

Clinical characterization of *ERBB2* exon 20 insertions and heterogeneity of outcomes responding to afatinib in Chinese lung cancer patients

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Purpose: *ERBB2* exon 20 insertions (20ins) have been identified as oncogenic drivers in lung cancers. Lung cancer patients with 20ins benefit from afatinib. However, response heterogeneity was observed in patients harboring different 20ins subtypes. In this study, we interrogated clinical characteristics in *ERBB2*-mutated Chinese lung cancer and investigated the clinical outcomes of specific *ERBB2* 20ins in response to afatinib.

Experimental design: In this study, we retrospectively collected genomic profiling data of 7,520 lung cancer patients sequenced using next-generation sequencing in a Clinical Laboratory Improvement Amendments-certified laboratory. We analyzed the clinical and molecular features of patients harboring *ERBB2* 20ins and evaluated clinical outcomes of 19 patients with clinical records after afatinib treatment.

Results: *ERBB2* 20ins were identified in 2.27% (171/7,520) of this lung cancer cohort. It occurred with a high proportion in females with adenocarcinoma histology. *ERBB2* 20ins was mutually exclusive with other well-established lung cancer oncogenic driver mutations. The most frequently appearing subtype was Y772_A775dup (69.6%) and several novel insertion subtypes were also identified. The correlations of specific 20ins subtypes and survival were investigated. The presence of a glycine at position 778 in *ERBB2* was suggested to be a common feature of drug sensitivity mutations. Patients harboring G778_P780dup (G778) subtype achieved longer median progression-free survival and median overall survival than other 20ins (non-G778) subtypes (median progression-free survival, 10 vs 3.3 months, $P=0.32$; median overall survival, 19.7 vs 7 months, $P=0.16$). Moreover, we presented the first clinical case of a lung squamous cell carcinoma patient harboring *ERBB2* 20ins who achieved partial response to afatinib.

Conclusion: This study interrogated the characteristics of *ERBB2* 20ins in a large cohort from single ethnicity and demonstrated the response heterogeneity to afatinib among different *ERBB2* 20ins subtypes. Further studies in a larger cohort are needed to investigate the underlying molecular mechanisms and clinical response of different *ERBB2* 20ins subtypes.

Keywords: *ERBB2*, lung cancer, afatinib, survival, heterogeneity

Introduction

Genetic alterations of human epidermal growth factor receptor 2 (*ERBB2*, also known as *HER2*), occurring in 2%–4% of lung cancers, have been identified as an oncogenic driver.^{1–4} *HER2* protein, encoded by *ERBB2* gene, drives cell proliferative and activates PI3K/AKT and MEK/ERK downstream signaling pathways.⁵ In-frame insertions of *ERBB2* exon 20 (20ins), resulting in constitutive activation of the downstream PI3K/AKT signaling, initiate the protumorigenic signaling cascade.^{2,3,6} *ERBB2* 20ins

were predominantly observed in females, nonsmokers, and adenocarcinoma patients.^{3,7,8} No clinical case of *ERBB2* 20ins has been reported in lung squamous cell cancer. The most frequently occurring subtype is Y772_A775dup, accounting for approximately 60% of all 20ins in non-small-cell lung cancer.^{3,9}

With the rapid advancement of genomic profiling technologies and targeted therapy, several *ERBB2* inhibitors have been approved for the treatment of *ERBB2*-positive breast cancer, such as trastuzumab, lapatinib, and pertuzumab.^{10–15} However, the development of *ERBB2*-targeted therapy in lung cancer was not very successful in the beginning when the first drug trastuzumab showed negative result in a Phase II trial, which revealed that combination therapy of trastuzumab and chemotherapy failed to produce additive effect in *ERBB2*-positive lung cancer patients.¹⁶ Despite the dismal results from several studies, some case series have reported that lung cancer patients with *ERBB2* 20ins can benefit from afatinib. It is an ATP-competitive anilinoquinazoline derivative which can covalently bind to and irreversibly block signaling from HER2.¹⁷ The response heterogeneity suggests that *ERBB2* mutant patients do not represent a homogenous group. Very limited studies have investigated what contributes to such heterogeneity. In this study, we investigated whether different *ERBB2* 20ins subtypes are responsible for treatment heterogeneity by correlating specific 20ins subtypes to survival in 171 lung cancer patients.

Materials and methods

Patient information

We retrospectively screened 7,520 lung cancer patients (from eight hospitals) whose tissue or plasma samples were sequenced in a Clinical Laboratory Improvement Amendments-certified genomic profiling laboratory (Burn-ing Rock Biotech, Guangzhou, People's Republic of China), from October 2015 to January 2018. Their sequencing results were reviewed with the intent to study the association between *ERBB2* exon 20 insertions distribution and prognosis after afatinib treatment. A total of 171 patients with *ERBB2* exon 20 insertions were identified. The study was approved by the institutional review board of Chinese PLA General Hospital.

Preparation of tissue DNA and plasma cfDNA

Tissue DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Circulating cfDNA was recovered

from 4 to 5 mL of plasma by using the QIAamp Circulating Nucleic Acid kit (Qiagen). Quantification of DNA was done with the Qubit 2.0 fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA).

Next-generation sequencing library preparation

DNA shearing was performed with a Covaris M220 focused ultrasonicator (Covaris, Inc., Woburn, MA, USA) followed by end repair, phosphorylation, and adaptor ligation. Fragments with a size of 200–400 bp were selected by using Agencourt AMPure beads (Beckman Coulter, Fullerton, CA, USA) followed by hybridization with capture probes baits, hybrid selection with magnetic beads, and PCR amplification. A high-sensitivity DNA assay was performed to assess the quality and size of the fragments, and indexed samples were sequenced on a Nextseq500 sequencer (Illumina, Inc., San Diego, CA, USA) with paired-end reads. Our profiling panels, consisting of 8, 56, 168, or 295 cancer-related genes, were designed and validated for identification of base substitutions, insertions, deletions, copy-number variations, and gene fusion.

Sequencing data analysis

The sequencing data in the FASTQ format were mapped to the human genome (hg19) using BWA aligner 0.7.10.¹⁸ Local alignment optimization, variant calling, and annotation were performed using GATK 3.2, MuTect, and VarScan,^{19,20} respectively.

Statistical analysis

Kaplan–Meier analysis was used to estimate the survival functions, and log-rank test to determine the difference of survival curve between groups. A *P*-value of <0.05 was considered statistically significant. All these survival analyses were conducted in software R (Version 3.3.3, The R Foundation, Vienna, Austria) with survival package (Version 2.41-3; <https://cran.r-project.org/web/packages/survival/index.html>).

Results

Patient characteristics

Sequencing results of tissue or plasma samples obtained from 7,520 lung cancer patients were analyzed, and 171 patients were identified with *ERBB2* 20ins, resulting in an incidence of 2.27%. Clinical features of patients harboring *ERBB2* 20ins mutations are summarized in Table 1. Patients harboring *ERBB2* 20ins had a median age of 56 years, ranging from 23

Table 1 Summary of baseline clinical characteristics of patients with *ERBB2* 20ins

Patient characteristics	<i>ERBB2</i> 20ins	Afatinib treatment
Total	171	19
Gender		
Female	106 (62%)	12 (63%)
Male	65 (38%)	7 (37%)
Age (years)		
Median	56	57
Range	23–86	41–86
Histological types		
Adenocarcinoma	136	18
Adenosquamous carcinoma	1	0
SCC	1	1
Unknown	33	0

Abbreviations: 20ins, exon 20 insertions; SCC, squamous cell carcinoma.

to 86 years. Among them, 38% (65/171) were males and 62% (106/171) were females. A vast majority of them were diagnosed with adenocarcinomas, except for one adenosquamous carcinoma and one squamous cell carcinoma (SCC).

Genomic characteristic overview of the *ERBB2* 20ins patients

To investigate the prevalence and composition of *ERBB2* 20ins, capture-based targeted sequencing was performed using various panels covering critical exons and introns of lung cancer-related genes. Among the 171 *ERBB2* 20ins-positive patients, five patients were profiled using 295 cancer-related genes panel. Fifty-three patients were sequenced using a panel consisting of 168 lung cancer-related genes. Thirty-five patients were assessed using a 56-gene panel, and for the remaining 78 patients a panel consisting seven well-known lung cancer driver genes plus *KRAS*, a well-established prognostic factor, was used.

A total of 432 mutations were identified from the 171 patients harboring *ERBB2* 20ins, including 139 single-nucleotide variants (SNVs), 172 insertions and deletions (Indels), 83 copy-number amplifications, and 38 frameshift or splice variants. Among them, *TP53* ranked as the most frequently mutated gene in this cohort, occurring in 51.6% (48/93) patients who were sequenced using panels covering *TP53*. All *ERBB2* 20ins patients did not carry concurrent classic non-small-cell lung carcinoma driver genes (including *EGFR*, *BRAF*, *MET*, *ROS1*, *ALK*, *RET*, and *KRAS*), which is consistent with previous reports, confirming the strong mutual exclusivity of *ERBB2* with other driver oncogenes. Moreover, we found 11.7% (20/171) patients with *ERBB2* 20ins harboring concomitant *ERBB2* amplification.

ERBB2 mutation subtypes overview

Of the 171 patients harboring *ERBB2* 20ins, 13 insertion subtypes were identified. They included 12-base-pair insertions (Y772_A775dup, A775_G776insSVMA, A775_G776insVVMA, A775_G776insYVMS, Y772_V773insM-MAY), 9-bp insertions (G778_P780dup), 3-bp insertions (A775_G776insC), and deletions combined with insertions (G776delinsVC, G776delinsLC, G776delinsVV, G776delinsAVGC, G776delinsIC, G776_V777delinsCVC). The nomenclature of these exon 20 insertions strictly followed the Human Genome Variation Society guidelines. The structural schematic diagrams of those exon 20 insertion subtypes and corresponding insert positions are demonstrated in Figure 1A.

The most frequently appearing subtype was a duplication of 12-bp which resulted in an insertion of YVMA at codon 775 (Y772_A775dup) (Figure 1B). This subtype was detected in 119 patients (69.6%), consistent with previous reports. The second most frequently occurring *ERBB2* 20ins subtype in our cohort was G778_P780dup, an in-frame 9-bp insertion, which was identified in 18 patients (10.5%). Moreover, several rare or novel *ERBB2* 20 insertions were identified in this cohort. The Integrative Genomics Viewer screenshots of these rare or novel subtypes are shown in Figure 2.

Correlation of specific 20ins subtypes and survival

To interrogate the clinical outcomes of different *ERBB2* 20ins in response to afatinib, we selected 19 *ERBB2* 20ins patients who received afatinib as monotherapy with detailed survival information. Kaplan–Meier survival analysis revealed that patients with *ERBB2* G778_P780dup mutations achieved longer median progression-free survival (PFS) (10.0, 3.7, 1.5, 2.5 months, respectively, $P=0.69$; Figure 3A) and median overall survival (OS) (19.7, 7.0, 1.5, 11.1 months, respectively, $P=0.44$; Figure 3B) than those harboring Y772_A775dup, G776delinsVC, and other subtypes. However, the differences were not statistically significant owing to the limited patient number of each subgroup.

The glycine at *ERBB2* 778 site was reported to be a drug-sensitive mutation, which may facilitate inhibitor binding to *ERBB2*.²¹ Therefore, we stratified our *ERBB2* 20ins patients into two subgroups to compare survival between G778 patients (G778_P780dup) and non-G778 patients (other amino acids at 778 due to insertion). The Kaplan–Meier analysis revealed that patients with G778 displayed longer median PFS (10.0 vs 3.3 months, $P=0.32$; Figure 3C) and median OS (19.7 vs 7.0 months,

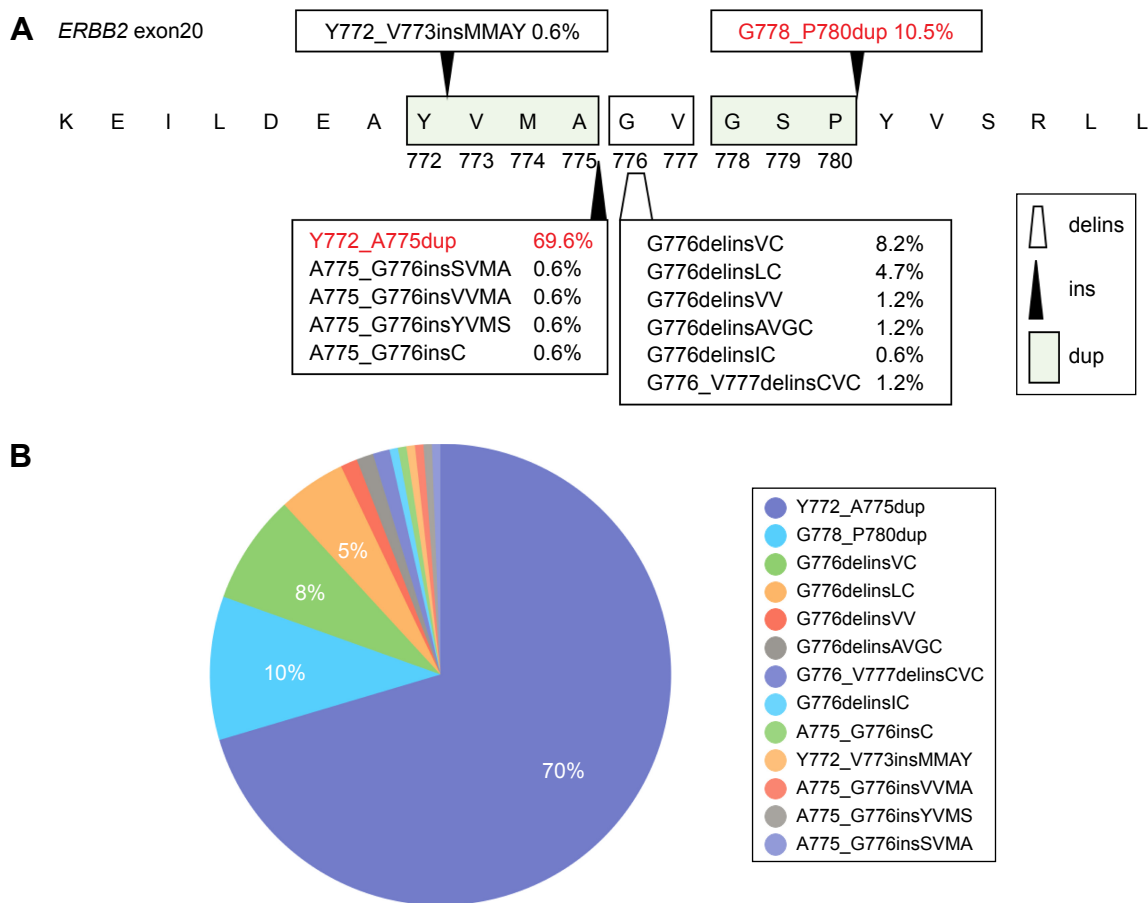


Figure 1 Structure and distribution of *ERBB2* 20ins.

Notes: (A) Schematic diagram demonstrates the amino acids change of 20ins subtypes in *ERBB2* kinase domain. The black arrows indicate the insertion position and trapezoid shape presents the position of deletion combined with insertion. (B) The pie chart presents the proportion of each 20ins subtype in the 171 cohort.

Abbreviations: 20ins, exon 20 insertions; delins, deletion and insertion; dup, duplicate; ins, insertion.

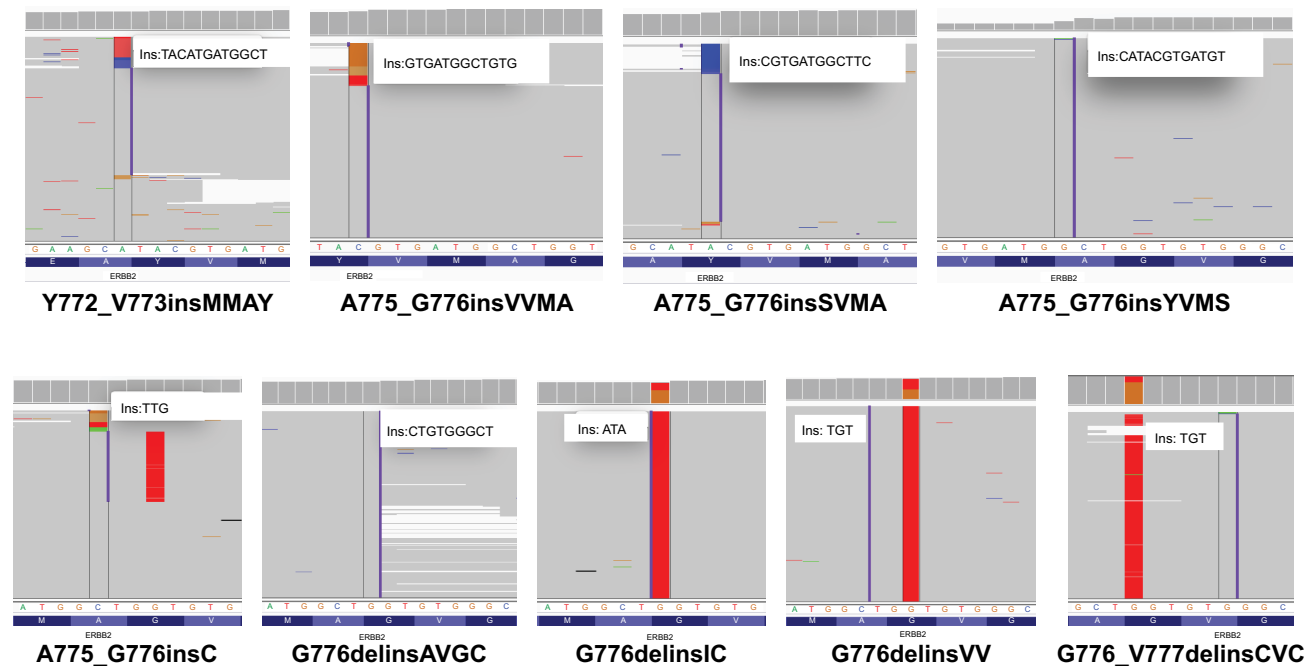


Figure 2 The Integrative Genomics Viewer screenshots displayed the reads of rare and novel 20ins subtypes identified by NGS.

Abbreviations: 20ins, exon 20 insertions; ins, insertion; NGS, next-generation sequencing.

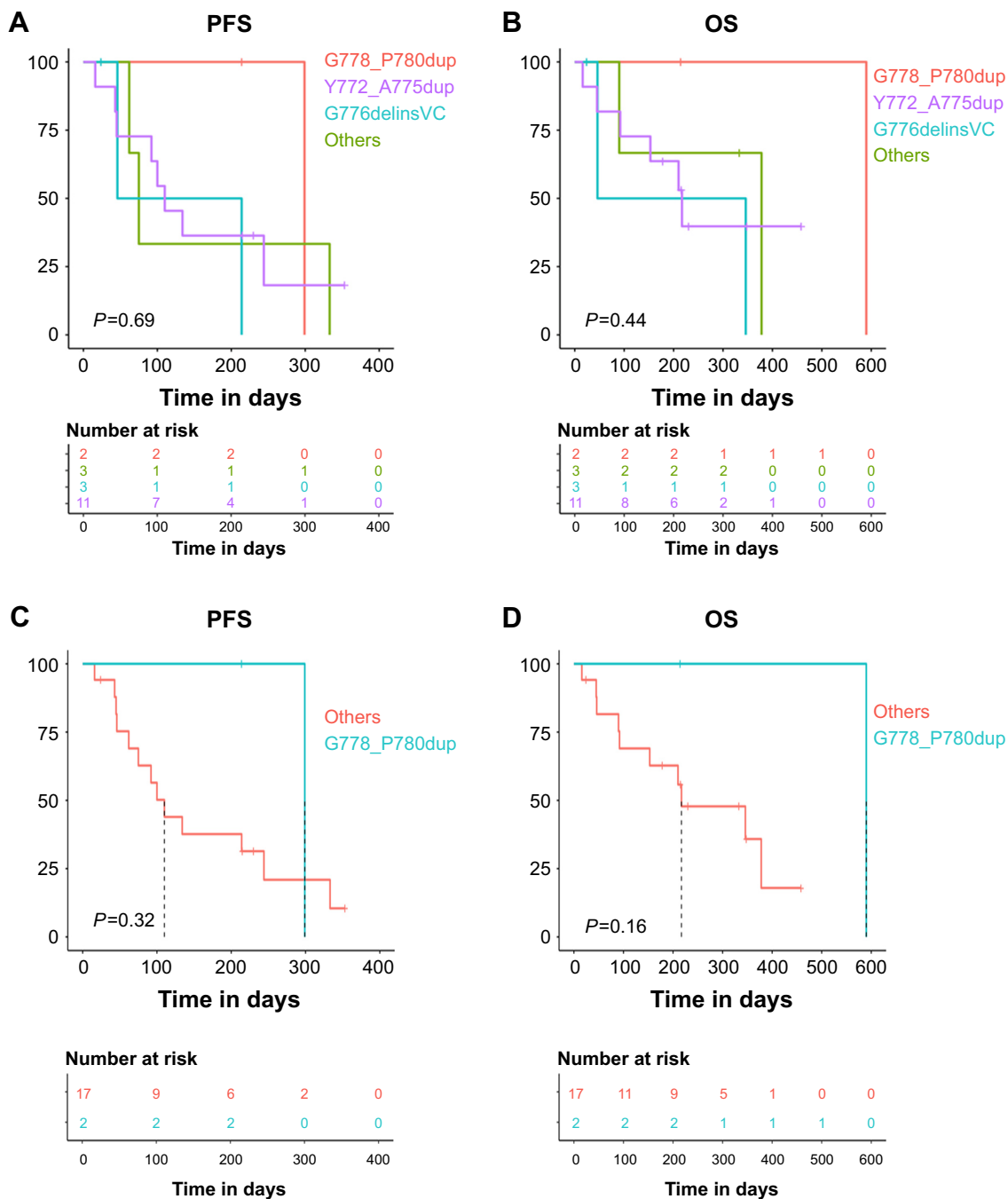


Figure 3 Correlation of 20ins subtypes and PFS.

Notes: (A, B) Association of distinct 20ins subtypes and PFS/OS. (C, D) Glycine at ERBB2 778 site was correlated with longer trend of PFS/OS.

Abbreviations: 20ins, exon 20 insertions; delins, deletion and insertion; dup, duplicate; OS, overall survival; PFS, progression-free survival.

$P=0.16$; Figure 3D) than non-G778 patients. But due to the small cohort size of G778 patients, the differences did not reach statistically significant. This result warrants further exploration in large cohorts to better understand this phenomenon.

Overall outcomes of afatinib treatment of the ERBB2 20ins patients

In the cohort of 19 patients receiving afatinib treatment, consisting of 18 lung adenocarcinomas and 1 squamous cell lung

cancer, 14 patients reached progressive disease (PD) and the other 5 patients were still benefiting from afatinib treatment to date. The median PFS of the 14 patients was 4.5 months and median OS was 11.5 months after afatinib monotherapy. The overall response rate in this cohort was 15.8% (3/19) and disease control rate was 68.4% (13/19). Among them, three patients achieved partial response (PR) to afatinib treatment, ten patients were in stable disease (SD) after afatinib, whereas six patients only had PFS period records and response details were

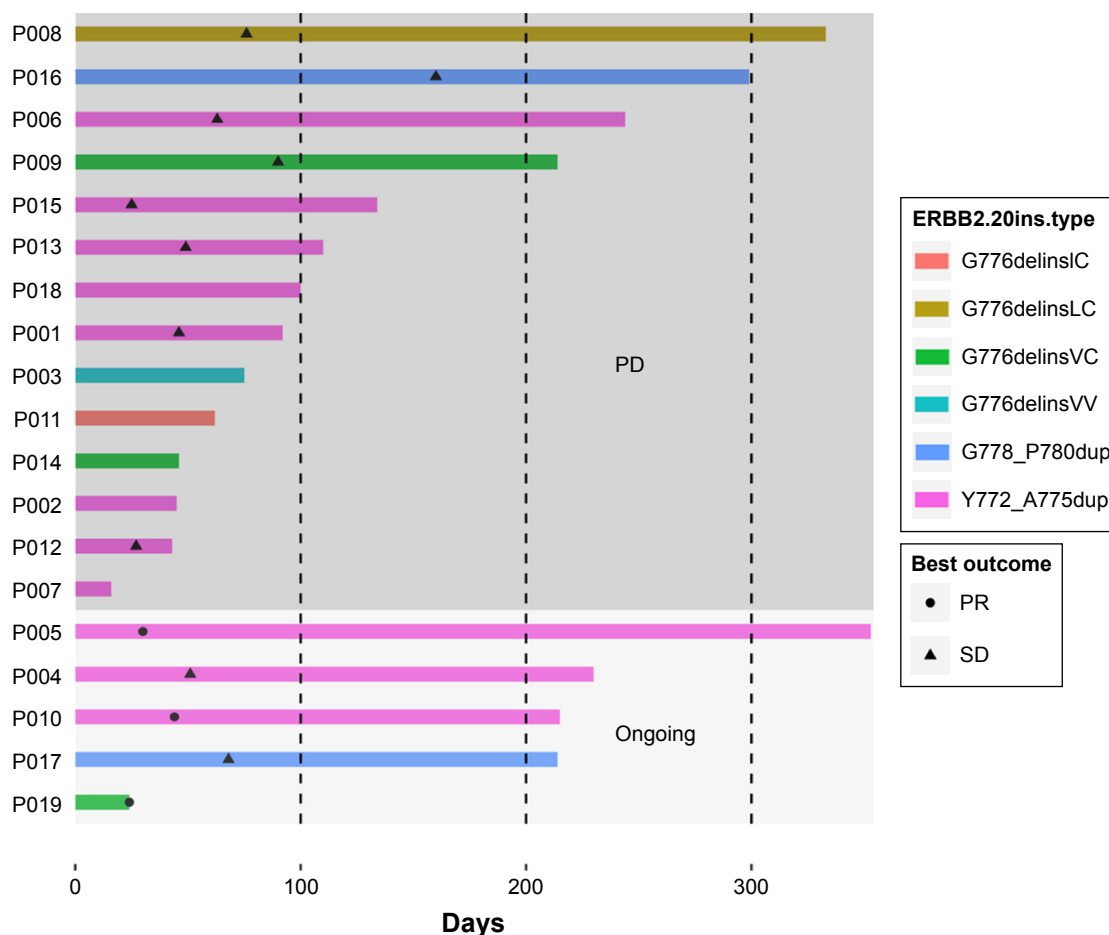


Figure 4 PFS information for each *ERBB2* 20ins patient treated with afatinib.

Notes: Each line represents a patient. The length of each line indicates the period of PFS. Each color presents each 20ins subtype. Solid circle means the time when patient achieved PR and solid triangle indicates the time of SD achievement. P008–P019 are patient ID numbers.

Abbreviations: 20ins, exon 20 insertions; delins, deletion and insertion; dup, duplicate; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease.

not available due to rapid disease progression. The overall outcomes of afatinib treatment of the 19 patients harboring *ERBB2* 20ins are demonstrated in a swimmer's plot in Figure 4.

For the eleven *Y772_A775dup* patients, eight patients finally achieved PD, with a median PFS of 3 months. The other three patients were still under afatinib treatment, with two achieving PR and one with SD. Two patients harboring *G778_P780dup* insertion had SD to afatinib treatment and displayed durable response. One had a PFS of 299 days, while another had not developed PD in the last visit after 214 days of treatment. As for rare subtypes of *ERBB2* 20ins, the patient with *G776delinsVV* had a PFS of 75 days; another patient with *G776delinsLC* achieved SD with a PFS of 333 days. The patient detected with *G776delinsIC* obtained a PFS of 62 days. Two of the three *G776delinsVC* patients displayed very different responses, with a PFS of 214 and 46 days, respectively. Another *G776delinsVC* patient achieved PR to afatinib in the last visit at day 24 and was still under treatment. Taken together, our study demonstrated that patients with

different *ERBB2* 20ins subtypes obtained diverse clinical response to afatinib. Due to the limited sample size, larger cohorts are needed to validate our findings.

A squamous cell lung carcinoma patient harboring *ERBB2* 20ins achieved PR to afatinib

To the best of our knowledge, no *ERBB2* 20ins has been reported in lung SCC. In our cohort, we identified a SCC patient harboring *ERBB2* 20ins. A 47-year-old female patient with no history of smoking presented to the outpatient clinic with intermittent cough and thoracalgia. Chest computed tomography scan revealed a mass located in right lung. Computed tomography-guided biopsy followed by hematoxylin–eosin staining revealed the representative histological appearance of lung SCC (Figure 5A). She was diagnosed with stage IV SCC with bone and brain metastasis. Genomic profiling was performed and revealed the presence of *ERBB2* exon 20 insertion *G776delinsVC*. The patient was administered afatinib (40 mg,

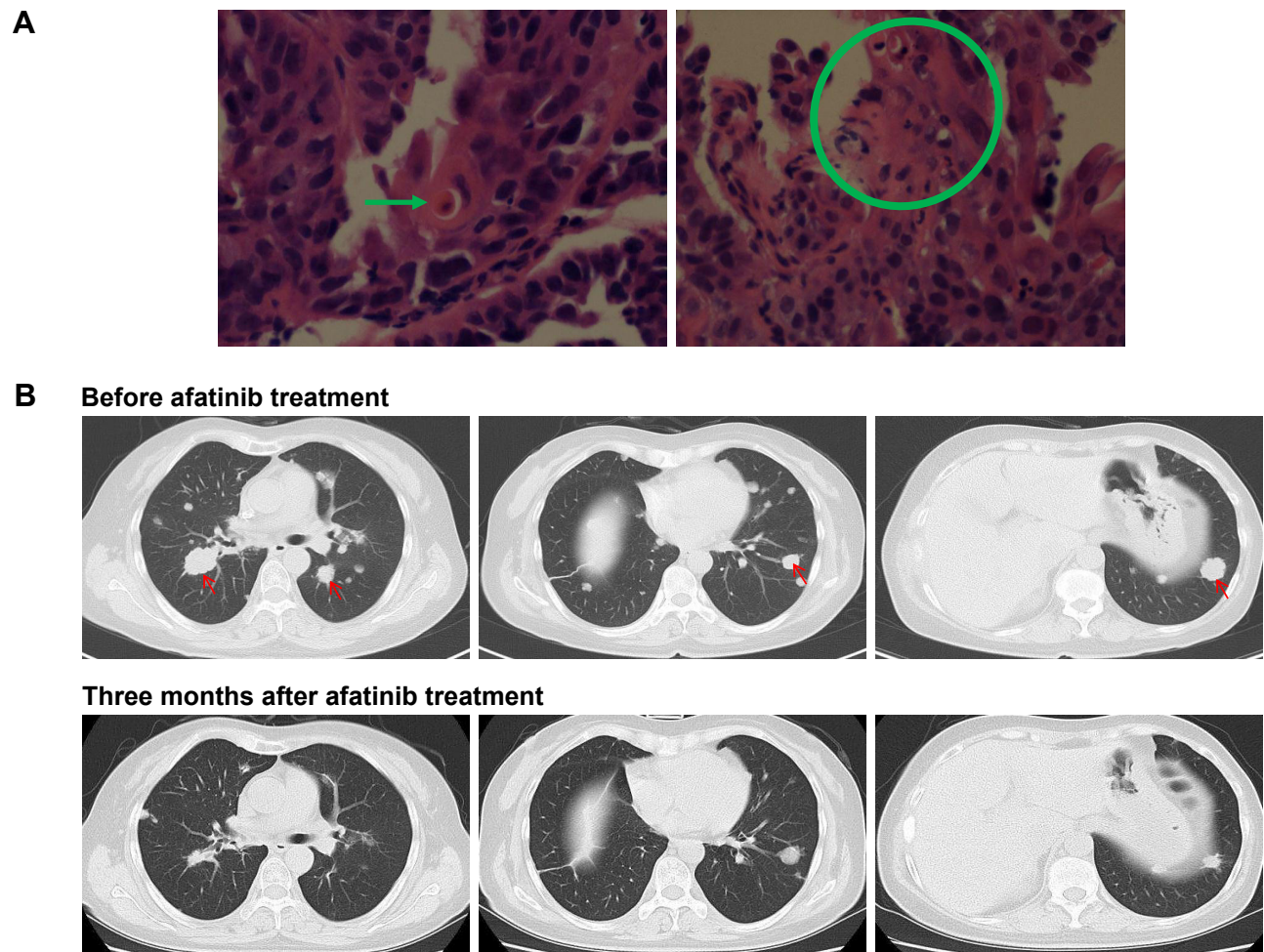


Figure 5 A lung SCC patient was identified as harboring *ERBB2* 20ins and responded to afatinib.

Notes: (A) Hematoxylin–eosin staining revealed the histological appearance of SCC. The green arrow and a green circle indicate typical histological appearance of SCC. (B) Responses of an *ERBB2* 20ins patient with lung SCC to afatinib. Computed tomography scans of the chest were obtained at baseline and after 3 months treatment of afatinib. The red arrows indicate tumor lesion.

Abbreviations: 20ins, exon 20 insertions; SCC, squamous cell carcinoma.

QD). Three months later, she achieved PR based on Response Evaluation Criteria In Solid Tumors (RECIST) 1.1, evident by tumor shrinkage (Figure 5B) and alleviation of symptoms, such as coughing, thoracalgia, and dyspnea. Collectively, this case is the first report of an SCC patient harboring *ERBB2* 20ins and showing benefit from afatinib.

Discussion

In this study, we retrospectively interrogated the clinical and molecular characteristics of *ERBB2* 20ins in a large Chinese lung cancer cohort. We identified several novel *ERBB2* 20ins subtypes which were first detected in lung cancer patients. Moreover, we investigated the correlations of *ERBB2* 20ins subtypes and clinical outcomes to afatinib treatment. In addition, we presented the first case of a lung SCC patient harboring *ERBB2* 20ins and achieving PR to afatinib.

Our study revealed that *ERBB2* 20ins demonstrated a female preponderance and adenocarcinoma predominance,

in an agreement with published data.^{3,7,8} In this study, 62% of the patients were female, and nearly all the *ERBB2* 20ins occurred in adenocarcinoma, except for one in SCC and one in adenosquamous. The insertion YVMA at codon 775 (Y772_A775dup) was the most frequently occurring subtype, accounting for 70% of all 20ins in our cohort, consistent with previous publications, which reported a prevalence of 60%.^{3,9} As *ERBB2* mutations were reported to be mutually exclusive with major mutations in *EGFR* and *KRAS*,^{1,2} *ERBB2* 20ins in all the 171 patients in our study displayed mutual exclusivity with other well-established oncogenic driver mutations, including *EGFR*, *KRAS*, *BRAF*, *MET*, *ALK*, *ROS1*, and *RET*. In addition, we found that 11.7% (20/171) *ERBB2* 20ins patients harbored concurrent *ERBB2* amplification. To date, the molecular association of *ERBB2* mutation and gene amplification in lung cancers has not been distinctly defined. Li et al⁸ reported that *ERBB2* mutation and gene amplification did not overlap in lung cancer and may present distinct

clinical entities. However, their coexistence was reported in other studies.^{7,22,23} Thus, further efforts are needed to interrogate the underlying association between *ERBB2* mutation and amplification.

Previous studies have reported response heterogeneity to afatinib with different *ERBB2* 20ins subtypes.^{17,24} G778_P780dup were reported to be sensitive to *ERBB2* targeted tyrosine kinase inhibitors than other 20ins subtypes in vitro, and the presence of a glycine at *ERBB2* position 778 was supposed to be a common feature among the drug-sensitive insertion subtypes.²¹ In this study, the survival analysis revealed that patients harboring G778_P780dup (with glycine at site 778) displayed favorable PFS and OS compared with those with other insertion subtypes, which further supports the in vitro finding. Kosaka et al²¹ reported an adenocarcinoma patient with *ERBB2* G778_P780dup achieving significant tumor shrinkage for 7 months when afatinib was given as monotherapy. In our cohort, two patients were identified as harboring G778_P780dup insertion at exon 20. They both responded to afatinib treatment and achieved long durable response. One patient achieved a PFS of 299 days, and another is still benefiting from afatinib in the latest reexamination at day 214 after treatment. This observation further confirmed the notion that glycine778 indicates favorable clinical benefits to afatinib. Both biophysical and structural analyses will be required to further investigate the underlying mechanism, and additional clinical validations in larger cohorts will be necessary to confirm our clinical findings.

There were still limitations in this study. The small population size of cohort for survival correlation studies inevitably induced bias into the analysis and cannot fully reflect the overall correlation. Thus, we were not able to adequately illustrate the correlation of different 20ins subtypes and survival after afatinib treatment. Despite this limitation, our study improved the knowledge of *ERBB2* 20ins-driven non-small-cell lung cancer in terms of clinical outcomes to afatinib. Our study might be helpful to provide guidance for clinicians to design a more precise clinical strategy for different patients based on specific *ERBB2* 20ins subtypes.

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Disclosure

Xinru Mao, Jianxing Xiang, Ke Ma, Bing Li and Tengfei Zhang were employed by Burning Rock Biotech, Guangzhou, Guangdong, People's Republic of China, at the time

of the study. The authors report no other conflicts of interest in this work.

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