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EGFR exon 19 insertion EGFR-K745_E746insIPVAIK and others with rare XPVAIK amino-acid insertions: preclinical and clinical characterization of the favorable therapeutic window to all classes of approved EGFR kinase inhibitors

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Abstract

Background: The epidermal growth factor receptor (EGFR)-K745_E746insIPVAIK and others with XPVAIK amino-acid insertions are exon 19 insertion mutations, which, at the structural modeling level, resemble EGFR tyrosine kinase inhibitor (TKI)-sensitizing mutants. An important

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unmet need is the characterization of therapeutic windows plus clinical outcomes of exon 19 XPVAIK amino-acid insertion mutations to available EGFR TKIs.

Methods: We used preclinical models of EGFR-K745_E746insIPVAIK and more typical EGFR mutations (exon 19 deletion, L858R, L861Q, G719S, A763_Y764insFQEA, other exon 20 insertion mutations) to probe representative 1st (erlotinib), 2nd (afatinib), 3rd generation (osimertinib), and EGFR exon 20 insertion active (mobocertinib) TKIs. We also compiled outcomes of *EGFR* exon 19 insertion mutated lung cancers—from our institution plus the literature—treated with EGFR TKIs.

Results: Exon 19 insertions represented 0.3–0.8% of all *EGFR* kinase domain mutation in two cohorts (n=1772). Cells driven by EGFR-K745_E746insIPVAIK had sensitivity to all classes of approved EGFR TKIs when compared to cells driven by EGFR-WT in proliferation assays and at the protein level. However, the therapeutic window of EGFR-K745_E746insIPVAIK driven cells was most akin to those of cells driven by EGFR-L861Q and EGFR-A763_Y764insFQEA than the more sensitive patterns seen with cells driven by an EGFR exon 19 deletion or EGFR-L858R. The majority (69.2%, n=26) of patients with lung cancers harboring *EGFR*-K745_E746insIPVAIK and other mutations with rare XPVAIK amino-acid insertions responded to clinically available EGFR TKIs (including icotinib, gefitinib, erlotinib, afatinib and osimertinib), with heterogeneous periods of progression-free survival. Mechanisms of acquired EGFR TKI resistance of this mutant remained underreported.

Conclusions: This is the largest preclinical/clinical report to highlight that EGFR-K745_E746insIPVAIK and other mutations with exon 19 XPVAIK amino-acid insertions are rare but sensitive to clinically available 1st, 2nd, and 3rd generation as well as EGFR exon 20 active TKIs; in a pattern that mostly resembles the outcomes of models with EGFR-L861Q and EGFR-A763_Y764insFQEA mutations. These data may help with the off-label selection of EGFR TKIs and clinical expectations of outcomes when targeted therapy is deployed for these *EGFR* mutated lung cancers.

Keywords

lung cancer; EGFR exon 19 insertion; K745_E746insIPVAIK; afatinib; osimertinib; erlotinib; mobocertinib

Introduction:

Precision oncology is one of the cornerstones of the management of advanced lung cancer[1,2]. *Epidermal growth factor receptor* (*EGFR*) kinase domain mutations are some of the most prevalent driver oncogenes linked to approved oral targeted therapies[3]. However, there is significant heterogeneity among the common (exon 19 deletions/indels, L858R), less common (exon 20 insertions, L861Q, G719X, S768I) and rare (A763_Y764insFQEA, delE709_T710insX, E709X, kinase domain duplications, kinase domain fusions) EGFR mutants as it relates to preclinical sensitivity to EGFR tyrosine kinase inhibitors (TKIs) plus clinical outcomes[4–15]. As of 2023, 1st generation reversible (gefitinib, erlotinib), 2nd generation irreversible (afatinib, dacomitinib), 3rd generation covalent mutation-selective (osimertinib) and EGFR exon 20 insertion mutation active (mobocertinib) EGFR TKIs have

completed the long haul of clinical development into regulatory approval in the United States[16–18].

One of the least studied rare subtypes of EGFR mutations are EGFR exon 19 insertions centered around amino-acids K745 to E746 with usually in-frame addition of six amino-acids to the structure of the kinase domain (Fig. 1)[19]. Prior reports have disclosed that these mutants seldom exceed 0.5–1% of all identified EGFR mutations (Fig.1A)[4,20], with the most common variant the nucleotide changes that lead to the exact amino-acid insertions of *EGFR*-K745_E746insIPVAIK (Fig.1B)[19]. *EGFR*-I744_K745insKIPVAI generates the identical protein. Other reported variants include *EGFR*-K745_E746insTPVAIK and *EGFR*-K745_E746insVPVAIK[19]. Limited structural modeling reports have proposed a mechanism of kinase activation that hinges on the EGFR-L747 to EGFR-P747 amino-acid structural change that occurs with the aforementioned mutants that both activates the kinase domain and creates a therapeutic window to EGFR TKIs in relation to wild-type (WT) EGFR (Fig.1C)[19].

However, important unmet preclinical and clinical needs include the full characterization of therapeutic windows of EGFR-exon 19 XPVAIK amino-acid insertions to all classes of available EGFR TKIs and the compilation of clinical outcomes with the same inhibitors when given to patients with lung cancers harboring these mutations. In this report, we provide a comprehensive characterization of EGFR exon 19 insertion mutations to close the aforementioned gap of knowledge.

Materials and Methods:

Drugs

Erlotinib, afatinib, osimertinib (LC Laboratories) and mobocertinib (MedChemExpress) were dissolved in dimethyl sulfoxide (DMSO, Fisher Scientific) at 10 mM and stored at −80°C before dilutions.

EGFR gene sequencing

RNA was extracted using Direct-zol RNA MiniPrep Plus Kit (Zymo Research). cDNA was transcribed from 1 μg of total RNA using High Capacity cDNA RT kit (Applied Biosystem). cDNA was used as a template for subsequent PCR amplifications of *EGFR* genes. EGFR exons 18 to 21 were amplified and sequenced. National Center for Biotechnology Information (NCBI) reference sequence NM 005228.5 was used as a reference for *EGFR* sequence analysis.

Cell lines and reagents

Ba/F3 murine cells and Bosc23 cells were kindly gifted by Dr. Daniel G. Tenen at Beth Israel Deaconess Medical Center (USA). The parental Ba/F3 cells were maintained in Roswell Park Memorial Institute-1640 (RPMI) medium (Corning) supplemented with 10% fetal bovine serum (FBS) (Corning), 1% penicillin and streptomycin (PS) (Corning), and 5% myelomonocytic leukemia, macrophage-like, Balb/C Mouse cells (WEHI)-conditioned medium as the source of interleukin-3 (IL-3). In the case of EGFR-WT driven Ba/F3 cells,

10 ng/mL of EGF (PeproTech) was added. Bosc23 cells were maintained in Dulbecco's modified eagle medium (DMEM) (Corning) supplemented with 10% FBS and 1% PS. All cells were grown at 37°C in a humidified atmosphere with 5% CO_2 and tested for absence of mycoplasma contamination (MycoAlert Mycoplasma Detection Kit, Lonza) prior to experiments (initiated within the initial 1 to 4 passages).

EGFR mutant constructs

EGFR exon 18 to exon 21 mutant constructs were introduced into the human *EGFR* WT sequence in the context of the MigR1 retrovirus vector (Addgene) using Q5® Site-Directed Mutagenesis Kits (New England BioLabs) as described previously[7,8,21,22]. The mutant *EGFR* constructs used in this study were K745_E746insIPVAIK, delL747_P753insS (exon 19 deletion), L858R, G719S, L861Q, A763_Y764insFQEA and V769_D770insASV. G719S and L861Q mutant constructs were kindly gifted from Dr. Hiroyuki Yasuda at Keio University (Japan)[23]. The oligonucleotide sequences used to K745_E746insIPVAIK are CGTCGCTATCAAGGAATTAAGAGAAGCAAC (forward) and GGAATTTTAATAGCGACGGGAATTTTAAC (reverse), using NEB changer (New England BioLabs). The resulting constructs were confirmed by nucleotide sequencing.

Generation of EGFR mutant driven Ba/F3 cell lines

Bosc23 cells were transfected with 15 μg of *EGFR* WT and mutant constructs using TransIT-X2® Dynamic Delivery System (Mirus Bio). The supernatant of Bosc23 cells containing MigR1 retrovirus was collected 2 days after transfection. Retronectin (Takara) was applied to Falcon petri dishes (Fisher Scientific). To infect Ba/F3 cells, the retrovirus and Ba/F3 cells were incubated for 24 hours on these plates. The infection was repeated twice. To select *EGFR* expressing Ba/F3 cells, green fluorescent protein (GFP) positive cells were sorted by FACSAria (Beckton Dickinson BioSciences). The sorted cells were grown in RPMI with 10% FBS. All EGFR mutants were able to drive Ba/F3 proliferation in the absence of IL-3. IL-3 independent Ba/F3 cells were used for further experiments, as described in prior reports[7,8,21,22]. After establishment, sequencing analysis was performed to confirm the mutant *EGFR*.

Cell proliferation assays

Cell viability was determined by CellTiter 96 aqueous one solution proliferation kit (Promega) for Ba/F3 cells. 10,000 cells were plated in 96-well plates and then treated in RPMI with indicated EGFR TKIs at 10 different concentrations for 3 days. A one solution reagent containing a mix of a tetrazolium compound (MTS) and an electron coupling reagent (PES) was added at the end of the 72-hour period and incubated for 2 hours. The number of living cells in each well was measured by using a 96-well plate reader to record the absorbance at 490 nm, which directly correlated to the number of living cells oxidizing the MTS solution. Inhibitory proliferation curves and the 50% inhibitory concentration (IC_{50}) were generated using GraphPad Prism 7 (GraphPad Software). Preclinical therapeutic window was calculated using logarithm of IC_{50} of EGFR mutants compared to EGFR-WT with values below zero indicating sensitivity (i.e., favorable therapeutic window) and values above zero indicating resistance (i.e., unfavorable therapeutic window) to each EGFR TKI tested.

Immunoblotting

Cells were treated with indicated EGFR TKIs for 6 hours at various concentrations. Cytoplasmic proteins were isolated via cell lysis for western blotting, as detailed in prior reports[7,8,21,22]. Protein extracts were then subjected to electrophoresis on 7.5% to 10% SDS polyacrylamide gels, with 30 to 45 mg of protein being added to each well. Samples were transferred to polyvinylidene difluoride membranes (MilliporeSigma), which were blocked then with 5% nonfat milk in phosphate-buffered saline with tween (PBST). Primary incubation with antibodies for the proteins of interest in a mix of 5% bovine serum albumin (BSA) and 0.02% sodium azide in PBST was performed overnight at 4º C. Total EGFR (Santa Cruz Biotechnology), β-actin (Cell Signaling) antibodies, phospho-EGFR antibody (pY1068) (Cell Signaling), total ERK (Cell Signaling) and phospho-ERK (Cell Signaling) were at 1:1000 dilution. Total AKT (Cell Signaling) and phospho-AKT (Cell Signaling) were at 1:500. Secondary incubation was performed for 2 hours at room temperature using horseradish peroxidase- (HRP) conjugated goat-anti-rabbit IgG and goat-anti-mouse IgG (Bio-Rad) antibodies at 1:5000 dilution in 5% nonfat milk. Membrane films were exposed and developed using enhanced chemiluminescence (ECL) Prime Western Blotting detection reagent Pierce ECL Western Blotting Substrate, 500 mL Kit (Thermo Scientific). Chemiluminescence assays for detecting the target proteins were performed using an Amersham Imager 600 (GE Healthcare).

Clinical and tumor genomic data collection

The frequency of *EGFR* mutations was calculated using three separate cohorts of cases, including our own institutional database (Fig.1). Genotype frequency, clinical, radiographic and survival outcomes used for this study were obtained from an ongoing Institutional Review Board approved protocol at Beth Israel Deaconess Medical Center. Additional genotype-inhibitor data were obtained through a literature review of studies published in PubMed and other databases, as well as oncology meeting abstracts using the search field “EGFR exon 19 insertion” plus “IPVAIK”. Response evaluation criteria in solid tumors (RECIST) was used, when provided. Progression-free survival (PFS) and overall survival (OS) were calculated in months, from the time of initiation of an EGFR TKI, when provided.

Results:

Frequency and *EGFR* mutation patterns for exon 19 insertions in lung cancer

We analyzed a large cohort from a commercial sequencing vendor[20], our own institutional database of *EGFR* mutated lung cancer and the Catalogue Of Somatic Mutations In Cancer (COSMIC) compendium catalogue[24]. EGFR exon 19 insertions between amino-acids K745 and E746 were reported in less than 0.8% of all *EGFR* mutated lung cancer cases (Fig.1A). The COSMIC catalogue (21 mutants) enumerated that the majority (81%) of EGFR exon 19 PVAIK insertions were of nucleotide sequences that generated equivalent mutants to *EGFR*-K745_E746insIPVAIK, while less common variants included *EGFR*-K745_E746insTPVAIK (14%) and *EGFR*-K745_E746insVPVAIK (5%) (Fig.1B). In view of these findings, we generated preclinical models driven by EGFR-K745_E746insIPVAIK for further experimentation.

Preclinical characterization of EGFR-K745_E746insIPVAIK and its pattern of sensitivity to EGFR TKIs in comparison to other EGFR mutants

Cells driven by EGFR-K745_E746insIPVAIK had sensitivity to all classes of representative EGFR TKIs tested (erlotinib, afatinib, osimertinib, mobocertinib) when compared to cells driven by EGFR-WT in proliferation assays (Fig.2A); thus, these cells displayed a favorable therapeutic window (IC_{50} below that of EGFR-WT) to EGFR TKIs. However, the preclinical therapeutic window to all drugs tested was less robust than that of cells driven by an EGFR-exon 19 deletion (the indel EGFR-delL747_P753insS) or EGFR-L858R by 1 to 2 or more logarithms of mutant IC_{50}/WT IC_{50} (Fig.2A). To better highlight these differences, the dose-dependent inhibition of proliferation for cells harboring *EGFR*-K745_E746insIPVAIK or *EGFR*-delL747_P753insS treated with erlotinib or osimertinib consistently disclosed more than 10 to 20-fold higher doses of drug needed to fully inhibit EGFR-K745_E746insIPVAIK dependent cells (Fig.2B). To further highlight both the EGFR TKI sensitivity of EGFR exon 19 insertion positive cells and the less robust patterns of inhibition when compared to EGFR-delL747_P753insS positive cells, we studied the intracellular signaling response to TKI therapy (Fig.2C). The active form of EGFR, as measured by one of the many phosphorylated epitopes of the protein, was inhibited by sub-micromolar doses of EGFR TKIs but again EGFR-K745_E746insIPVAIK dependent cells required 10-fold higher concentrations to achieve similar levels of inhibition (Fig.2C). The same pattern was observed with downstream signaling cascades mediated by the mitogen-activated protein kinase (ERK1 and ERK2) and the PI3K-AKT pathways (Fig. 2C).

The preclinical therapeutic window of our model driven by EGFR-K745_E746insIPVAIK was more similar to the pattern we observed for cells with EGFR-A763_Y764insFQEA for all EGFR TKIs tested or cells with EGFR-L861Q for erlotinib, osimertinib and mobocertinib (Fig.2A). The pattern was also not that dissimilar to that of EGFR-G719S dependent cells for erlotinib, osimertinib and mobocertinib (Fig.2A). Cells with one of the most common EGFR exon 20 insertion mutants (A767_V769dupASV) had unfavorable therapeutic windows to erlotinib, afatinib and mobocertinib, as expected from our prior experience and the structural conformation of this mutant's kinase[5,7,8,22,23]. Using the recent structure-function analysis of preclinical subgroups of EGFR mutations to predict their putative clinical response to various EGFR TKIs, both EGFR-L861Q and EGFR-A763_Y764insFQEA mutants are considered to cluster as "classical-like" mutations[11]—a subgrouping that would also apply to EGFR-K745_E746insIPVAIK and presumably to other exon 19 XPVAIK insertion mutants.

Clinical responses to EGFR TKIs in patients with *EGFR* exon 19 insertion mutated lung cancer

To better understand if the aforementioned preclinical results with EGFR-K745_E746insIPVAIK would translate into responses to approved EGFR TKIs in the clinical sphere, we concluded an extensive literature search to identify 23 separate individuals[25–38] and added 3 unpublished cases from our institutional cohort of *EGFR* mutated lung cancer (Table 1). These 26 patients with tumors harboring *EGFR* exon 19 insertion mutations (that lead to amino-acid changes that represent either EGFR-K745_E746insIPVAIK, EGFR-K745_E746insTPVAIK or EGFR-K745_E746insVPVAIK)

were treated with 1st generation (icotinib, gefitinib, erlotinib; n=20), 2nd generation (afatinib; n=3) and 3rd generation (osimertinib; n=3) EGFR TKIs. The clinical and pathologic characteristics were similar to those of other *EGFR* mutated lung cancers[4–15], with predominance of adenocarcinoma histology and never smokers (Table 1).

The majority of patients responded to EGFR TKIs. For 1st generation EGFR TKIs, 13 out of 20 (65%) of cases had a partial response (PR). For the 2nd generation TKI afatinib, 2 out of 3 (66%) had a PR. For the 3rd generation TKI osimertinib, 3 out of 3 (100%) had a PR (Table 1). Very few cases had progressive disease as the best tumor assessment (Table 1). The periods of tumor burden control for cases with PR and stable disease (SD) on EGFR TKI therapy were variable. For icotinib, gefitnib and erlotinib, the PFS varied from 4 to 50 months (Table 1). For afatinib and osimertinib, data on PFS was more limited but some cases had PFS that equaled or exceed 9 months (Table 1).

The calculated median PFS for the 26 cases receiving any type of EGFR TKI was 12 months (95% confidence interval [CI], 8–18 months). The calculated median OS for the whole cohort after initiation of an EGFR TKI was 12.5 months (95% CI, 9–19 months).

Mechanisms of acquired EGFR TKI resistance of this mutant remained underreported in the literature review and our compilation of cases without a genomic aberration identified in the few rebiopsy reports despite the growing use of clinical rebiopsy samples in academic centers[32]. As examples, our internal case 14 (Table 1) did not have on-target *EGFR* resistant mutations at time of progression tumor rebiopsy and our internal case 26 (Table 1) of *EGFR*-K745_E746insIPVAIK mutated lung adenocarcinoma that responded to osimertinib for 9 months lacked identifiable new on-target or off-target genomic aberrations at liquid and tumor rebiopsy.

Discussion:

To the best of our knowledge, the current report represents the largest preclinical and clinical report to highlight that EGFR-K745_E746insIPVAIK and other EGFR mutations with rare exon 19 XPVAIK amino-acid insertions (Fig.1B) are sensitive to clinically available 1st, 2nd, and 3rd generation, as well as EGFR exon 20 active TKIs (Fig.2A), and are responsive to icotinib, gefitinib, erlotinib, afatinib or osimertinib in patients with lung cancer (Table 1). We were able to determine that these *EGFR* exon 19 insertion changes are relatively uncommon, accounting for less than 1% of all cases of *EGFR* genomic aberrations in lung cancer (Fig.1A). The main amino-acid changes cluster into an insertion of around 6 amino-acids between the location of EGFR-K745 and EGFR-E746 of the WT EGFR structure, with insertions of IPVAIK (close to 80% of cases), TPVAIK (less than 15%) or VPVAIK (5% of cases) following the WT EGFR IPVAIK amino-acid stack of I740 to K745 (Fig.2B). Most comprehensive genomic profiling assays used for lung cancer genotyping[1,39] should be capable of identifying all the aforementioned mutations.

The mechanism of activation of these primary EGFR exon 19 insertions is glanced at from structural modeling efforts in lieu of a known crystal structural report. The current understanding of the structure-function of EGFR-K745_E746insIPVAIK and other

mutants is anchored on the EGFR-P747 amino-acid equivalent structural change from WT EGFR-L747 of the mutants (Fig.1C), which is predicted to alter the formation of the hydrophobic core of the EGFR kinase domain (involving amino-acids EGFR-F723, −M766, −L777, −L788, −L858, −L861 and −L862 that are key to stabilizing the inactive kinase state) and henceforth activate the mutant EGFR in a manner that generates a favorable therapeutic window to most approved EGFR TKIs[19]. This mechanism of activation shares similarities with the proposed structure-function models currently understood to explain EGFR activation and favorable therapeutic windows to EGFR TKIs of the EGFR-A763_Y764insFQEA exon 20 mutant and of the EGFR-L861Q exon 21 mutant[5]. Within the proposed structure-function subgrouping to better classify *in vitro* sensitivity of structurally-defined categories of EGFR mutants, there are a few clusters including classical-like (exon 19 deletions/indels, L858R, L861Q, A763_Y764insFQEA) with broad sensitivity to multiple generations of EGFR-directed TKIs, P-loop αC-helix compressing (PACC) mutants (such as G719X and S768I) with most sensitivity to 2nd generation irreversible EGFR TKIs, T790M-like hydrophobic core mutants (EGFR-T790M plus other mutations) and exon 20 loop insertions[11]. Our preclinical data would support EGFR exon 19 insertions (EGFR-K745_E746insIPVAIK) as another subgroup that clusters within the classical-like cohort.

It is notable that EGFR structure-function models and preclinical cell line models have translated remarkably faithfully to clinical outcomes. The most sensitive preclinical mutations with the lowest inhibitory concentrations and widest therapeutic windows—exon 19 indels and L858R (Fig.2A)—also have the best clinical outcomes. Patients with advanced *EGFR*-exon 19 indels or *EGFR*-L858R mutated lung adenocarcinoma treated with 1st, 2nd and 3rd generation EGFR TKIs have radiographic response rates (i.e., ORR) that exceed 60–80% and that can be prolonged (PFS times that exceed 12–18 months)[40,41,17]. The most robust overall survival with monotherapy has been achieved with use of 1st line osimertinib with a reported median OS of more than 3 years[42]. The next best annotated mutations are EGFR-L861Q and EGFR-G719X. These have intermediate preclinical sensitivity patterns (Fig.2A) and less stellar clinical outcomes. Patients with advanced *EGFR*-G719X mutated lung adenocarcinoma have the best reported clinical activity with 2nd generation EGFR TKIs such as afatinib (with ORR of >60%, PFS >12 months and OS >24 months) with more limited activity of 1st or 3rd generation TKIs (with ORR of <55% and PFS <9 months)[43–45]. For patients with EGFR-L861Q mutated lung adenocarcinoma, the ORR exceeds 50–70% (PFS >8 months and OS >17 months) to 1st, 2nd and 3rd generation EGFR TKIs[43–45]—with the most robust activity seen with osimertinib[43]. The data for the unique exon 20 insertion mutant EGFR-A763_Y764insFQEA is not that dissimilar to EGFR-L861Q. The largest clinical series compilation disclosed ORR >60%, PFS>5 months and OS>20 months to all classes of EGFR TKIs[21]. Unlike these latter cohorts of classical-like and PACC mutants, typical EGFR exon 20 insertion mutations do not have a therapeutic window to 1st, 2nd and 3rd generation EGFR TKIs or clinical responses to these drugs[21]; with only modest preclinical and clinical activity to EGFR exon 20 insertion active TKIs such as mobocertinib[8,18,22]. Our database (Table 1) would support that the preclinical clustering of EGFR exon 19 insertions into classical-like mutants best matches a clinical pattern seen with EGFR-L861Q or EGFR-A763_Y764insFQEA. Indeed, we see ORR >65% to 1st,

2nd and 3rd generation EGFR TKIs and PFS times more than 11 months for cases with *EGFR*-K745_E746insIPVAIK and similar mutated lung adenocarcinomas (Table 1).

The one area that our large database is unable to address are mechanism of resistance to EGFR TKIs in *EGFR* exon 19 insertion mutated lung cancer, as none of the cases either reported rebiopsy specimens or identified a new genetic/epigenetic event. It is well known from most classical-like and PACC EGFR mutated lung cancers that the three most common mechanisms of resistance include: on-target resistance (i.e., EGFR-T790M to 1st/2nd generation EGFR TKIs or EGFR-C797S to 3rd generation EGFR TKIs), off-target resistance (i.e., amplification, mutation or another mechanism of activation of bypass driver oncogenes) or epigenetic histological transformation[46–50]. One can only speculate that *EGFR* exon 19 insertion mutated lung cancers would display similar patterns of resistance.

Conclusion:

Ours is the largest preclinical and clinical report of EGFR-K745_E746insIPVAIK and other mutations with exon 19 XPVAIK amino-acid insertions. We are able to conclude that these mutants are relatively rare (<1% of all *EGFR* mutations) but sensitive to clinically available 1st, 2nd, and 3rd generation as well as EGFR exon 20 active TKIs. The preclinical and clinical pattern mostly resembles the outcomes of *EGFR*-L861Q or *EGFR*-A763_Y764insFQEA mutated lung cancers. We believe the data provide here may help with the off-label selection of EGFR TKIs in real-world settings and in the definition of clinical expectations of outcomes when targeted therapy is deployed for *EGFR* exon 19 insertion mutated lung cancers.

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Highlights

- EGFR-K745_E746insIPVAIK and others with XPVAIX amino-acid insertions are exon 19 insertion mutations (<0.8% of all *EGFR* mutations), which, at the structural modeling level, resemble EGFR inhibitor-sensitizing mutants
- An important unmet need is the characterization of therapeutic windows plus clinical outcomes of exon 19 XPVAIX amino-acid insertion mutations to available EGFR TKIs.
- Preclinical models of EGFR-K745_E746insIPVAIK had sensitivity to all classes of approved EGFR TKIs when compared to cells driven by EGFR-WT in proliferation assays and at the protein level but therapeutic windows most akin to EGFR-L861Q and EGFR-A763_Y764insFQEA than the more sensitive patterns seen with cells driven by an EGFR exon 19 deletion or EGFR-L858R
- This is the largest preclinical/clinical report to highlight that EGFR-K745_E746insIPVAIK and other mutations with exon 19 XPVAIX amino-acid insertions are rare but sensitive to clinically available 1st, 2nd, and 3rd generation as well as EGFR exon 20 active TKIs

A

EGFR mutation	Reference 20 cohort (n=1510) frequency (%)	BIDMC cohort (n=262) frequency (%)
exon 19 insertions between K745 and E746	0.3	0.8
exon 19 deletions/indels between E746 and P753	44	46
L858R	31	34
exon 20 insertions/indels between A763 and C775	13	6
G719X	6	8
L861Q	2.5	4
S768I	2.5	0.4
E709_T710delins/ E709X	0	0.4
rearrangement/ fusion	0.4	0
exon 18-25 kinase domain duplication	0.3	0.4

B

Amino-acid position	_PVAI insertion (n=21) frequency (%)	740	741	742	743	744	745	746	747	748	749	750	751	752	753
EGFR-WT		I	P	V	A	I	K	E	L	R	E	A	T	S	P
Amino-acid position		740	741	742	743	744	745	ins	ins	ins	ins	ins	ins	746	747
EGFR-K745_E746insIPVAIK	81	I	P	V	A	I	K	I	P	V	A	I	K	E	L
EGFR-K745_E746insTPVAIK	14	I	P	V	A	I	K	T	P	V	A	I	K	E	L
EGFR-K745_E746insVPVAIK	5	I	P	V	A	I	K	V	P	V	A	I	K	E	L

C

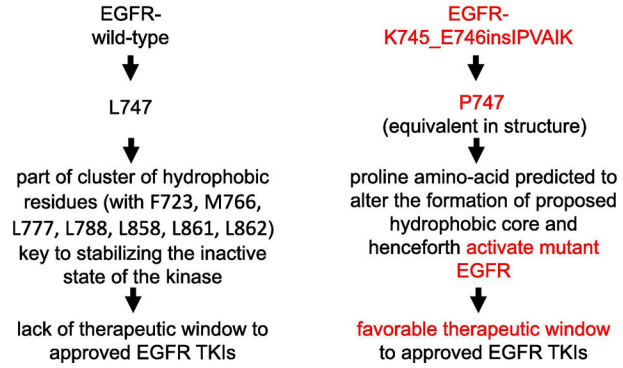

Figure 1. EGFR exon 19 deletions in context of other genomic aberrations.

(A) Frequency of common *EGFR* mutations and *EGFR*-K747_E746insIPVAIK and other variants in two separate cohorts. The larger cohort represents data from the commercial vendor Foundation Medicine[20] and the smaller from our institutional cases from Beth Israel Deaconess Medical Center (BIDMC) between years 2004 to 2021. (B) Display of amino acid sequence of wild-type EGFR compared to the different variants of EGFR-K745_E746ins_XPVAIK mutations based on reference [19]. Frequency of the different XPVAIK insertions curated from the COSMIC database is displayed. (C) Theoretical mechanism of the sensitivity of the EGFR-K747_E746insIPVAIK mutation that details the change that may allow for sensitivity to approved EGFR TKIs based on structural modeling from reference [19]. There is no published crystal structure for this mutant. The proposed mechanism of activation depicted is similar to mechanisms described for EGFR-A763_Y764insFQEA and EGFR-L861Q.

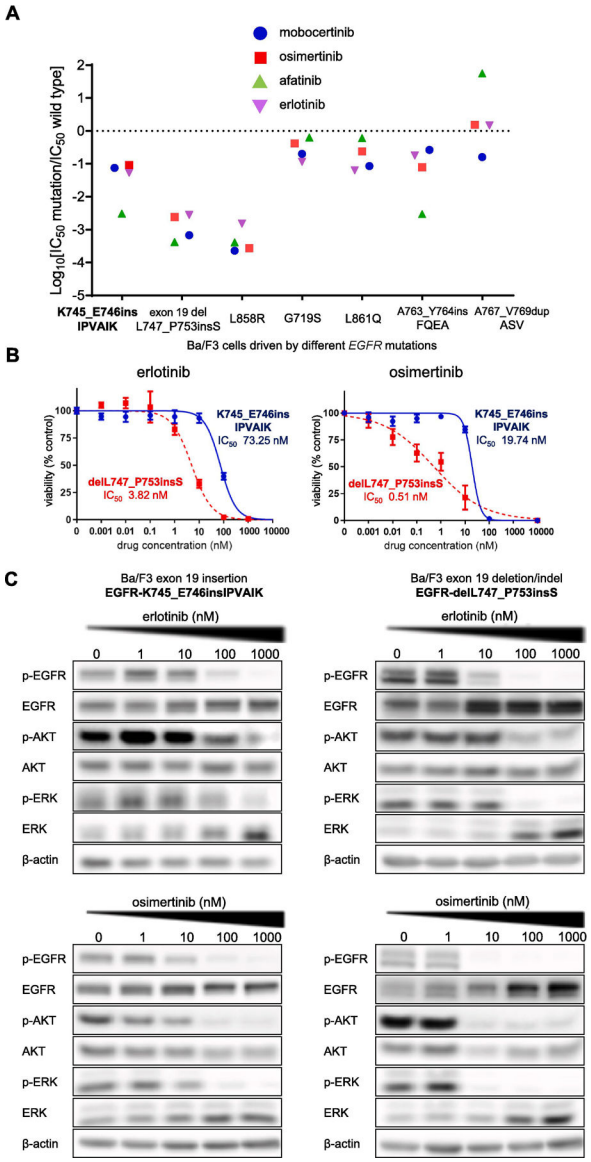

Figure 2. Ba/F3 system isogenic preclinical models of EGFR mutations to probe EGFR inhibitors.

(**A**) Therapeutic window of different EGFR-TKIs to EGFR mutants. Cells were plated at a density of 10,000 cells per well (96-well plates) and grown over 3 days after treatment. Logarithm of the 50% inhibitory concentration (IC50) of EGFR mutants compared to EGFR-WT is plotted with 3 separate experiments used to generate IC50. Values below zero (0) indicate sensitivity, while values above 0 indicate resistance to EGFR-TKIs. The therapeutic window of Ba/F3 cells with EGFR-K745_E746insIPVAIK are contrasted with other mutations.

(**B**) Dose-response proliferation assays (percent viability) for patient-derived lung cancer cell lines harboring EGFR-K745_E746insIPVAIK and EGFR-delL747_P753insS after exposure to increasing concentrations of EGFR TKIs. Three separate experiments were used to generate IC_{50}, and standard deviations are depicted in vertical bars. (**C**) Western blotting of Ba/F3 cells driven by EGFR-K745_E746insIPVAIK and EGFR-delL747_P753insS. Cells

were treated with the EGFR TKIs for 6 hours at the indicated ascending concentrations. pEGFR, phosphorylated EGFR at position 1068, total EGFR, pAKT, phosphorylated AKT, total AKT, phosphorylated ERK, total ERK and β-actin (loading control) are exhibited.

Table 1.

Clinical, pathological characteristics and response to EGFR TKIs of patients with tumors harboring *EGFR* exon 19 insertion mutations: K745_E746insIPVAIK and others.

Case number	Ref. (number)	EGFR mutation/ histology	EGFR TKI (line of therapy)	Dose	Response RECI ST	Percent change target lesion(s)	PFS (months)	OS(months from start of EGFR TKI)	sex/ age(years)/ ethnicity/PS	smoking history (pack-years)
1	25	I740_K745dupI PVAIK*/ adenocarcinoma	Icotinib (1st)	NA	PR	NA	13	NA	M/74/ Asian/NA	NA
2	31	I744_K745insKIPVAI*/ adenocarcinoma	Gefitinib (1st)	250 mg	PR	− 43.7%	4	10	M/68/ Asian/NA	50
3	31	I744_K745insKIPVAI*/ adenocarcinoma	Gefitinib (1st)	250 mg	SD	NA	5	NA	F/51/ Asian/NA	0
4	33	I744_K745insKIPVAI*/ adenocarcinoma	Gefitinib (1st)	NA	PR	NA	5	9	F/42/ Asian/NA	0
5	36	I744_K745insKIPVAI*/ adenocarcinoma	Gefitinib (1st)	250 mg	PR	-70%	5	NA	F/56/ white/NA	0
6	36	I744_K745insKIPVAI*/ adenocarcinoma	Gefitinib (2nd)	250 mg	SD	NA	>9	NA	F/48/ white/NA	0
7	36	I744_K745insKIPVAI*/ adenocarcinoma	Gefitinib (1st)	250 mg	SD	NA	>11	NA	F/54/ white/NA	0
8	27	I744_K745insKIPVAI*/ adenocarcinoma	Gefitinib (1st)	250 mg	PR	NA	12	18	F/48/ white/2	0
9	33	I744_K745insKIPVAI*/ adenocarcinoma	Gefitinib (2nd)	NA	SD	NA	22	32	M/46/ Asian/NA	15
10	37	I744_K745insKIPVAI*/ adenocarcinoma	Gefitinib (1st)	NA	SD	NA	24	NA	F/37/ Asian/NA	0
11	33	I744_K745insKIPVAI*/ adenocarcinoma	Erlotinib (2nd)	NA	PD	NA	2	11	F/47/ Asian/NA	0
12	26	delI744_K745in sKIPVAI*/ adenocarcinoma	Erlotinib (2nd)	150 mg	PR	NA	7	9	F/36/ white/NA	NA
13	current BIDM C	K739_I744dup KIPVAI*/ adenocarcinoma	Erlotinib (1st)	150 mg	PR	− 30.4%	7	11	F/59/ white/3	0
14	current BIDM C	K739_I744dup KIPVAI*/ adenocarcinoma	Erlotinib (2nd)	100 mg	PR	− 31.2%	8	12	F/68/ white/0	10
15	34	K745_E746insI PVAIK/ adenocarcinoma	Erlotinib (1st)	150 mg	PR	NA	9	NA	F/39/ Arab/3	0
16	37	E746_L747insV PVAIK/ adenocarcinoma	Erlotinib (1st)	NA	PR	NA	16	NA	F/60/ Asian/NA	0
17	29	I740_K745dupI PVAIK*/ adenocarcinoma	Erlotinib (3rd)	150 mg	PR	NA	16	>48	F/39/ white/NA	0
18	28	K745_E746insI PVAIK/ adenocarcinoma	Erlotinib (1st)	150 mg	SD	NA	18	24	F/55/ Asian/1	0
19	19	K745_E746ins PVAIK/ adenocarcinoma	Erlotinib (1st)	NA	PR	NA	19	NA	NA	NA
20	19	K745_E746insIPVAIK/ adenocarcinoma	Erlotinib (1st)	NA	PR	NA	50	NA	NA	NA

Case number	Ref. (number)	EGFR mutation/ histology	EGFR TKI (line of therapy)	Dose	Response RECI ST	Percent change target lesion(s)	PFS (months)	OS(months from start of EGFR TKI)	sex/ age(years)/ ethnicity/PS	smoking history (pack-years)
21	35	K745_E746insIPVAIK/ adenocarcinoma	Afatinib (1st)	40 mg	PD	NA	1	4	F/75/ Asian/1	NA
22	30	I740_K745dupIPVAIK*/ adenocarcinoma	Afatinib (2nd)	30 mg	PR	NA	13	21	M/63/ Asian/NA	45
23	19	K745_E746insI PVAIK/ adenocarcinoma	Afatinib (1st)	NA	PR	NA	14	NA	NA	NA
24	38	I740_K745dupI PVAIK*/NSCLC NOS	Osimertinib (1st) +bevacizumab	NA	PR	NA	>1	NA	F/58/ Asian/NA	NA
25	38	I740_K745dupI PVAIK*/NSCLC NOS	Osimertinib (1st)	NA	PR	NA	>2	NA	F/67/ Asian/NA	0
26	current BIDM C	K745_E746insI PVAIK/ adenocarcinoma	Osimertinib (1st)	80 mg	PR	− 31.6%	9	>13	F/73/ white/0	2

EGFR, epidermal growth factor receptor; PS, ECOG performance status; TKI, tyrosine kinase inhibitor; RECIST, Response evaluation in solid tumors version 1.1; PR, partial response; SD, stable disease; PD, progressive disease; PFS, progression-free survival; OS, overall survival; NSCLC NOS, non-small-cell lung cancer not otherwise specified; NA, not available; +, ongoing survival for PFS or OS. For OS, it was assumed survival was ongoing (>) when report did not specify otherwise. When extrapolating from written or graphic data from publications, we rounded response change or months to nearest full value. * indicates mutations that have the same amino-acid sequence as EGFR-K745_E746insIPVAIK