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Efficacy of EGFR tyrosine kinase inhibitors in patients with non–small cell lung cancer with *EGFR* exon 19 insertions: clinical-genomic, preclinical analysis through LC-SCRUM-Asia (multi-institutional genomic screening registry)

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Abstract

Background: *EGFR* exon 19 insertions (*EGFR*ex19ins) are rare *EGFR* mutations. Their clinical-genomic characteristics and outcomes with EGFR-tyrosine kinase inhibitors (TKIs) remain uncertain.

Methods: We evaluated the clinical-genomic characteristics and outcomes of EGFR-TKIs for *EGFR*ex19ins in the multi-institutional prospective lung cancer genomic screening project (LC-SCRUM-Asia). We also studied preclinical Ba/F3 models expressing *EGFR*-K745_E746insIPVAIK (Ba/F3-IPVAIK) to investigate their sensitivity to 1st-, 2nd-, 3rd-generation, and *EGFR* exon 20 insertion-active TKIs.

Results: In LC-SCRUM-Asia, 16,204 NSCLC patients were enrolled from March 2015 to December 2023. *EGFR*ex19ins were detected in 13 samples (0.1% of NSCLC). The median age was 72 years (range, 38–80); most patients were female (77%), had adenocarcinoma (92%), and were never-smokers (62%). Twelve patients (93%) had *EGFR*-K745_E746insIPVAIK, while one (7%) had *EGFR*-K745_E746insVPVAIK. The most frequent co-mutation was *TP53* (62%);

no patients had other driver alterations. Six patients (46%) tested positive for *EGFR* exon 19 deletions with PCR-based Cobas EGFR test, likely due to cross-reactivity arising from sequence homology. Twelve patients received EGFR-TKIs; five (42%) experienced partial response. In the preclinical study, Ba/F3-IPVAIK showed the highest sensitivity to 2nd-generation EGFR-TKIs compared to other EGFR-TKIs. Structural studies supported these consistent results. When broken down by EGFR-TKI generations, response rates for 1st-, 2nd-, and 3rd-generation TKIs were 50% (1/2), 80% (4/5), and 0% (0/5), respectively. The median PFS for 1st-, 2nd-, and 3rd-generation TKIs were 8.7 (95% CI, 7.4–NR), 14.7 (95% CI, 8.0–NR), and 4.4 (95% CI, 3.4–NR) months, respectively.

Conclusion: Our preclinical, structural, and clinical findings indicate 2nd-generation EGFR-TKIs are more effective for *EGFR*ex19ins compared to other TKIs.

Keywords

Non-small cell lung cancer; Afatinib; Osimertinib; EGFR; I740_K745dup; K745_E746insIPVAIK

Introduction

The clinical outcome of patients with major *EGFR* mutations (exon 21 L858R or exon 19 deletions) have significantly improved owing to treatment with 1st-, 2nd-, or 3rd-generation tyrosine kinase inhibitors (TKIs) [1–5]. While other *EGFR* mutations in the kinase domain (exons 18–21) have also been identified as oncogenic drivers of NSCLC, patients with uncommon *EGFR* mutations exhibit varied and diminished responses to EGFR-TKIs [6,7]. As of 2024, the uncommon *EGFR* mutations with treatments approved by the US Food and Drug Administration (FDA) include *EGFR* S768I, L861Q, and G719X. For these mutations, afatinib is effective based on the prospective studies [8,9]. Additionally, amivantamab (EGFR/MET bispecific antibody) is approved for exon 20 insertions [10]; other *EGFR* exon 20 insertion active TKI (sunvozertinib, zipalertinib) and pan-HER TKI (poziotinib) are currently under clinical development [11–15].

One of the least reported rare subtypes of *EGFR* mutations is *EGFR* exon 19 insertion, centered around amino acids K745 to E746, which typically results in in-frame addition of six amino acids to the kinase domain structure [16–18]. Prior studies have revealed that the prevalence of these mutants is 0.1–0.4% of NSCLC [16–18]. The most common variant is EGFR- c.2217_2234dup (K745_E746insIPVAIK [identical to I740_K745dup]), and EGFR-c.2214_2231dup (I744_K745insKIPVAI), all of those generating the same mutant protein). Although some case series and case reports have described the clinical-genomic characteristics of patients with *EGFR* exon 19 insertions—predominantly non-smoking females who are sensitive to EGFR–TKIs—no prospective study evaluated a cohort of patients with *EGFR* exon 19 insertions [16–21]. Furthermore, the differential therapeutic efficacies of 1st-, 2nd-, and 3rd-generation, as well as in-development EGFR–TKIs, remain undetermined based on integrated preclinical, structural, and clinical cohort analyses.

Herein, we analyzed NSCLC cases using the integrated clinical and genomic data of the lung cancer genomic screening project (LC-SCRUM-Asia), to evaluate the clinical-genomic characteristics. We also examined preclinical models of *EGFR*-K745_E746insIPVAIK and

major *EGFR* mutations to investigate the efficacy of 1st-, 2nd-, 3rd-generation, and *EGFR* exon 20 insertion active TKIs. Additionally, we performed structural analyses to validate these in vitro findings. Finally, the clinical efficacy of approved EGFR-TKIs was evaluated in the LC-SCRUM-Asia cohort.

Materials and method

Multi-institutional lung cancer genomic screening project (LC-SCRUM-Asia)

LC-SCRUM-Asia (UMIN000010234) is multi-institutional prospective observational study to screen lung cancer patients with targetable gene alterations for the clinical development of novel targeted therapies [22–28]. As of March 2024, LC-SCRUM-Asia has involved over 300 institutions in Japan, enrolling nearly 20,000 lung cancer patients. The inclusion criteria of this study include patients to be at least 16 years old with clinical stage II to IV or recurrent lung cancer, an ECOG performance status of 0 to 1, and adequate organ function. In this screening, we included a targeted next-generation sequencing (NGS) system as previously described [22–28]. The gene list for each panel is summarized in Supplemental Table 1. As some genes were analyzed solely by Oncomine Comprehensive Assay (OCA) (Thermo Fisher Scientific, Waltham, MA, USA) and not by Oncomine Precision Assay (OPA) (Thermo Fisher Scientific, Waltham, MA, USA), the mutation fractions for those genes were determined only from genes detected by OPA. Furthermore, a multiplex PCR test using the AmoyDx® Pan Lung Cancer (PLC) Polymerase chain reaction (PCR) test (Amoy Diagnostics Co., Ltd., Xiamen, China) was implemented in parallel with these analyses [29]. The residual DNA samples with *EGFR* exon 19 insertions were also tested with the cobas® EGFR Mutation Test v2 (Roche Molecular System, Branchburg, NJ, USA). All analyses were conducted as central tests at a Clinical Laboratory Improvement Amendments (CLIA)-certified clinical laboratory (SRL, or LSI Medience, Tokyo, Japan). Clinical data, including baseline patient characteristics, treatment regimens, therapeutic outcomes, and patient prognoses, were collected and updated annually using the electronic data capture system of LC-SCRUM-Asia. Tumor responses were assessed according to RECIST, version 1.1, by investigators. The database has been previously described in detail [22–28].

This study is registered with the University Medical Hospital Information Network (UMIN) Clinical Trial Registry (UMIN000010234). The study protocol was approved by the Ethics Committee of the National Cancer Center Hospital East (Chiba, Japan) (2023–223). All specimens and data were collected after obtaining comprehensive written informed consent from the patients.

Tyrosine kinase inhibitors

Gefitinib, erlotinib, afatinib, and osimertinib were obtained from LC Laboratories (Woburn, MA, USA). Mobocertinib, sunvozertinib, and zipalertinib were obtained from MedChemExpress (Monmouth Junction, NJ, USA). Dacomitinib and poziotinib were obtained from Selleckchem (Houston, TX, USA) and Adooq BioScience (Irvine, CA, USA), respectively. All TKIs were dissolved in dimethyl sulfoxide at 10 mM and stored at −80°C.

Cell lines and reagents

Ba/F3 murine cells and Bosc23 cells were kindly provided by Dr. Daniel G. Tenen of Beth Israel Deaconess Medical Center, USA. The parental Ba/F3 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI) medium (Corning, Corning, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Corning, Corning, NY, USA), 1% penicillin and streptomycin (PS) (Corning, Corning, NY, USA), and 5% myelomonocytic leukemia, macrophage-like, Balb/C Mouse cells (WEHI)-conditioned medium, which served as the source of interleukin-3 (IL-3). For the EGFR-WT driven Ba/F3 cells, 10 ng/mL of EGF (PeproTech, Cranbury, NJ, USA) was added. Bosc23 cells were preserved in Dulbecco's Modified Eagle Medium (DMEM) (Corning, Corning, NY, USA) supplemented with 10% FBS and 1% PS. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO2 and were tested for the absence of mycoplasma contamination (MycoAlert Mycoplasma Detection Kit, Lonza, Basel, Switzerland) before experiments.

EGFR mutant constructs

EGFR exon 19 mutant constructs were introduced into the human EGFR WT sequence within the MigR1 retrovirus vector (Addgene, Watertown, MA, USA) using Q5® Site-Directed Mutagenesis Kits (New England BioLabs, Ipswich, MA, USA) as previously described [17,30–33]. The mutant *EGFR* constructs used in this research included K745_E746insIPVAIK (exon 19 insertion) and Del E746_A750 (exon 19 deletion). The oligonucleotide sequences employed for K745_E746insIPVAIK were CGTCGCTATCAAGGAATTAAGAGAAGCAAC (forward) and GGAATTTTAATAGCGACGGGAATTTTAAC (reverse) utilizing NEB changer (New England BioLabs, Ipswich, MA, USA). All resulting constructs were verified by nucleotide sequencing using ACATCTCCGAAAGCCAAC (forward) and CTTGATAGCGACGGGAAT (reverse), spanning the whole EGFR-kinase domain.

Generation of Ba/F3 cell line models

Bosc23 cells were transfected with 15 μg of EGFR WT and mutant constructs utilizing the TransIT-X2® Dynamic Delivery System (Mirus Bio, Madison, WI, USA). The supernatant from Bosc23 cells containing the MigR1 retrovirus was collected 2 days post-transfection. Retronectin (Takara Bio Inc., Shiga, Japan) was applied to Falcon petri dishes (Thermo Fisher Scientific, Waltham, MA, USA). For infecting Ba/F3 cells, the retrovirus and Ba/F3 cells were co-incubated for 24 hours on these dishes. This infection process was repeated twice. To isolate EGFR-expressing Ba/F3 cells, green fluorescent protein (GFP) positive cells were sorted using FACSAria (Becton Dickinson BioSciences, Franklin Lakes, NJ, USA). The sorted cells were cultivated in RPMI supplemented with 10% FBS. All EGFR mutants demonstrated the ability to drive Ba/F3 proliferation without the need for IL-3. As outlined in previous reports, IL-3-independent Ba/F3 cells were utilized for subsequent experiments [17,30–33]. Upon establishment, sequencing analysis was conducted to validate the mutant *EGFR*.

Cell proliferation assays

Cell viability was assessed using the CellTiter 96 aqueous one solution proliferation kit (Promega, Madison, WI, USA) for Ba/F3 cells. 10,000 cells were seeded in 96-well plates and subsequently treated in RPMI with specified EGFR-TKIs across 10 different concentrations for 3 days. At the culmination of the 72-hour period, a one solution reagent comprising a tetrazolium compound (MTS) and an electron coupling reagent (PES) was introduced and incubated for 2 hours. The viable cell count in each well was gauged using a 96-well plate reader, measuring the absorbance at 490 nm, which is directly proportional to the number of live cells oxidizing the MTS solution. Inhibition proliferation curves and the 50% inhibitory concentration (IC_{50}) were plotted using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). The preclinical therapeutic window was determined by calculating the logarithm of IC_{50} values for *EGFR* mutants relative to *EGFR*-WT. Values below zero signified sensitivity (denoting a favorable therapeutic window), whereas values exceeding zero represented resistance (denoting an unfavorable therapeutic window) to the tested EGFR-TKIs.

Structural Modeling

To elucidate the pathogenicity of missense variants around *EGFR* K745, we utilized the AlphaMissense tool, an adaptation of AlphaFold (DeepMind, London, UK) that has been fine-tuned using human and primate variant population frequency databases to predict the pathogenicity of missense variants [34,35]. Subsequently, we generated 3D visualizations of *EGFR* exon 19 insertions (K745_E746insIPVAIK) using the ColabFold implementation of AlphaFold2 (DeepMind, London, UK), provided by Neurosnap Inc. (Wilmington, DE, USA), a computational biology platform for research (https://neurosnap.ai/) [36,37].

The structure available in the Protein Data Bank (PDB) served as the primary template for modeling the complex of WT EGFR protein with 1st-, 2nd-, 3rd-generation, and *EGFR* exon 20 insertion active TKIs. The coordinates for wild-type (WT) EGFR/afatinib complex (PDB id: 4G5J), EGFR/osimertinib complex (PDB id: 4ZAU), WT EGFR/gefitinib complex (PDB id: 2ITY), and WT EGFR/mobocertinib complex (PDB id: 7T4I) were further remodeled using PyMOL software (Schrödinger, New York, NY, USA, http://www.pymol.org/pymol). Cavity calculations were performed with CavitOmiX (v. 1.0, 2022, Innophore, San Francisco, CA, https://innophore.com/cavitomix/), using AI models from OpenFold (powered by NVIDIA (Santa Clara, CA, US)'s BioNeMo service), DeepMind (London, UK)`s AlphaFold, and Meta (Menlo Park, CA, US)'s ESMFold. The hydrophobicity module of the VASCo program was used to analyze the hydrophobicity of the cavities [38]. The Cavities were calculated using a modified LIGSITE algorithm [39].

Statistical analysis

Descriptive statistics were used to summarize baseline characteristics, with the $\chi 2$ and Wilcoxon rank-sum tests applied to categorical and continuous variables, respectively. Response to initial EGFR-TKI therapy was evaluated according to RECIST 1.1 criteria. Progression-free survival (PFS) of initial EGFR-TKI therapy was defined as the duration from the start of treatment to disease progression or death from any cause, and censored at the last follow-up prior to disease progression or death. Overall survival (OS) was defined

as the duration from the start of first-line systemic treatment for stage IV or recurrence of disease to death from any cause, and censored at the last follow-up prior to death. Survival curves were estimated using the Kaplan-Meier method and compared using the log-rank test. All statistical analyses were performed using R, version 4.1.3 (R Project for Statistical Computing), and significance was set at a 2-sided $P < .05$.

Results

Prevalence of *EGFR* exon 19 insertions

Of the total 16,204 NSCLC patients enrolled from March 2015 to December 2023 (Figure 1A), tumor specimens from 15,226 (94%) patients were successfully analyzed by targeted NGS (OCA/ OPA). Among them, *EGFR* mutations were detected in 3,269 patients (21%), of which exon 19 insertions were found in 13 patients (0.1% of NSCLC and 0.4% of *EGFR*-mutated NSCLC) by NGS analyses. Twelve patients (93%) had EGFR-K745_E746insIPVAIK (c.2217–2234dup), and the other had EGFR-K745_E746insVPVAIK (c.2219–2236dup).

Clinico-genomic characteristics of *EGFR* exon 19 insertions

Table 1 and Table 2 summarize the baseline characteristics of the patients with *EGFR* exon 19 insertions. The median age was 72 years, and 10 patients (77%) were female. All participants were Asian. Eight patients (62%) were never-smokers, and five were former smokers. Twelve (93%) had adenocarcinoma histology, and one was characterized as adenosquamous carcinoma. At enrollment, 11 patients had stage IV disease (85%), and two had recurrence disease (15%). Three patients (23%) had brain metastasis. No distinct differences in clinical characteristics were observed compared to patients with major *EGFR* mutations from LC-SCRUM-Asia (Table 1).

The most frequent co-existing gene alterations were *TP53* mutation (8/13, 62%), followed by *EGFR* amplification (2/13, 15%), *CDKN2B* amplification (2/13, 15%), *PIK3CA* mutation (1/13, 8%), and *FGFR* mutation (1/13, 8%) (Figure 1B). Importantly, none of the patients showed the other oncogenic driver alterations.

The concordance between *EGFR* mutation detection assays

All 13 samples with *EGFR* exon 19 insertions identified by OCA/OPA were tested for *EGFR* mutations by AmoyDx PLC PCR test, as well as cobas® EGFR Mutation Test v2, both of which do not cover *EGFR* exon 19 insertions. As expected, all 13 samples were negative for EGFR mutations by the AmoyDx PLC PCR test. However, 46% (6/13) of samples (from patient numbers 1, 6, 7, 8, 12, and 13 in Table 2—one with p.K745_E746insVPVAIK and five with p.K745_E746insIPVAIK) tested positive for *EGFR* mutations but were incorrectly identified as having an exon 19 deletion by the cobas® EGFR Mutation Test v2. This misdiagnosis may result from cross-reactivity with primers designed for exon 19 deletion mutations (c.2235_2248delinsAATTC, p.E746_A750>IP; c.2237_2255>T, p.E746_S752delinsV), which share sequence homology with the insertion sequences (Supplemental Figure 1).

Preclinical characterization of *EGFR*-K745_E746insIPVAIK and its pattern of sensitivity to EGFR-TKIs compared to *EGFR* exon 19 deletion

Next, we sought to assess sensitivity to FDA-approved and investigational EGFR-TKIs in cells driven by *EGFR*-K745_E746insIPVAIK. To this end, we used Ba/F3 expressing *EGFR*-K745_E746insIPVAIK (Ba/F3-IPVAIK) as described previously [17]. As a reference, we also generated and used Ba/F3 cells expressing wild-type *EGFR* (Ba/F3-WT) or *EGFR*-delE746_A750 (Ba/F3-Del19). The IC_{50} of all tested EGFR-TKIs in Ba/F3-IPVAIK were lower than those in Ba/F3-WT, displaying favorable therapeutic windows (the ratio of mutant IC_{50} to WT IC_{50}) to EGFR-TKIs (Figure 2A). The preclinical therapeutic window to 1st-(gefitinib, erlotinib) and 3rd- (osimertinib) generation EGFR-TKIs and exon20 insertions active EGFR-TKIs (mobocertinib, sunvozertinib, and zipalertinib) in Ba/F3-IPVAIK was less robust than that of Ba/F3-Del19 by 2 to 4 or more logarithms of mutant IC_{50}/WT IC_{50} (Figure 2B and Supplemental Figure 2). Interestingly, the preclinical therapeutic window to the 2nd-generation TKIs (afatinib, dacomitinib, poziotinib) in Ba/F3-IPVAIK cells was as favorable as that in Ba/F3-Del19 cells (Figure 2B and Supplemental Figure 2). The IC_{50} of 2nd-generation EGFR-TKI was ranging 0.57–0.95 nM, which was much lower than that of other EGFR-TKIs (IC_{50} range: 1st-generation, 61–158 nM; 3rd-generation, 13.8 nM; exon 20 insertion active, 1.78–21.3 nM). Taken together, these data suggest that all tested EGFR-TKIs have favorable therapeutic windows, and 2nd-generation EGFR-TKIs may be much more effective for tumor cells driven by *EGFR*-K745_E746insIPVAIK.

Structure modeling of *EGFR* exon 19 insertions in complex with EGFR-TKIs

To validate these in vitro findings, we performed *in silico* analysis. First, we analyzed functional hotspots to elucidate the pathogenicity of missense variants around EGFR K745, using AlphaMissense predictions for EGFR protein (Figure 3A). This analysis demonstrated that single amino acid substitutions around K745 predominantly confer pathogenicity to missense variants. The amino acid residue at codons 740–746 was located in the beta-strand. Subsequently, we predicted the structures of *EGFR*-K745_E746insIPVAIK and WT EGFR, using AlphaFold2 (Figure 3B). Finally, we analyzed protein-drug interactions to validate the differential efficacy of EGFR-TKIs. The crystal structures of the WT EGFR kinase domain complexed with afatinib, osimertinib, gefitinib, and mobocertinib were depicted in space-filling models in Figure 3C–F and Supplemental Figure 3A–D. Previous structure-function analyses have classified *EGFR*-K745_E746insIPVAIK as a P-loop α-Chelix compressing (PACC) mutant, which affects the orientation of the P-loop or αC-helix [7]. *In silico* analysis of the interaction of afatinib with *EGFR*-K745_E746insIPVAIK shows that afatinib maintained interaction points within the protein cavity predicted by CavitOmiX (Figure 3C–D). In contrast, the interaction of osimertinib, gefitinib, and mobocertinib with PACC mutations predicted that alterations in the P-loop's orientation shift the positions of TKI stabilization points, causing the indole ring of osimertinib to tilt away from the P-loop and destabilizing drug binding within the predicted protein cavity (Figure 3C–F, Supplemental Figure 3A–D).

Efficacy of EGFR-TKIs in patients with *EGFR* exon 19 insertions

To investigate whether the preclinical and *in silico* results with *EGFR*-K745_E746insIPVAIK described above can be implemented into clinical settings, we analyzed 12 patients with *EGFR* exon 19 insertions (11 patients, K745_E746insIPVAIK; one patient, I745_K746 insVPVAIK; Table 2), who received EGFR-TKI treatment. Of 12 patients treated with EGFR-TKIs, 9 (75%) patients received EGFR-TKI as first-line treatment (gefitinib, n = 1; erlotinib, n = 1; afatinib, n = 4; osimertinib, n = 3). The other three (25%) patients received EGFR-TKI as 2nd or 3rd line treatment (afatinib, n = 1; osimertinib, n = 2). The overall response rate (ORR) and disease control rate (DCR) of EGFR-TKIs were 42% (5/12), and 83% (10/12), respectively. When broken down by EGFR-TKI generation, the ORR for 1st-, 2nd-, and 3rd-generation TKIs were 50% (1/2), 80% (4/5), and 0% (0/5), respectively (Figure 4A). Swimmers plot that details the clinical progression of patients treated with EGFR-TKIs is shown in Figure 4B. The median PFS and OS of patients treated with EGFR-TKI were 7.2 months (95%CI, 6.0–NR [not reached]) and 20.1 months (95%CI, 16.3–NR) with a median follow-up of 34.1 months, respectively (Figure 4C and 4D). When broken down by EGFR-TKI generation, the median PFS for the 1st-, 2nd-, and 3rd-generations were 8.7 (95% CI, 7.4–NR), 14.7 (95% CI, 8.0–NR), and 4.4 (95% CI, 3.4–NR) months (Figure 4E). Representative cases treated with afatinib and osimertinib were shown in Supplemental Figures 4A and 4B. The median OS was not reached in the 2nd-generation TKI cohort, 25.5 months (95% CI, 21.4–NR) in the 1st-generation TKI cohort, and 15.9 months (95% CI, 9.3–NR) in the 3rd-generation TKI cohort (Figure 4F).

Discussion

In this study, we highlighted the clinical-genomic features of patients with NSCLC harboring *EGFR* exon 19 insertions. To the best of our knowledge, our cohort with *EGFR* exon 19 insertions is the largest prospectively observational cohort to date, allowing us to characterize this rare population and analyze the response of EGFR-TKIs based on EGFR-TKI generations. We determined that *EGFR* exon 19 insertions are exceedingly rare, representing only 0.1% of all NSCLC cases. Moreover, this is the first report showing that 2nd-generation EGFR-TKIs are more effective than other EGFR-TKIs for patients with *EGFR* exon 19 insertions, consistent with our *in vitro* and *in silico* studies.

The majority of patients with *EGFR* exon 19 insertions were female, never-smokers, with a predilection for having brain metastases, a pattern similar to major *EGFR* mutations. While several previous reports have findings consistent with our study, these were often based on collections of published case reports or original papers where each cohort consisted of a limited number of stage IV NSCLC patients, introducing bias to the interpretation of proportions [6,17,21,40,41]. Moreover, the majority (93%) of exon 19 insertion variants were K745_E746insIPVAIK, and one patient (7%) had K745_E746insVPVAIK. This aligns with previous studies where K745_E746insIPVAIK accounted for 85% and K745_E746insVPVAIK for 11% [6].

In our cohort, patients treated with 2nd-generation EGFR-TKIs exhibited better ORR, PFS, and OS than those treated with 1st- and 3rd-generation EGFR-TKIs. Our preclinical

and structural analyses showed that 2nd-generation EGFR-TKIs were more effective than 1st-, 3rd-, and exon 20 insertion-active TKIs for *EGFR* exon 19 insertion-positive tumors. Robichaux and colleagues proposed preclinical structure-function-based subgroups (classical-like, T790M-like hydrophobic core mutants, PACC mutants, and exon 20 loop insertions), where *EGFR*-K745_E746insIPVAK was classified into the PACC mutations group [7]. Additionally, *EGFR*-K745_E746insIPVAIK was reported to be more sensitive to afatinib compared to gefitinib in experimental models [18]. Aligning with these prior studies, both our clinical, preclinical, and structural results strongly supported that EGFR-TKIs are effective for NSCLC harboring *EGFR* exon 19 insertions, and 2nd-generation EGFR-TKIs are the most effective among several generation EGFR-TKIs.

We validated all 13 samples detected by OCA/OPA, using AmoyDx PLC and Cobas EGFR test. Though AmoyDx PLC and Cobas EGFR test do not cover *EGFR* exon 19 insertion, 46% (6/13) samples were positive for *EGFR* exon 19 deletion by Cobas EGFR test. Interestingly, the DNA sequence of the exon 19 deletion mutation (c.2235_2248delinsAATTC, p.E746_A750>IP), which is detected by the Cobas EGFR test but not by the AmoyDx PLC test, closely resembles the exon 19 insertion sequence (c.2217–2234dup, p.K745_E746insIPVAIK) (Supplementary Figure 1). Similarly, the DNA sequence of another exon 19 deletion (c.2237_2255>T, p.E746_S752delinsV) closely resembles the exon 19 insertion sequence (c.2219–2236dup, p.K745_E746insVPVAIK) (Supplementary Figure 1). Thus, the Cobas primer may cross-react with these insertion sequences, resulting in an incorrect exon 19 deletion diagnosis. As mentioned above, since *EGFR* exon 19 insertions are sensitive to EGFR-TKIs and can act as targetable driver mutations, we should not overlook patients with *EGFR* exon 19 insertions. It is important to emphasize the limitation of PCR detection, where the range of variants detected is limited. Furthermore, our results also suggest that patients with *EGFR* exon 19 deletions detected by Cobas EGFR test may include those with *EGFR* exon 19 insertions, which is not so sensitive to 3rd-generation EGFR-TKI compared with *EGFR* exon 19 deletions. Therefore, there is an increasing need to apply NGS testing for a more precise diagnosis of targetable oncogenic drivers and their variants to treat patients with more adequate molecular targeted agents.

This study has several limitations. Firstly, our study was a real-world observational investigation, and it lacked specifications regarding the timing or frequency of radiological assessments for treatment responses. Second, while this represents the largest cohort of *EGFR* exon 19 insertions to date, the sample size remains small. This constrains our ability to interpret the subgroup analysis of EGFR-TKI generations accurately. Further international collaborative databases are needed.

In conclusion, *EGFR* exon 19 insertions are exceedingly rare, accounting for 0.1% of NSCLC and 0.4% of EGFR-mutated NSCLC. Our preclinical, structural, and clinical findings suggest that 2nd-generation EGFR-TKIs are more effective than other EGFR-TKIs in treating patients with *EGFR* exon 19 insertions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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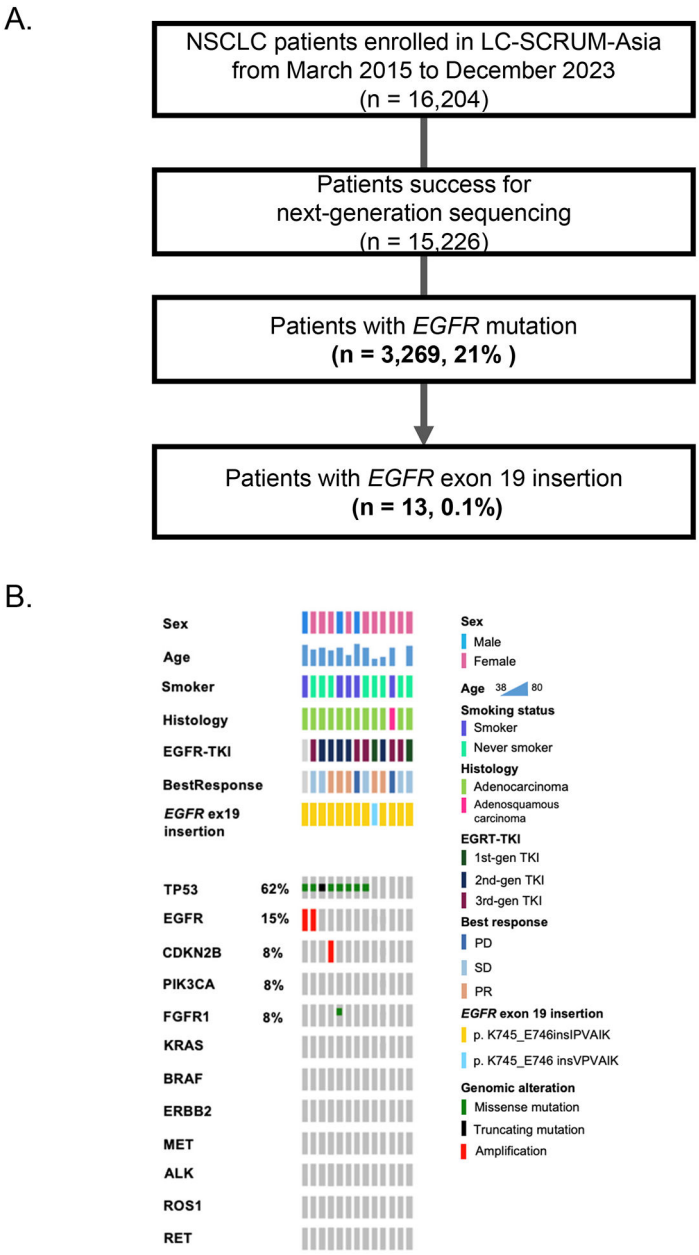

Figure 1:

Study flowchart and oncoprint for patients with *EGFR* exon 19 Insertion. (A) Study flowchart. (B) Oncoprint of oncogenic alterations in pretreated samples with frequency.

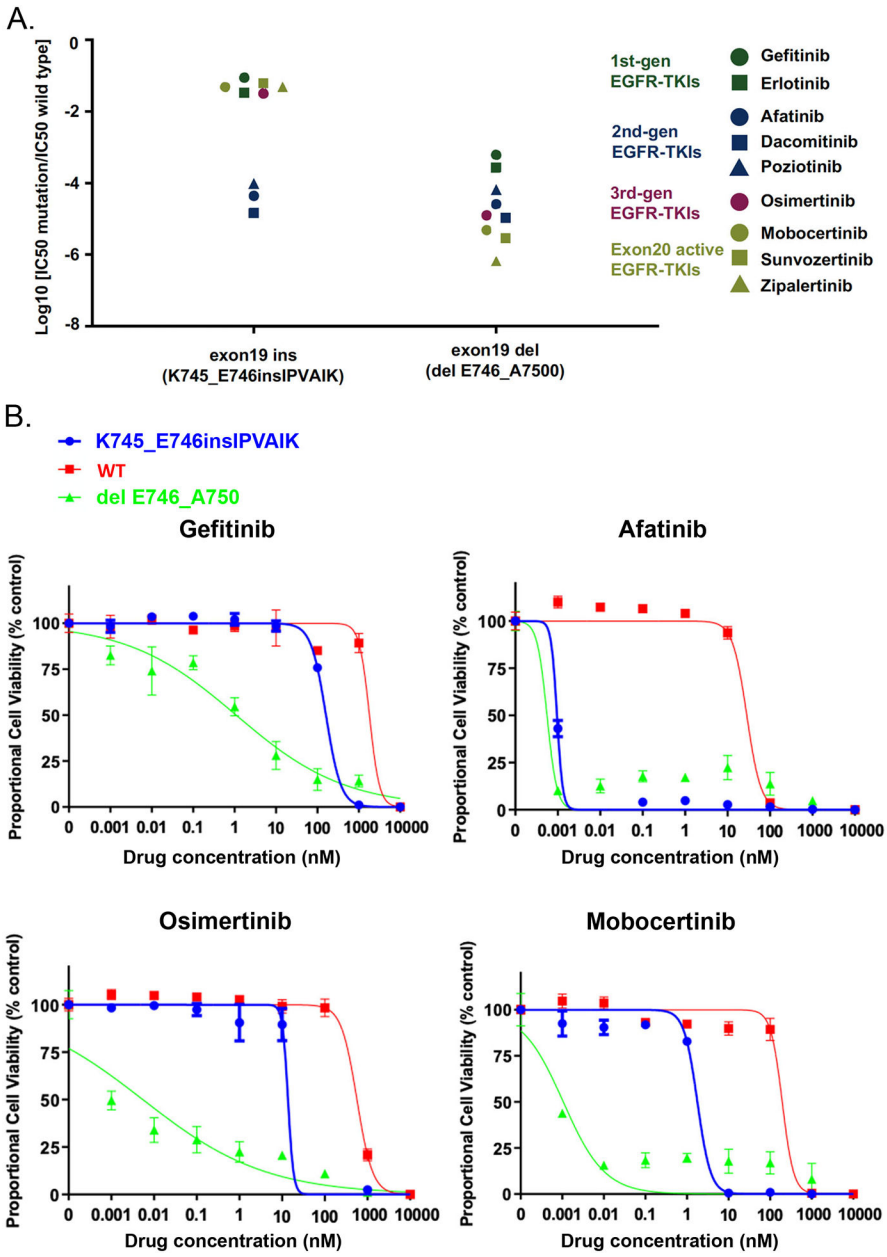

Figure 2:

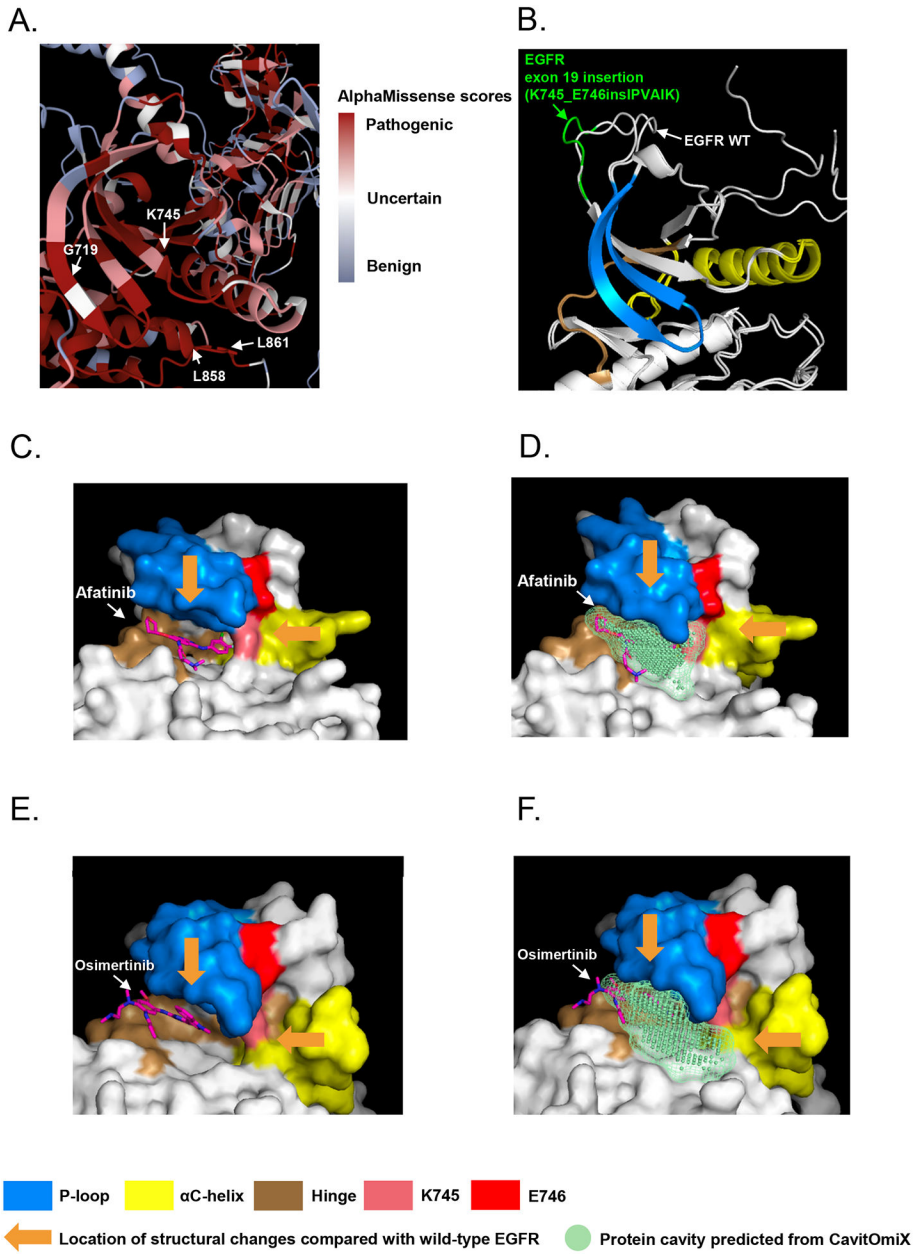

Figure 3:

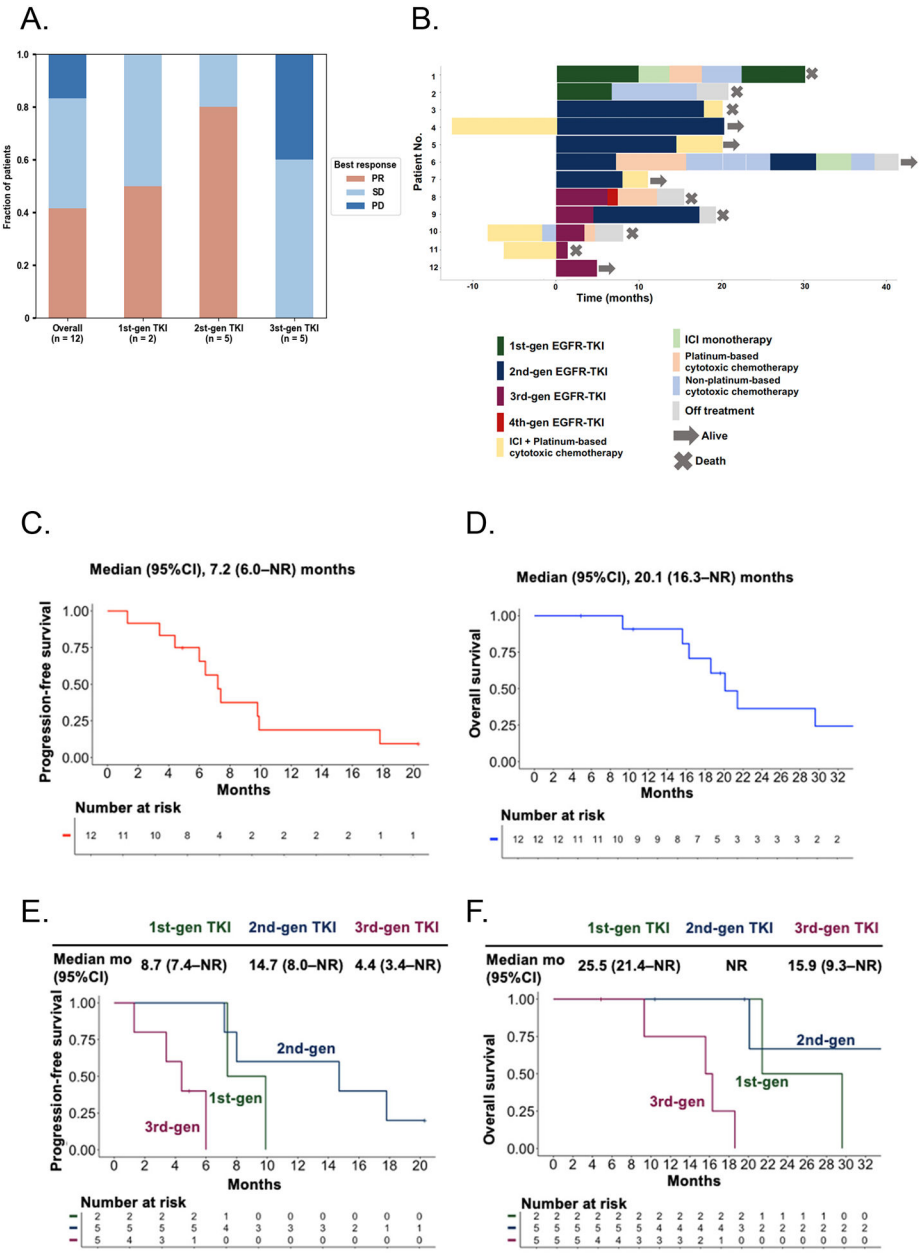

Figure 4:

Table 1:

Clinicopathological characteristics of patients with *EGFR* exon 19 insertion.

	***EGFR* exon 19 insertion (n = 13)**	**Major EGFR mutations (L858R, exon 19deletion) (n = 1,359)[a]**	***P* value**
Median age (range), year	72 (38–80)	68 (26–97)	0.77
Sex, n (%)			
Male	3 (23)	524 (39)	0.39
Female	10 (77)	835 (61)	
Smoking history, n (%)[b]			
Never	8 (62)	795 (58)	1.0
Former	5 (38)	558 (42)	
Histological subtypes, n (%)			
Adenocarcinoma	12 (93)	1293 (95)	1.0
Others[c]	1 (7)	66 (5)	
Disease stage, n (%)			
IV	11 (85)	1080 (79)	0.34
Recurrence	2 (15)	127 (9)	
II, III	0 (0)	152 (12)	
Brain metastasis, n (%)			
Yes	3 (23)	360 (26)	1.0
No	10 (77)	999 (74)	

[a] Cohort from LC-SCRUM-Asia: March 2015 to April 2021.

[b] Among patients with major *EGFR* mutations, the smoking history of six patients was unknown.

[c] In patients with *EGFR* exon 19 insertion, one patient had adenosquamous carcinoma. Among patients with major *EGFR* mutations, two had adenosquamous carcinoma, four had pleomorphic carcinoma, four had large cell carcinoma, and 56 had NSCLC-NOS (not otherwise specified).

Table 2:

Details of the patients with *EGFR* exon 19 insertion.

Pt. No.	Sex	Age	Smoking	PS	Stage	Histology	Amino acid change	Amoy Dx PLC	Cobas *EGFR* test	EGFR-TKI	Best response	PFS[a]	OS[a]
1	F	51	Never	1	IV	ADC	p. I745_K746 insVPVAIK	Negative	*EGFR* exon 19 deletion	1L Erlotinib	PR	9.9	30
2	F	76	Never	0	IV	ADC	p. K745_E746insIPVAIK	Negative	Negative	1L Gefitinib	SD	7.4	21
3	F	67	Never	0	IV	ADC	p. K745_E746insIPVAIK	Negative	Negative	1L Afatinib, Bevacizumab	PR	18	20
4	F	55	Never	0	IV	ADC	p. K745_E746insIPVAIK	Negative	Negative	2L Afatinib	PR	20+	34+
5	M	72	Former	1	IV	ADC	p. K745_E746insIPVAIK	Negative	Negative	1L Afatinib	PR	15	20+
6	F	73	Never	1	IV	ADC	p. K745_E746insIPVAIK	Negative	*EGFR* exon 19 deletion	1L Afatinib	SD	7.2	43+
7	F	58	Former	1	IV	ADC	p. K745_E746insIPVAIK	Negative	*EGFR* exon 19 deletion	1L Afatinib	PR	8.0	11+
8	F	69	Never	1	IV	ADC	p. K745_E746insIPVAIK	Negative	*EGFR* exon 19 deletion	1L Osimertinib	SD	6.0	16
9	F	72	Never	1	IV	ADC	p. K745_E746insIPVAIK	Negative	Negative	1L Osimertinib	SD	4.4	19
										2L Afatinib	SD	13	
10	F	72	Former	0	IV	ASC	p. K745_E746insIPVAIK	Negative	Negative	3L Osimertinib	PD	3.4	16
11	M	80	Former	0	Recurrence	ADC	p. K745_E746insIPVAIK	Negative	Negative	2L Osimertinib	PD	1.3	9.3
12	F	38	Never	0	IV	ADC	p. K745_E746insIPVAIK	Negative	*EGFR* exon 19 deletion	1L Osimertinib	SD	4.9+	4.9+
13	M	77	Former	1	Recurrence	ADC	p. K745_E746insIPVAIK	Negative	*EGFR* exon 19 deletion	NA			

[a]+ indicates treatment is ongoing.

Abbreviations: 1L, first-line; 2L, second-line; 3L, third-line; ADC, adenocarcinoma; ASC, adenosquamous carcinoma; EGFR, epidermal growth factor receptor; F, female; M, male; NA, not available; OS, overall survival; PD, progressive disease; PFS, Progression-free survival; PR, partial response; PS, performance status; PLC, pan lung cancer; Pt. No., patient number; SD, stable disease; TKI, tyrosine kinase inhibitor.