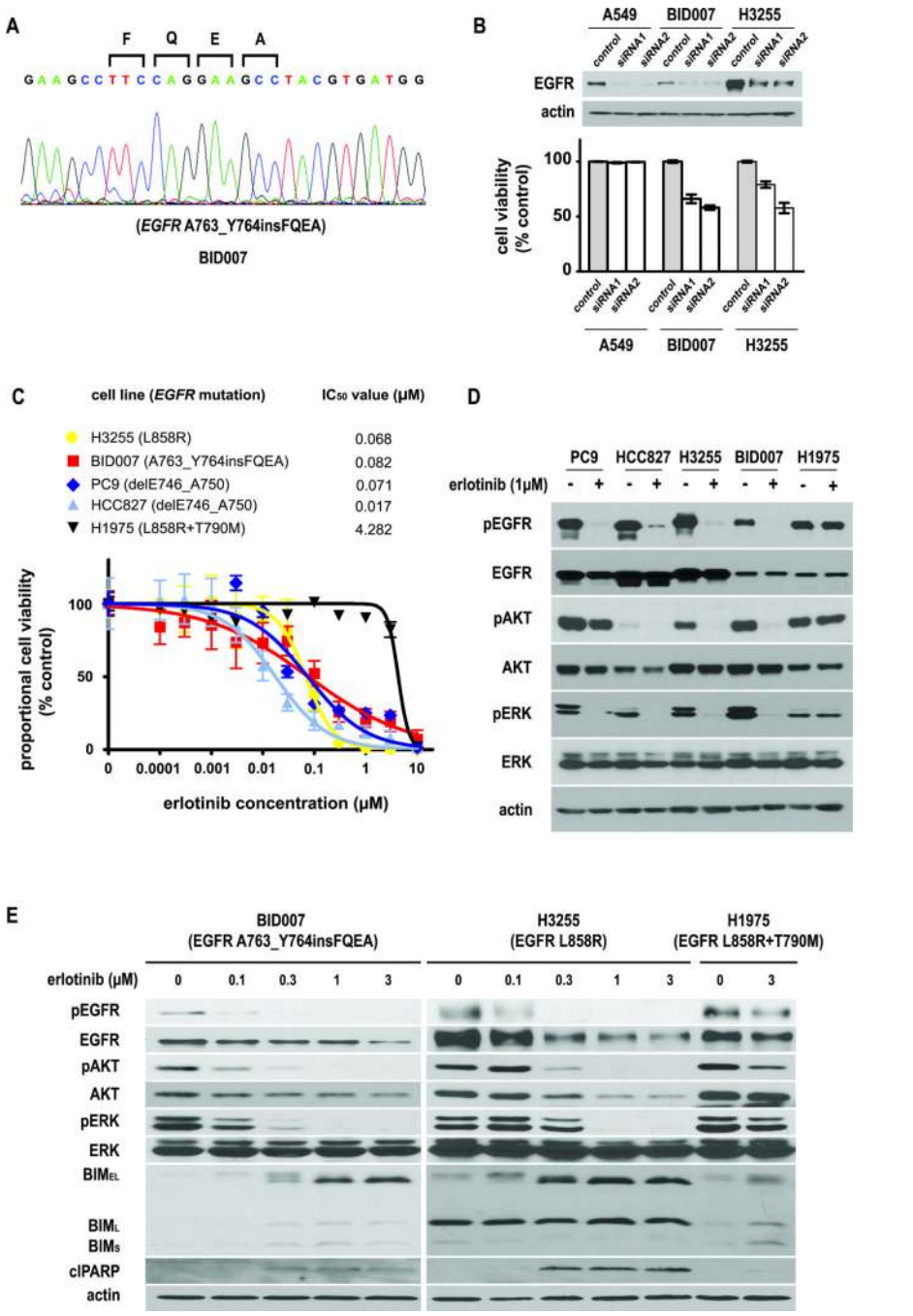


Figure 3.

BID007, a cell line expressing *EGFR*-A763_Y764insFQEA. A. Sequence of BID007's DNA confirms *EGFR*-A763_Y764insFQEA. B. siRNA knockdown of *EGFR* in NSCLC cell lines A549, BID007, and H3255 cells for 72 hours (n=3). EGFR protein was detected by immunoblotting. Compared to A549 cells, siRNA1 and siRNA2 inhibited proliferation in BID007 (p=0.005 and p=0.001, respectively) and H3255 (p=0.003 and p=0.003, respectively). C. Dose-dependent cell growth inhibition of H3255, BID007, HCC827, H1975 and PC9 cells. The cells were treated with the indicated doses of erlotinib for 72 hours. Error bars indicate standard deviation (n=3). Calculated average IC_{50} values of H3255, BID007, HCC827, H1975 and PC9 cells are shown (n=3). D. Inhibition of EGFR

signaling by erlotinib in NSCLC cell lines. PC9, HCC827, H3255, BID007, and H1975 cells were treated with or without 1 μM erlotinib for 6 hours. Phosphorylation of EGFR, AKT, and ERK proteins was detected by immunoblotting. E. Dose-response of erlotinib in BID007, H3255, and H1975 cells. The cells were treated with indicated doses of erlotinib for 24 hours. Immunoblotting was done against the indicated proteins (EGFR, AKT, ERK, clPARP and BIM).

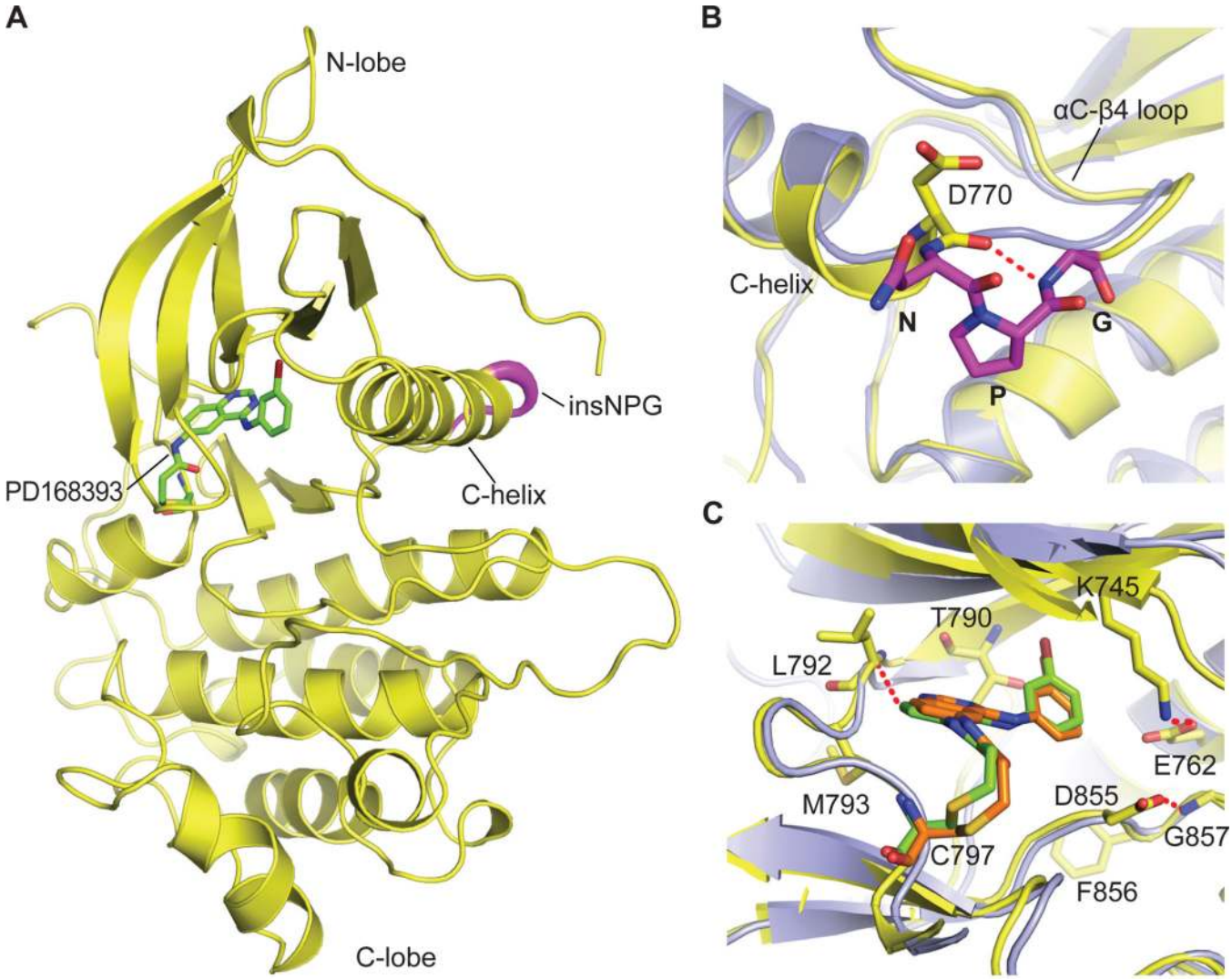


Figure 4.

Implications of the crystal structure of the EGFR exon 20 insertion D770_N771insNPG (insNPG). A. Crystal structure of the insNPG mutant. The inhibitor PD168393, covalently bound to Cys797 (C797), is shown in stick form with carbon atoms colored green. The inserted NPG sequence is highlighted in magenta. B. Detailed view of the NPG insertion. The insNPG structure is shown in yellow with the inserted residues in magenta in stick form and is superimposed on the L858R structure (blue ribbon). C. Superposition of the active site region of the insNPG and L858R mutants bound to inhibitor PD168393. The compound binds in an essentially identical manner in both structures, forming a covalent bond with C797.

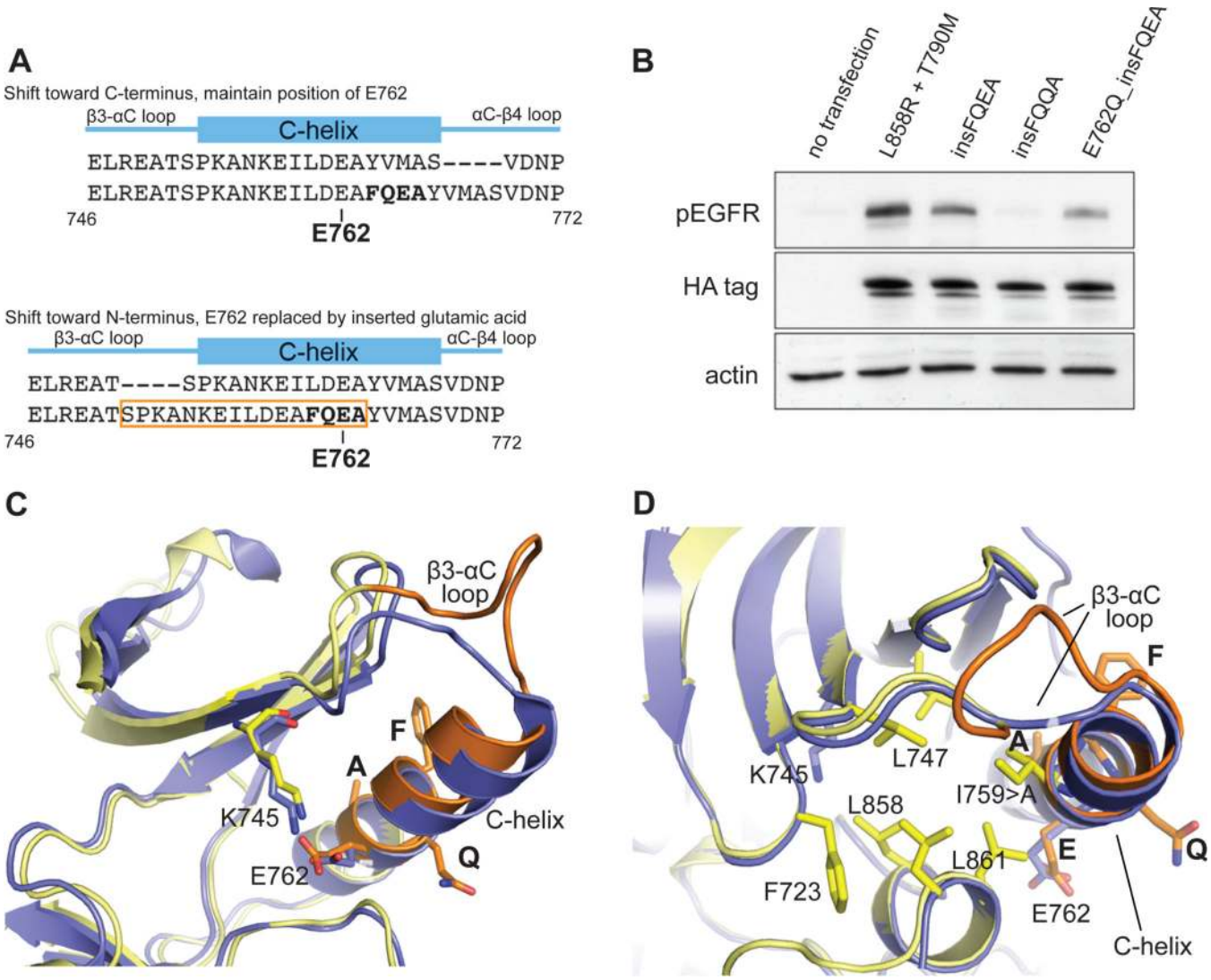


Figure 5.

Homology modeling of the TKI-sensitive EGFR-A763_Y764insFQEA mutation (insFQEA). A. Two plausible alignments of the wild-type (WT) and insFQEA sequences in the region of the C-helix. The inserted FQEA residues (bold) could shift the register of the C-helix in the C-terminal direction (upper panel) or in the N-terminal direction (lower panel) and maintain a glutamic acid in the position of Glu762, a key active site residue. A C-terminal shift would be expected to lengthen the loop following the C-helix (αC-β4), while an N-terminal shift would lengthen the loop preceding it (β3-αC). B. EGFR mutant constructs introduced into Cos-7 cells. EGFR phosphorylation and presence of transfected constructs (as measured by HA tag levels) are depicted. Four different constructs, insFQEA, insFQQA (E->Q mutation in the inserted sequence), E762Q_insFQEA (E->Q mutation in the endogenous E762) and L858R + T790M, were transiently transfected into COS-7 cells. Phosphorylation of EGFR and HA tag were detected by immunoblotting. Mutagenesis of the respective glutamic acid residues indicates a shift in the N-terminal direction; and the region that would be altered relative to WT, is boxed in orange (lower panel A). C. Homology model of the insFQEA mutant (yellow) superimposed on the WT EGFR structure (blue ribbon) in the active conformation. The inserted residues are labeled in bold, and the shifted sequence is colored orange (corresponding to the boxed region in panel A); the glutamic

acid residue in the FQEA insertion assumes the position of E762, and is positioned to form a salt bridge with K745. D. Homology model of the insFQEA mutant in the inactive conformation (yellow, with the structurally altered region in orange) superimposed on the WT EGFR structure in the inactive conformation (drawn from PDB ID: 1XKK). A cluster of hydrophobic residues (yellow side chains) is important for the stability of the inactive state; L858 and L861 are part of this cluster. The insFQEA insertion will shift an alanine residue into the position of I759 in this cluster (I759>A). The insertion is also expected to alter the length and conformation of the β3-αC loop, which is the site of exon 19 deletion mutations.

Table 1

Enzyme kinetic parameters of wild-type (WT) and mutant epidermal growth factor receptor (EGFR) kinases.

EGFR kinase	fold activity vs. WT	K_m [ATP] (μM) average (SD)	K_i [gefitinib] (nM) average (SD)	K_i/K_m (× 10^{-3})
WT	1.00	4.98 (± 1.2)	16.4 (± 0.2)	3.29
L858R	33.12	68.5 (± 3.4)	6.4 (± 0.8)	0.09
D770_N771insNPG	4.93	36.8 (± 7.4)	25.7 (± 4.3)	0.69
A763_Y764insFQEA	8.96	19.2 (± 0.9)	2.5 (± 0.3)	0.13

WT, wild type; EGFR, epidermal growth factor receptor; $K_{m[ATP]}$, Michaelis constant for ATP; K_i, inhibition constant; SD, standard deviation; ±, indicates the standard deviation within parentheses for K_{m}[ATP] and $K_{i,}$ [gefitinib]. All experiments were performed in triplicate (n=3).