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Extensive functional evaluation of exon 20 insertion mutations of *EGFR*

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ABSTRACT

Objectives: Exon 20 insertion mutations of epidermal growth factor receptor (EGFR) have been identified as oncogenic mutations in general; however, the functional relevance of each remains largely uninvestigated. Herein, we comprehensively assessed the functional significance of insertion mutations of *EGFR* exon 20.
Materials and methods: The transforming potential and drug sensitivities of 25 *EGFR* recurrent mutants, including twenty-one exon 20 insertions, were evaluated using the mixed-all-nominated-in-one method.
Results: The sensitivity of *EGFR* exon 20 insertions to EGFR tyrosine kinase inhibitors (TKIs) was generally lower than that of the L858R mutation or exon 19 deletions. The results were also confirmed through an *in vivo* drug test. All of the exon 20 insertions were resistant to gefitinib and afatinib, whereas several mutants were sensitive to osimertinib. *EGFR* exon 20 insertions exhibited relatively good responses to poziotinib and mobocertinib.
Conclusions: *EGFR* exon 20 insertions were shown to have different degrees of sensitivity to EGFR TKIs. This extensive assessment of *EGFR* exon 20 insertions may provide a fundamental database for aiding in a customized mode of therapy for cancers having insertional mutations within exon 20 of *EGFR*, although the clinical impact of preclinical data should be validated by clinical evidence in the future.

1. Introduction

Epidermal growth factor receptor (EGFR) is a transmembrane receptor protein belonging to a family of four related proteins (EGFR, HER2, HER3, and HER4). Upon ligand binding, each receptor forms a dimer that becomes activated through autophosphorylation and evokes intracellular signaling [1]. The two main signaling pathways that are activated by EGFR are the RAS–RAF–MEK–MAPK and the PI3K–AKT pathways.

EGFR is constitutively activated through somatic mutations in different types of cancers such as non-small cell lung cancers (NSCLC), wherein EGFR was first identified as the source of cancer cell proliferation signals [2–4]. EGFR tyrosine kinase inhibitors (TKIs), such as gefitinib, afatinib, and osimertinib, have led to better management and outcome of NSCLC patients with *EGFR* activating mutations [5–9]. *EGFR* mutations are present in approximately 10–30 % of NSCLC [10,11] and predominantly found in female, non-smoking, adenocarcinoma patients and in those of East Asian descent [12,13]. Common mutations are in-frame deletions around the Leu–Arg–Glu–Ala motif (residues 746–750) of exon 19 (45 % of all *EGFR* mutations), and the Leu858Arg (L858R) point mutation in exon 21 (40 %) [14]. There is an ethnic difference in the *EGFR* mutation prevalence among NSCLC; the rate is 30 % in East Asians and 15 % in Western Europeans [12,15–17]. While uncommon mutations are comprised of G719X, S768I, L861Q, exon 20 insertions and complex mutations, 5 %–10 % of all EGFR mutations are exon 20 insertions that are known to be oncogenic [13,18–22]. Patients with *EGFR* exon 20 insertions fundamentally have a shorter survival time compared with those who have common *EGFR* mutations due to the general lack of sensitivity to *EGFR* kinase inhibitors [19]. The sensitivity of several exon 20 insertions has been examined for EGFR TKIs [23–34]; however, little is known about the clinical significance of individual variants. Clinical trials have gathered the data about the activity of TKIs in NSCLC harboring uncommon *EGFR* mutations [35]. Only two (8.7 %) of 23 NSCLCs harboring *EGFR* exon 20 insertion mutations demonstrated a significant response to afatinib, indicating its limited efficacy.

Herein, we assessed recurrent insertion mutations within *EGFR* exon 20 present in the COSMIC database v89 (https://cancer.sanger.ac.uk/cosmic). Transforming activity and drug sensitivity for these mutants were comprehensively assessed using the mixed-all-nominated-in-one (MANO) method developed in our laboratory [36] that can measure

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the both characters in a high-throughput manner.

2. Materials and methods

2.1. Cell lines

Mouse 3T3 fibroblasts and human embryonic kidney (HEK) 293 T cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) supplemented with 10 % fetal bovine serum (FBS), 2 mmol/L glutamine (all from Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (P/S). Interleukin 3 (IL-3)–dependent murine pro-B cell line (Ba/F3) was cultured in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10 % FBS, 2 mmol/L glutamine, 1% P/S, and mouse IL-3 (20 U/mL; Sigma-Aldrich, St. Louis, MO, USA).

2.2. Establishment of retroviral vector with random barcodes

The pcx6 vector was set up by inserting random 10 base-pair (bp) DNA barcode sequences upstream of the start codon of the target genes into the pcx4 vector [37]. The barcode sequences are shown in **Supplementary Table S1.** Into the pcx6 vector, a full-length wild-type cDNA of human *EGFR* was inserted. Recurrent 25 mutants of *EGFR* (two exon 19 insertions, one exon 19 deletion, 21 exon 20 insertions, and one exon 21 missense mutation) reported in the COSMIC database v89 were selected for the study (**Supplementary Fig. S1, Supplementary Table S2**). Plasmids encoding *EGFR* variants were created through the GeneArt Gene Synthesis system (Thermo Fisher Scientific) whose sequences were confirmed by next generation sequencing. Three clones with individual barcodes were constructed per each variant to obtain triplicate data in each individual assay.

2.3. Retrovirus production and infection into 3T3 cells and Ba/F3 cells

The recombinant plasmids were transduced together with packaging plasmids (TaKaRa Bio, Shiga, Japan) into HEK293 T cells to create recombinant retroviral particles. The 3T3 cells were infected with ecotropic recombinant retroviruses in 12-well plates using 4 μg/mL of Polybrene (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. The Ba/F3 cells were seeded into retronectin-coated (TaKaRa Bio) 12-well plates and infected with retroviruses in the RMPI 1640 medium containing 20 U/mL of IL-3.

2.4. Focus formation assay

To assess anchorage-independent growth, 3T3 cells expressing various *EGFR* mutants were cultured in DMEM-F12 supplemented with 5% bovine calf serum for 2 weeks, then the cells were stained with the Giemsa solution.

2.5. The MANO method

As illustrated in **Supplementary Fig. S2,** the MANO method uses a retroviral vector that can stably integrate individual genes into the genome of assay cells, such as 3T3 or Ba/F3 cells, along with 10-bp barcode sequences. Individually transduced assay cells were all pooled and cultured in a competitive manner to evaluate their *in vitro* or *in vivo* transforming potential or drug sensitivity. At the end of the cultivating period, genomic DNA was obtained from cell lysates using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), followed by PCR amplification using primers, indices, and adaptor sequences of the Illumina technology (primer sequence is described in **Supplementary Table S3**). The obtained products were purified using AMPure beads (Beckman Coulter, Brea, CA, USA). The sequencing libraries were prepared using the NEB Next Q5 Hot Start HiFi PCR Master Mix (NEB), according to the manufacturer's instructions. Library quality was checked using a Qubit 2.0 fluorometer (Thermo Fisher Scientific) and the Agilent 2200 TapeStation system (Agilent Technologies). The library was sequenced on an Illumina MiSeq system with the Reagent Kit V2 (300 cycles), and 150-bp paired-end reads were created (the sequencing primer loaded into the MiSeq cartridge is shown in **Supplementary Table S4**). The barcode sequence 5′-TTAATTAAXXXXXXXXXXGGATCACT-3′ (where X denotes any nucleotide) was included in the sequencing results, and the number of each barcode per mutant was quantified.

2.6. TKI sensitivity assays using the MANO method

The Ba/F3 cells expressing each *EGFR* mutant were cultured in RPMI 1640 medium without IL-3. The transformed Ba/F3 cells which showed IL-3-independency were mixed in equal amounts and incubated for 4 d with the indicated concentrations of gefitinib, afatinib, and osimerinitib (100 pM–10 μM; LC Laboratories, Woburn, MA, USA). The experiment was conducted in triplicate. The number of each barcode was calculated using the MANO method. Considering the different doubling times of the transduced cells, dimethyl sulfoxide (DMSO)-treated cell mixtures were used as the reference control for normalizing each cell clone signal. The relative growth inhibition rate of each cell clone was calculated as follows: (average read number across triplicates/average read number of the DMSO control) × 100. The assessment of sensitivity was based on the IC_{90} of each drug according to previous reports [36,38].

2.7. PrestoBlue cell viability assay

The transformed Ba/F3 cells expressing each *EGFR* mutant were cultivated in 96-well plates (with 100 μL of culture medium per well) in the RPMI 1640 medium without IL-3, and gefitinib, afatinib, osimerinitib (1 nM to 10 μM), poziotinib (100 pM to 1 μM), and mobocertinib (1 nM to 10 μM) were added at different concentrations. Poziotinib and mobocertinib were purchased from Selleck Chemicals (Houston, TX, USA). Next, 10 μL of PrestoBlue (Thermo Fisher Scientific) was added to the plates 4 d after exposure to the TKIs, and fluorescence was measured (excitation 530 nm, emission 590 nm) after 3 h of incubation at 0.1 s. The assessment of sensitivity was determined by the IC_{90} of each drug based upon previous reports [27,36,38,39].

2.8. The in vivo MANO method

Individually transduced 3T3 cell clones were mixed in equal numbers, and 5.0×10^6 cells of the mixture (i.e., 2.0×10^5 cells from each of the 25 cell clones) were subcutaneously injected into 10 6-week-old female nude mice, according to the animal use protocol reviewed and approved by the Animal Ethics Committee of the National Cancer Research Center (Tokyo, Japan). Then, gefitinib and osimertinib were dissolved in 10 % DMSO, 10 % 2-hydroxypropyl-beta-cyclodextrin, and sterile ultrapure water. Each group of 10 mice were treated with gefitinib and osimertinib or the vehicle for 2 weeks. At day 20 after cell injection, all mice were sacrificed to extract the developed tumor. The tumor was mechanically homogenized, and each DNA was extracted. Highly resistant variants, such as the L858R_T790 M mutant, were expected to grow dominantly under the gefitinib treatment. Strong clonal selection of L858R_T790 M might deplete the other variants during a longer incubation time of *in vivo* assay. Therefore, highly resistant variants were not included as controls when using the *in vivo* MANO method. Green fluorescent protein (GFP) was used as a control variant. The relative abundance of each cell clone compared with the GFP cell clone was calculated using the MANO method.

2.9. Clinical activity of TKIs to exon 20 insertions

Clinical reports that demonstrated the sensitivity of exon 20

insertions to TKIs were collected and summarized. There are not enough data on each exon 20 insertion, but we analyzed the available data to assess the concordance between this study and the clinical information. For the overall response rate (ORR), we defined sensitive as more than 50 % patients showing partial response (PR) or long stable disease (over eight months), partially sensitive as 50 % or less but not less than 20 %, and resistant as less than 20 %, according to the Response Evaluation Criteria in Solid Tumors (RECIST) [24,40].

3. Results

3.1. Spectrum of EGFR exon 20 insertions identified in the COSMIC database

A total of 116 variants of insertion mutations within *EGFR* exon 20 are reported in the COSMIC database (**Supplementary Table S2**); however, only 19 of them are functionally annotated in the OncoKB database (https://www.oncokb.org/) (**Supplementary Table S5**). A total of twenty-one recurrent exon 20 insertions of *EGFR* were selected for the present study **(**Fig. 1 **and Supplementary Fig. S1).** All recurrent mutants were found across several cancer subtypes, and most of them were reported in lung cancer, followed by cancers of the upper respiratory and digestive tract, breast, urinary tract, and soft tissues (Fig. 1B). The exon 20 insertions are all located in the intracellular tyrosine kinase domain (Fig. 1C), and the most frequent one is V769_D770insASV (and the identical amino acid sequence A767_V769dup) reported in lung cancer. In the OncoKB data source, oncogenicity was annotated in only five out of 21 mutations (**Supplementary Table S5**).

3.2. Evaluation of exon 20 insertions with the MANO method

To assess the transforming potential of insertion mutations within *EGFR* exon 20, focus formation assay with 3T3 cells was conducted (Fig. 2). Almost all of the mutants showed transformed focus formation, except for wild-type *EGFR* and the P772_H773insPR, a mutant reported in a previous study without detailed annotation [41].

To evaluate the drug sensitivity of these mutants with the MANO method, the mixture of Ba/F3 cells expressing twenty-five different mutants (twenty-one exon 20 insertions, three exon 19 deletions, and L858R) were treated with gefitinib, afatinib, or osimertinib at different concentrations. The barcode count of the P772_H773insPR mutant disappeared from the cell mixture at the beginning of the TKI treatment, so this mutant was excluded from the later functional analysis. The degrees of sensitivity to gefitinib, afatinib, and osimertinib were evaluated based on cell viability that was calculated using the MANO method. As shown in Fig. 3A, the common driver mutations (E746_A750del and L858R) were sensitive to all three TKIs, confirming the validity of the MANO method. In contrast, exon 19 insertions (I744_K745insKIPVAI and K745_E746insVPVAIK) were partially sensitive to gefitinib and sensitive to afatinib and osimertinib. All exon 20 insertions, except D761_E762insEAFQ (and the identical amino acid sequence A763_Y764insFQEA), were resistant to gefitinib and afatinib, but sensitive or partially sensitive to osimertinib.

The PrestoBlue cell viability assay was performed to validate the sensitivity of these mutants to TKIs (Fig. 3B). EGFR L858R was sensitive to all three TKIs, whereas EGFR T790 M/C797S and KRAS G12 V (data not shown) were resistant to all three TKIs, confirming the validity of the MANO method. In concordance with the results of the MANO method, all exon 20 insertions were resistant to gefitinib and afatinib, but sensitive or partially sensitive to osimertinib. We also performed the assay using poziotinib and mobocertinib, which are inhibitors specific to exon 20 insertions, although they are currently under clinical investigation. According to the IC_{90} of poziotinib and mobocertinib, most of the exon 20 insertions showed sensitivity, while a few showed partial sensitivity **(Supplementary Fig. S3)**.

3.3. Evaluation of the sensitivity of EGFR mutants in vivo

Next, the effectiveness of each EGFR inhibitor was assessed through an *in vivo* assay. The 3T3 cells expressing 26 different constructs including the GFP control (**Supplementary Fig. S1**) were pooled, and injected en bloc into the flank of nude mice. The mice were then treated daily with oral administration of vehicle, gefitinib (100 mg/kg body weight), or osimertinib (20 mg/kg body weight) for 2 weeks (ten mice per group). The tumors were excised on day 20, and the relative percentage of each cell clone was quantitated using the MANO method. Fig. 4 shows the normalized fold changes in the read numbers of the TKI-treated group on day 20, compared with the vehicle-treated group. The barcode count of the P772_H773insPR mutant also disappeared from the 3T3 cell mixture and the data was not collected. EGFR exon 19 deletions

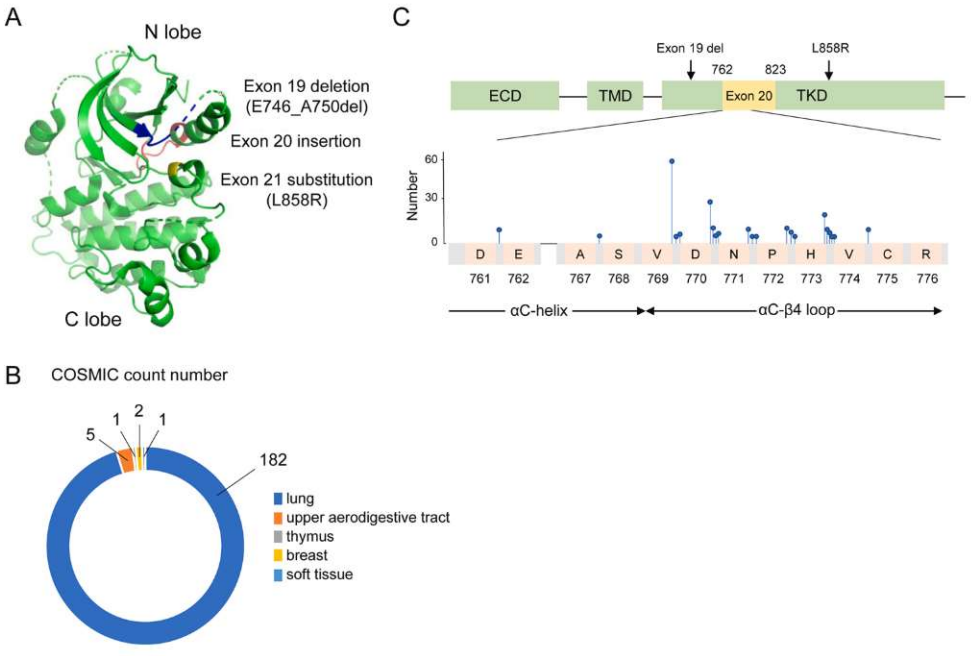


Fig. 1. The structure of EGFR and exon 20 insertion.
A. Protein structure of EGFR from Protein Data Bank (PDB). The structure is from PDB entry 4LQM. The position of exon 19 deletion (blue), exon 20 insertion (red), exon 21 substitution (yellow), N lobe, and C lobe are shown. **B.** The distribution of *EGFR* exon 20 insertions to tissues. *EGFR* exon 20 insertions were observed across several types of cancer. The number of each tissue indicates its sample count as reported in the COSMIC database. Almost all of the primary sites were in the lung. **C.** The structure of each domain of EGFR. Exon 19, 20, and 21 are all in the tyrosine kinase domain (upper). Lollipop plot shows the numbers of each variant reported in the COSMIC database (lower) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

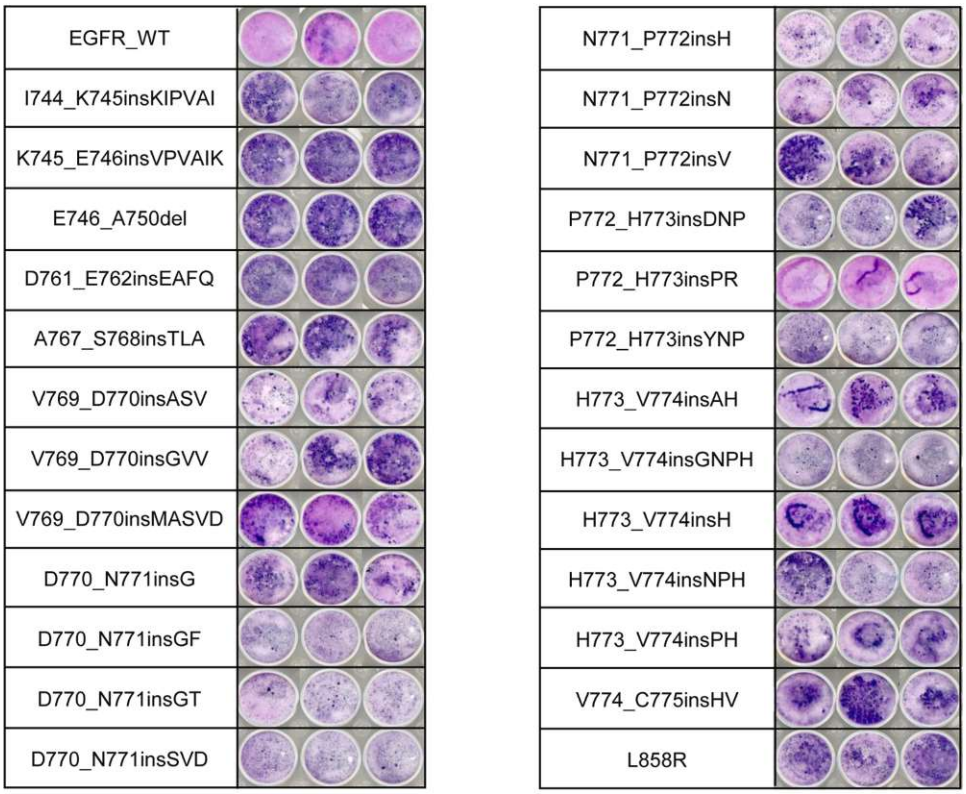


Fig. 2. Images of the focus formation assay using 3T3 cells.
The images represent 3T3 cells stained with Giemsa solution after performing focus formation assay. Individual variants with three different barcodes were prepared to obtain triplicate results. EGFR WT and P772_H773insPR are the only variants that did not show any focus formation.

and L858R were sensitive to both gefitinib and osimertinib. All *EGFR* exon 20 insertions except D761_E762insEAFQ were resistant to gefitinib. In contrast, fifteen out of twenty exon 20 insertions were sensitive to osimertinib.

3.4. Summary of TKI sensitivity profile of EGFR exon 20 insertions

Fig. 5 summarizes the estimated TKI sensitivity profiles of the insertion mutations in the present study. Based on this evaluation, all *EGFR* exon 20 insertions except for D761_E762insEAFQ were resistant to gefitinib and afatinib. However, several insertions were partially sensitive to osimertinib, although the sensitivity of exon 20 insertions were generally lower than that of L858R or exon 19 deletions. The cell number expressing the A767_S768insTLA, D770_N771insG, D700_N771insGT, N771_P772insH, or N771_P772insN mutants using the MANO method *in vivo*, showed >80 % reduction with the osimertinib treatment.

The clinical review of each mutation is summarized in Fig. 5 and **Supplementary Table S6.** Previous reports showed that patients with D770_N771insG and N771_P772insH mutants responded relatively well to osimertinib [23,26]. Only one case of D770_N771insG treated with gefitinib was reported and showed PD response [25].

4. Discussion

To our knowledge, this is the first report to comprehensively evaluate the sensitivity of exon 20 insertions to three generations of EGFR TKIs, although the function of some individual insertions have been previously reported. Moreover, few clinical reports have described the efficacy of osimertinib on exon 20 insertions. Our study reveals that each exon 20 insertion has a distinct sensitivity to different EGFR TKIs. All exon 20 insertions of *EGFR* investigated are located in the tyrosine kinase domain (Fig. 1B), and 90 % of them are in the αC-β4 loop that connects the αC-helix and β4 strand in the N-terminal adenosine triphosphate (ATP)-binding lobe of the kinase domain [42].

The MANO method is especially robust for *in vivo* assay, since the drug sensitivity of several variants can be evaluated in a single mouse carrying a heterogeneous tumor comprised of different mutants when the surrounding microenvironment is completely identical. It is well known that the overall success rate for anticancer agents in clinical development is under 10 %, and there is a gap between preclinical research and clinical feasibility [43]. Therefore, not only are *in vitro* experiments necessary, but verifying the drug effects in a live complex environment is essential. To date, no *in vivo* assessment of *EGFR* exon 20 insertions at this scale has been conducted.

Gefitinib and afatinib were ineffective against all exon 20 insertions, except for D761_E762insEAFQ, which stands at the position between exon 19 and 20. The inserted FQEA sequence alters the register of the C-helix toward the N-terminus, which leads to structural and kinetic alterations that resemble those seen with more common EGFR TKI–sensitizing mutations, like exon 19 indels and exon 21 L858R [44,45]. The evaluation of this variant in this study was consistent with that of previous clinical reports. In contrast, most of the insertions showed different degrees of sensitivity to osimertinib. Only a single residue difference may dramatically change kinase autophosphorylation, exhibiting long-range allosteric effects on ATP binding [42], which may cause the difference in the TKI sensitivity of individual mutants.

Emerging are novel EGFR TKIs targeting exon 20 insertions, and poziotinib, a smaller TKI with a more flexible structure, showed both *in vitro* and clinical efficacy (NCT03066206) [27]. In a phase 1/2 study of mobocertinib (TAK-788), an oral EGFR/ERBB2 inhibitor, 14 out of 26 lung cancer patients (54 %) harboring exon 20 insertions had a PR whereas 10 patients discontinued the treatment due to adverse effects [46]. In this study, exon 20 insertions exhibited relatively good responses to poziotinib and mobocertinib TKIs (Fig. 5) according to the PrestoBlue assay, as expected in a recent preclinical study [47]. In the

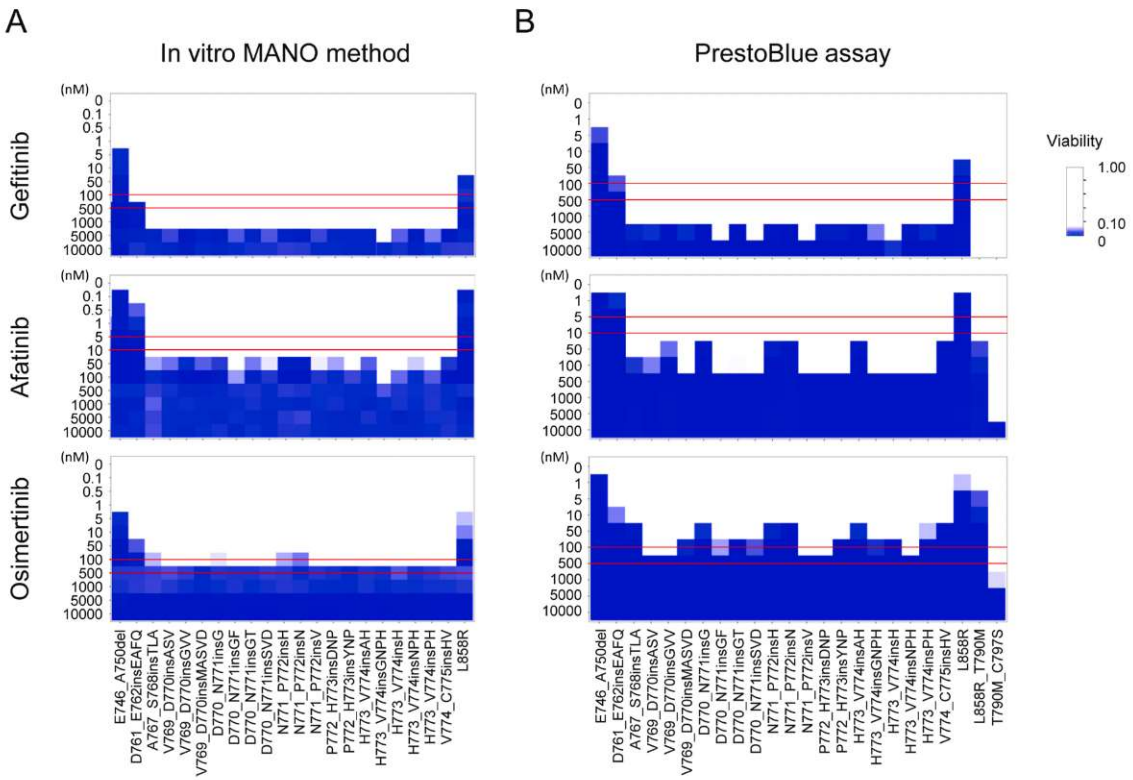


Fig. 3. The sensitivity of *EGFR* exon 20 insertions to TKIs.
A. *In vitro* MANO method. The Ba/F3 cells expressing EGFR variants (exon 19 deletion, insertions, 20 insertions, and L858R) were treated with DMSO or EGFR TKIs (gefitinib, afatinib, and osimertinib) at indicated concentrations for 4 d. All of exon 20 insertions, except D761_E762insEAFQ, were resistant to gefitinib and afatinib, whereas all variants were sensitive or partially sensitive to osimertinib. **B.** PrestoBlue cell viability assay. The Ba/F3 cells expressing EGFR variants were incubated with the indicated concentrations of TKIs for 4 d. In osimertinib, several exon 20 insertions, such as A767_S768insTLA, D770_N771insG, N771_P772insH, and N771_P772insN, were sensitive. Data are presented as mean (n = 6). The viabilities of cells with variants are color-coded. The red lines in the figure show the thresholds between sensitive and partially sensitive (the upper line), and between partially sensitive and resistant (the lower line) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

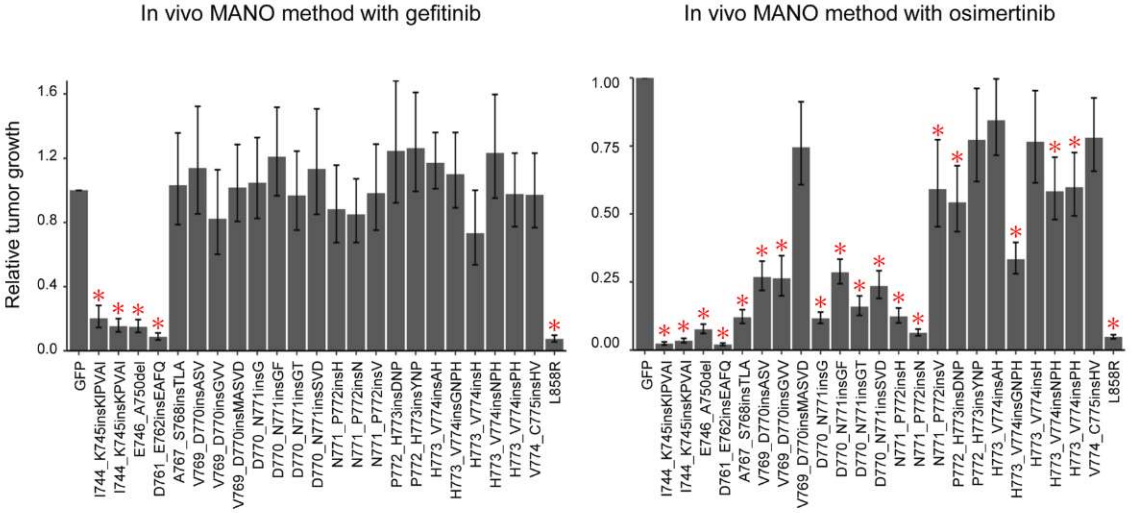
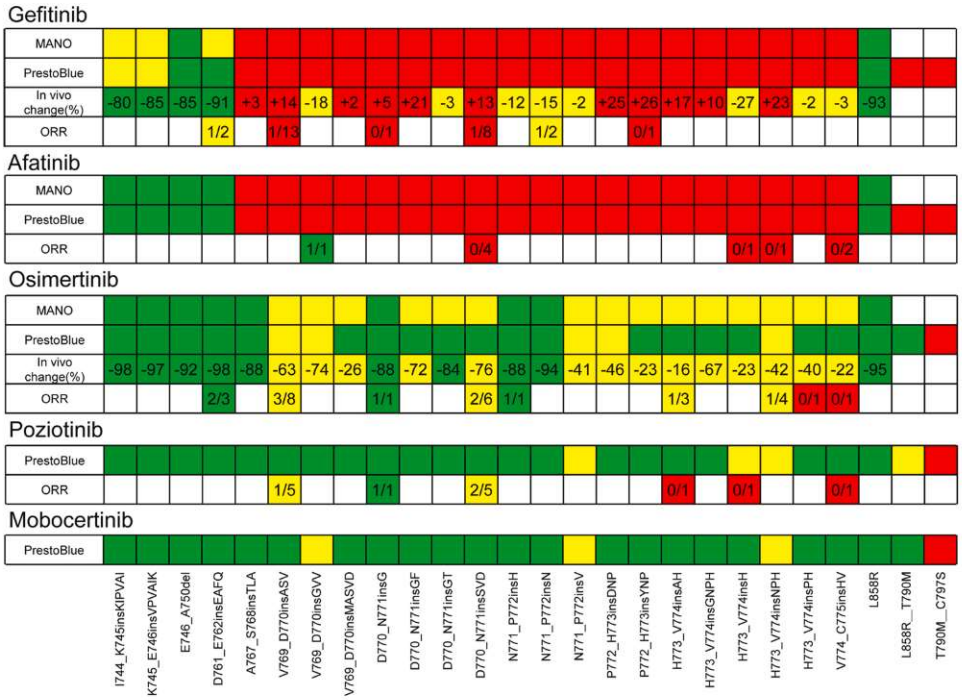


Fig. 4. *In vivo* MANO method using gefitinib and osimertinib.
The relative abundance of each cell clone, compared with GFP cell clone after treatment of gefitinib or osimertinib for 2 weeks, was calculated using the MANO method. Error bar shows the standard error with 10 mice. Asterisk indicates significance, compared with the GFP control (paired *t*-test, $p < 0.01$).

near future, these promising results may be validated in clinical trials.

The present study has several limitations. First, retroviral transduction of *EGFR* cDNAs into cell lines may result in overexpression of EGFR protein compared with endogenous EGFR levels. Especially, assessment on the transforming potential is difficult since overexpression of wild-type EGFR itself confers transformed foci in the 3T3 assay. Second, the experiment system with 3T3 and Ba/F3 cells to assess oncogenicity and drug sensitivity may be different from lung tissues. These two different cells may indeed produce different results in certain genes/mutants. Third, our experimental data are not yet



	Gefitinib/Osimertinib	Afatinib	Poziotinib/Mobocertinib
Sensiive	IC_{90} < 100 nM	IC_{90} < 5 nM	IC_{90} < 10 nM
Partially sensitive	100 nM ≦ IC_{90} ≦ 500 nM	5 nM ≦ IC_{90} ≦ 10 nM	10 nM ≦ IC_{90} ≦ 50 nM
Resistant	500 nM < IC_{90}	10 nM < IC_{90}	50 nM < IC_{90}

	In vivo change (%)	ORR (%)
Sensiive	% < -80	50 < %
Partially sensitive	0 ≦ % ≦ -80	20 ≦ % ≦ 50
Resistant	0 < %	% < 20

Fig. 5. Drug sensitivity assessment for EGFR mutants.
The drug sensitivity of EGFR mutants was evaluated using the *in vitro* MANO method in Ba/F3 cells, cell viability assay with PrestoBlue, *in vivo* MANO method using 3T3 cells, and clinical activity of each TKI to exon 20 insertions (Materials and methods 2.9). Drug sensitivity was categorized as sensitive, partially sensitive, or resistant based on the IC_{90} of each mutant for each drug and clinical activity. The number of ORR (%) means PR and/or long SD (over seven months) / total number of reported cases (**Supplementary Table S6**).

completely supported by clinical data. Although TKI efficacy to several exon 20 insertions can be confirmed through previous reports, most of them cannot be evaluated in the clinical setting because of their rarity. Fourth, there are extremely rare exon 20 insertions that we did not assess their drug sensitivity. Finally, our study did not take into account other gene mutations such as the *PIK3CA* mutation, which can possibly co-occur in cancers harboring *EGFR* mutations and may affect drug sensitivity [24,48].

In conclusion, a comprehensive evaluation of insertion mutants of *EGFR* exon 20 was successfully performed using the MANO method. The efficacy of five different EGFR TKIs on several mutants was first evaluated in our study. These preclinical data give us the opportunity to select the appropriate drug for individual patients harboring a rare exon 20 insertion, whose clinical data are insufficient. Given the different degrees of efficacy of EGFR TKIs, the MANO method may be useful in functionally evaluating variants of unknown significance, which could help determine the best treatment for cancers harboring exon 20 insertional mutations.

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CRediT authorship contribution statement

Takeshi Hirose: Investigation, Data curation, Writing - original draft. **Masachika Ikegami:** Software, Formal analysis. **Makoto Endo:** Supervision. **Yoshihiro Matsumoto:** Supervision. **Yasuharu Nakashima:** Supervision. **Hiroyuki Mano:** Writing - review & editing, Supervision, Funding acquisition. **Shinji Kohsaka:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2020.12.023.

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