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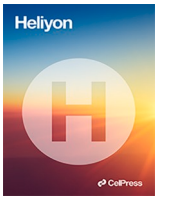

Research article

Genetic mutation profiling reveals biomarkers for targeted therapy efficacy and prognosis in non-small cell lung cancer

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A R T I C L E I N F O

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Outcome

A B S T R A C T

Introduction: The genetic heterogeneity of non-small cell lung cancer (NSCLC) with epidermal growth factor receptor (*EGFR*) mutations may affect clinical responses and outcomes to EGFR tyrosine kinase inhibitors (EGFR-TKIs). This study aims to investigate the genomic factors that influence the efficacy and clinical outcomes of first-line, second-line and third-line treatments in NSCLC and explore the heterogeneity of resistance mechanisms.

Materials and methods: This real-world study comprised 65 patients with *EGFR* mutant NSCLC. Molecular alterations were detected using a customized DNA panel before and after administering targeted therapy. The efficacy and prognosis of each treatment line were evaluated.

Results: In first-generation EGFR-TKIs treatment, gefitinib showed favorable efficacy compared to icotinib and erlotinib, particularly in patients with *EGFR* L858R mutations. The resistance mechanisms to first-generation EGFR-TKIs varied among different *EGFR* mutation cohorts and different first-generation EGFR-TKIs. In second-line EGFR-TKIs treatment, EPH receptor A3 (*EPHA3*), IKAROS family zinc finger 1 (*IKZF1*), p21 (RAC1) activated kinase 5 (*PAK5*), DNA polymerase epsilon, catalytic subunit (*POLE*), RAD21 cohesin complex component (*RAD21*) and RNA binding motif protein 10 (*RBM10*) mutations were markedly associated with poorer progression-free survival (PFS). Notably, *EPHA3*, *IKZF1* and *RBM10* were identified as independent predictors of PFS. The mechanisms of osimertinib resistance exhibited heterogeneity, with a higher proportion of non-*EGFR*-dependent resistant mutations. In third-line treatments, the combination of osimertinib and anlotinib demonstrated superior efficacy compared to other regimens. Glutamate ionotropic receptor NMDA type subunit 2A (*GRIN2A*) mutation was an independent risk indicator of shorter OS following third-line treatments.

Conclusions: Comprehending the tumor evolution in NSCLC is advantageous for assessing the efficacy and prognosis at each stage of treatment, providing valuable insights to guide personalized treatment decisions for patients.

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1. Introduction

Lung cancer is the predominant malignant tumor, holding the top position globally in terms of both its incidence and mortality [1]. This cancer is categorized into two distinct histological subtypes: non-small cell lung cancer (NSCLC), the most prevalent subtype, and small cell lung cancer (SCLC), recognized as the most aggressive subtype [2]. NSCLC constitutes the majority (85%) of all lung cancers and is primarily categorized into lung adenocarcinoma (LUAD), squamous cell carcinoma (SCC), and large cell carcinoma (LCC) [3]. In China, lung cancer ranks as the most prevalent cancer, with an estimated 0.82 million new cases reported in 2020 [4]. Additionally, it stands as the leading cause of death, accounting for 0.72 million deaths in the same year [4]. For *EGFR* mutation-positive NSCLC, epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) constitute a vital category of targeted therapies for tumors, and are the recommended standard first-line treatment for advanced NSCLC patients who have *EGFR* mutations [5]. Currently, there are three generations of TKIs available. First-generation TKIs can attain a median progression-free survival (PFS) of over 9 months. Representative drugs like gefitinib and erlotinib have demonstrated enhanced outcomes in comparison to chemotherapy, as demonstrated in various prospective randomized clinical trials [6–9]. In 2022, Chen et al. [9]. carried out a meta-analysis to compare first-line treatment strategies for NSCLC patients. Their findings indicated that second-generation EGFR-TKIs and third-generation EGFR-TKIs outperformed first-generation EGFR-TKIs in terms of PFS. Despite this significant progress, resistance to EGFR-TKIs still occurs in patients [10]. As a second-line therapy, acquired resistance mechanisms to osimertinib are mainly *EGFR*-dependent, while numerous unidentified *EGFR*-independent mechanisms are yet to be unveiled [11,12].

Indeed, there are notable differences between Asians and non-Asians when it comes to driver gene mutations in various types of cancer, especially in lung cancer [13–19]. Lung adenocarcinoma (LUAD) patients from eastern China exhibit a unique profile of mutations [20]. For instance, driver mutations like *EGFR* mutations are more prevalent among Asian populations compared to non-Asians. These differences in driver gene mutations can impact treatment strategies and outcomes, as well as contribute to the variations in response to targeted therapies. The use of a targeted DNA panel has proven invaluable in guiding personalized treatment decisions for NSCLC patients. Over the past decade, next-generation sequencing (NGS) testing has seen a growing role in clinical diagnosis and therapeutic interventions [21–25]. Gaining insight into the molecular alteration characteristics of NSCLC is instrumental in selecting personalized molecular targeted therapy or immunotherapy for patients.

Through a long-term single-center follow-up cohort, we emphasize the importance of understanding the tumor evolution and illustrate the practicality of utilizing DNA sequencing results to guide clinical treatment. We also emphasize the influence of genetic factors on the efficacy and outcomes of treatments at each line.

2. Methods and materials

2.1. Patient recruitment and sample collection

This study was conducted at Shanghai Chest Hospital from July 2013 to February 2019, recruiting a total of 65 patients diagnosed with NSCLC. Throughout the entire treatment course, these patients were diligently followed up, with the endpoint set for August 2023. None of these patients underwent surgical treatment, and the pathological diagnosis of their biopsy tissues was examined by two pathologists following the Union for International Cancer Control (UICC) classification of tumor node metastasis (TNM) for disease staging [26]. Patients with other malignant tumors were excluded. The collected clinical information includes age, sex, and smoking status. This study was approved by the Ethics Committee of Shanghai Chest Hospital (KS [Y] 19101). Informed consent was obtained from all participants.

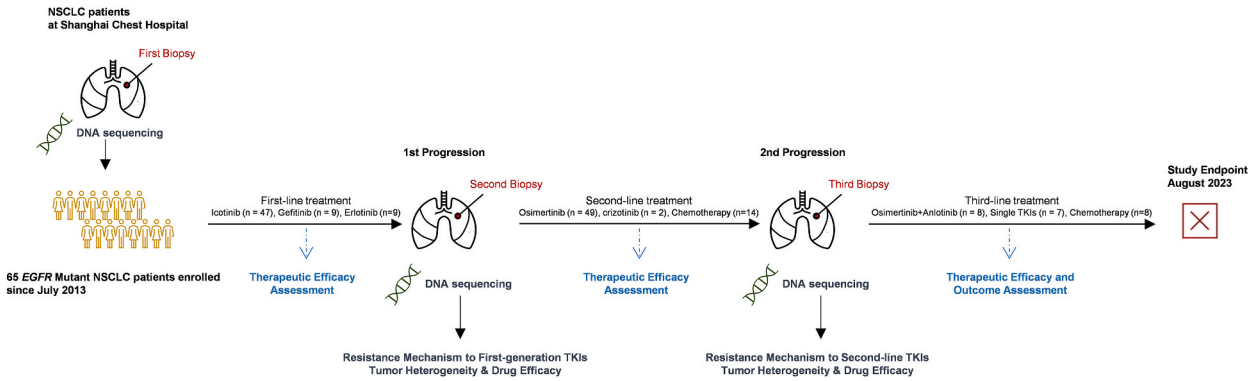

Fig. 1. Study design. A total of 65 patients with *EGFR* mutant NSCLC were enrolled. All patients received first-generation EGFR-TKIs. Upon developing resistance to first-generation TKIs, patients were treated with second-line treatments. Molecular changes in each biopsy were detected using a customized DNA panel. We studied the impact of tumor clonal evolution on the efficacy and clinical outcomes of first-line, second-line, and third-line treatments.

2.2. NGS-based genetic alterations detection and analysis

Lung Cancer Detection Panel (Singlera Genomics (Shanghai) Ltd., China) was performed to detect genetic alteration in first biopsy tissues and OncoAim® Panoramic Detection Panel (Singlera Genomics (Shanghai) Ltd., China) were used to detect gene mutations in second and third biopsy tissues. Study flow diagram was presented in Fig. 1. The genes covered in these two panels were exhibited in Supplementary Tables 1 and 2 DNA extraction was performed using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The quality of DNA was assessed through 1% agarose gel electrophoresis, and DNA concentration was determined using the Qubit dsDNA HS Assay kit along with the Qubit 3.0 fluorimeter (Life Technologies, Eugene, Oregon, USA) (Supplementary Fig. 1 and Supplementary Table 3). Library construction was carried out according to the Illumina standard library construction instructions (Illumina, Inc., California, USA) using 20 ng of DNA prepared with the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, USA). Subsequently, library products underwent 75 bp paired-end sequencing on the Illumina MiSeq platform. Raw data was aligned to the University of California at Santa Cruz (UCSC) human reference genome (GRCh37/hg19). Somatic alterations were defined as variations identified in tumor tissues but not in matched blood samples. Genomic variants were identified using the SnpEff tool [27].

2.3. Response to targeted therapy and outcome analysis

The clinical response to treatment was evaluated based on the Response Evaluation Criteria in Solid Tumors Version 1.1 (RECIST 1.1) [28]. Evaluations were performed every 3 months, starting one month after the initiation of treatment. PFS was defined as the duration from the start of treatment to disease progression or death and overall survival (OS) as the time from the start of first-line treatment to death. For individuals who did not experience disease relapse or death by the cutoff date, their information was censored at their last follow-up.

2.4. Statistical analysis

Mutational profiling was conducted using the MAF Visualization tools (maftools) in R (version 4.1.0). All statistical analyses were performed using SPSS (version 29.0). Categorical variables were analyzed using the Chi-Squared Test, while continuous variables were assessed using the Mann-Whitney *U* test. PFS and OS were analyzed using Kaplan-Meier survival analysis, and differences in survival were compared using the log-rank test. Statistical significance was considered when the *P*-value was less than 0.05.

Table 1
The clinical characteristics of 65 NSCLC patients.

Characteristics	Cohort (n = 65)
Age (year)	
Median (range)	60 (41–80)
Sex, n (%)	
Male	33 (50.8)
Female	32 (49.2)
Smoking status, n (%)	
Never	33 (50.8)
Ever or current	32 (49.2)
Histology, n (%)	
Adenocarcinoma	61 (93.8)
Squamous cell carcinoma	2 (3.1)
Adenosquamous carcinoma	2 (3.1)
TNM stage, n (%)	
I	0 (0)
II	0 (0)
III	7 (10.8)
IV	58 (89.2)
First-line treatment, n (%)	
Icotinib	47 (72.3)
Gefitinib	9 (13.8)
Erlotinib	9 (13.8)
Second-line treatment, n (%)	
Osimertinib	49 (75.4)
Crizotinib	2 (3.1)
Chemotherapy	14 (21.5)

NSCLC, Non-small cell lung cancer. TNM, Tumor Node Metastasis classification.

3. Results

3.1. EGFR mutations determine the clinical efficacy of first-generation EGFR-TKIs

Among the 65 treatment-naive patients diagnosed with NSCLC, there was an equal distribution of males and females and the majority of them exhibited adenocarcinoma histology (61/65) (Table 1). A total of 39 patients carried the *EGFR* 19Del mutations and 26 patients carried L858R mutations, and their baseline clinical characteristics were similar (Supplementary Table 4). The median PFS (mPFS) for first-generation EGFR-TKIs in 19Del cohort was 14.6 months, compared to 15.4 months in L858R cohort (HR: 0.68, 95% CI: 0.406–1.15, $P = 0.150$; Supplementary Fig. 2A). The median OS (mOS) of patients between the two groups were identical (mOS: 43.0 months vs. 39.0 months, HR: 0.95, 95% CI: 0.528–1.72, $P = 0.870$; Supplementary Fig. 2B). These patients received icotinib (n = 47, 72.3%), gefitinib (n = 9, 13.8%), and erlotinib (n = 9, 13.8%), respectively. The mPFS for gefitinib was 22.2 months, which was longer than icotinib and erlotinib cohorts, with mPFS of 15.6 months and 13.0 months, respectively, although no significant difference was observed (Fig. 2A). Patients with L858R mutations who received gefitinib had longer mPFS than those who received icotinib or elotinib (27.5 months vs. 15.6 months or 13.0 months, $P > 0.050$). Gefitinib showed a trend of longer mPFS in L858R cohort than in 19Del cohort (27.5 months vs. 10.3 months, $P = 0.056$). However, there was no such trend in icotinib or erlotinib treatment (15.6 months vs. 15.3 months, $P = 0.538$ or 13.0 months vs. 13.7 months, $P = 0.170$; Fig. 2B).

3.2. Resistant mutations in response to first-generation EGFR-TKIs vary across different EGFR mutations and different first-generation TKIs

Following the administration of first-generation EGFR-TKIs, patients who experienced disease progression underwent re-biopsy and NGS testing. The main resistance mechanism observed in 76.9% (50/65) of patients was conferred by *EGFR* T790 M. Additionally, 9.2% (6/65) of patients developed alternative pathway activation, including 6.2% (4/65) with *PIK3CA* mutations, two of whom also exhibited T790 M and small cell lung cancer (SCLC) transformation, respectively. Erb-b2 receptor tyrosine kinase 2 (*HER2*) amplifications were detected in two patients, with one patient also having T790 M. One case exhibited MET proto-oncogene, receptor tyrosine kinase (*MET*) amplification, accompanied by T790 M. Another case had SCLC transformation, and a third case showed a *BRAF* mutation. Furthermore, 13.8% (9/65) of patients had uncommon mutations (Fig. 3A).

We found substantial heterogenous resistance mechanisms between the tumors with *EGFR* 19Del mutations and tumors with *EGFR* L858R mutations. In comparison, *HER2* amplifications (5.1%, 2/39), *MET* amplification (2.6%, 1/39), and *BRAF* mutation (2.6%, 1/39) were observed exclusively in 19Del group (Fig. 3B). SCLC transformations 7.7% (2/26) were observed in L858R cohort (Fig. 3C). T790 M was more common in 19Del cohort compared to L858R cohort (84.6% vs. 65.4%, $P = 0.070$; Fig. 3D). Interestingly, apart from

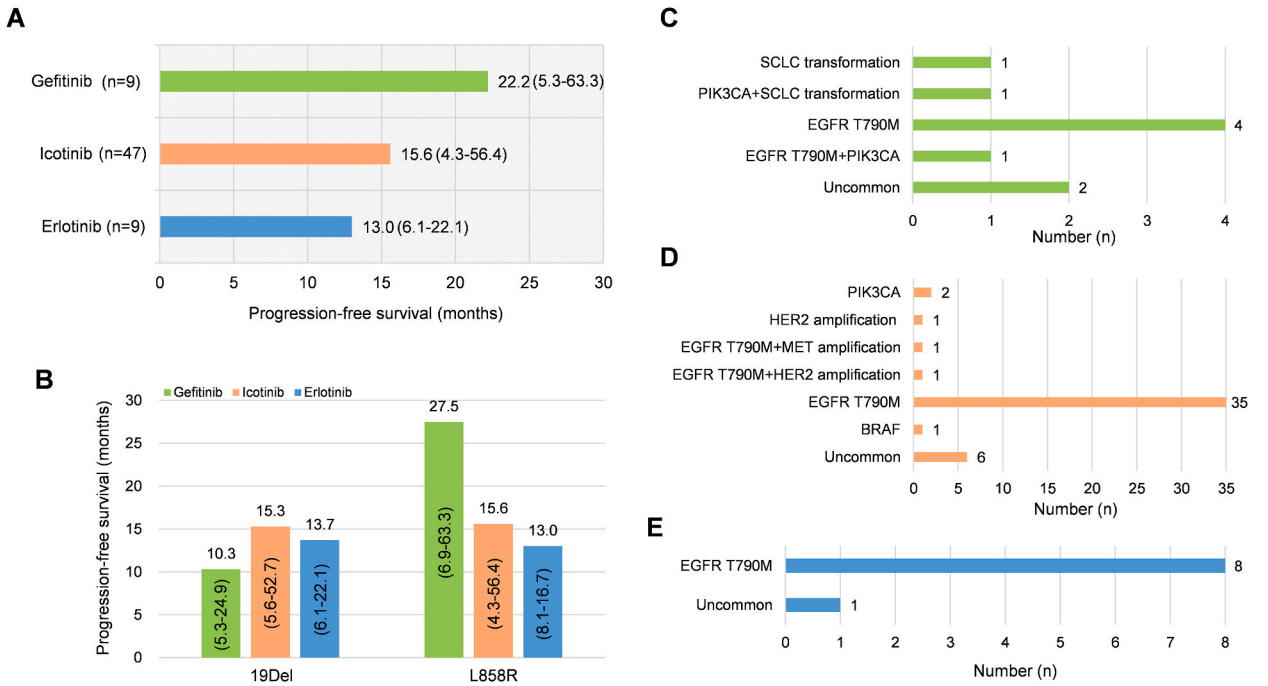

Fig. 2. Comparison of efficacy among three first-generation EGFR-TKIs. (A) The mPFS for gefitinib, icotinib, and erlotinib. (B) Comparison of efficacy among three first-generation EGFR-TKIs in patients with *EGFR* 19Del and *EGFR* L858R mutations. (C) The prevalence of common resistance mutations in patients treated with gefitinib. (D) Common resistance mutations in patients treated with icotinib. (E) Common resistance mutations in patients treated with erlotinib. The ranges of PFS for these three EGFR-TKIs are displayed in the figure. mPFS, median PFS.

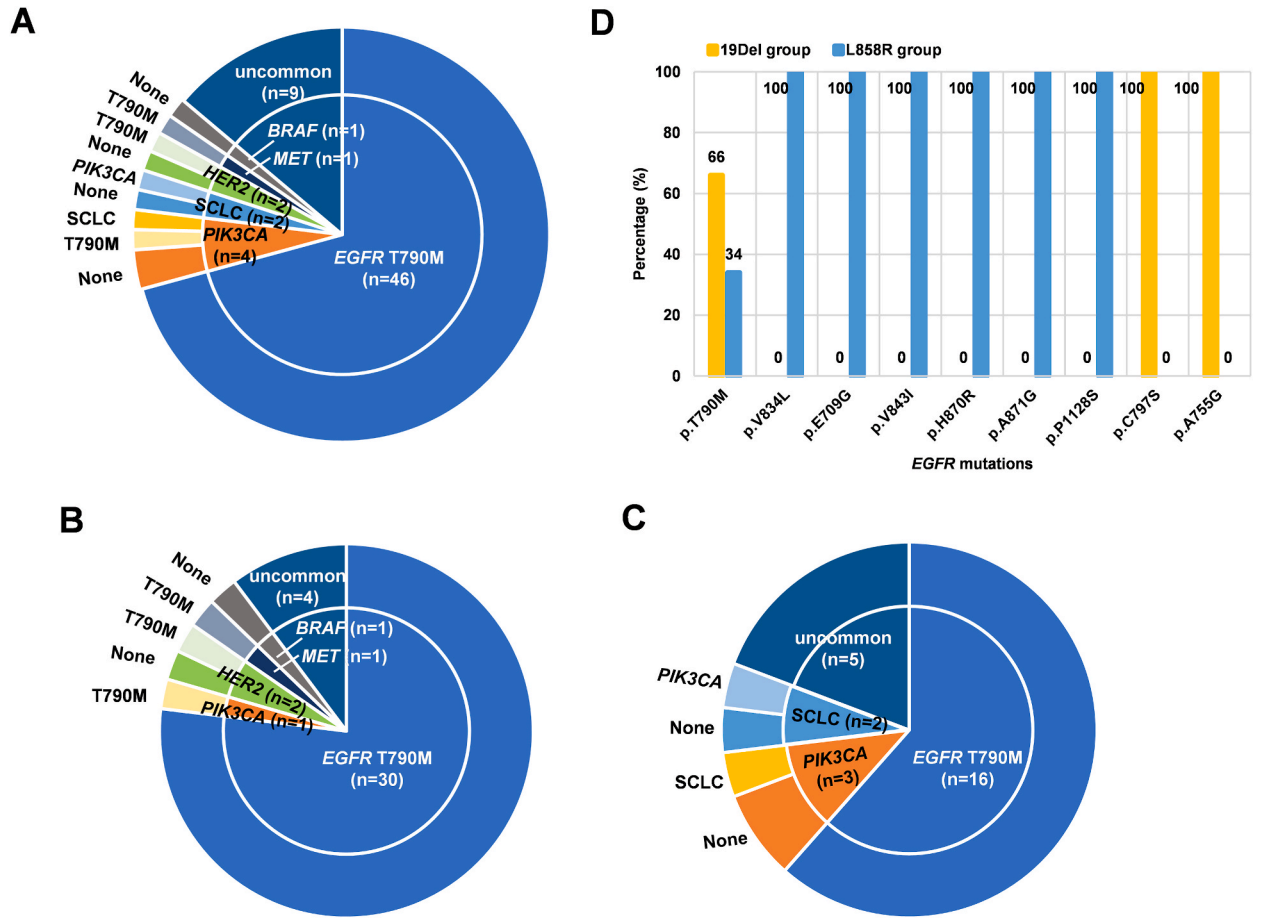

Fig. 3. Resistance mechanisms to first-generation EGFR-TKIs. (A) Distribution of common resistance mutations to first-generation EGFR-TKIs. (B) Distribution of common resistance mutations in *EGFR* 19Del cohort after developing resistance to first-generation EGFR-TKIs. (C) Distribution of common resistance mutations in *EGFR* L858R cohort after developing resistance to first-generation EGFR-TKIs. (D) Analysis of *EGFR* mutations in *EGFR* 19Del and *EGFR* L858R cohorts after developing resistance to first-generation EGFR-TKIs.

detecting *EGFR* C797S and A755G in 19Del cohort, the main "other" *EGFR* mutations were found in L858R cohort (Fig. 3D). Most of them have been reported as drug resistance or poor prognostic biomarkers in lung cancer (Table 2).

Next, we compared the tumor resistance mechanisms among subjects treated with different first-generation EGFR-TKIs. Histologic transformation was identified in patients treated with gefitinib, while *HER2* amplification, *MET* amplification, and *BRAF* mutation occurred in patients treated with icotinib. The common mutation detected in patients receiving erlotinib was only T790 M (Fig. 2C–E). The mPFS of patients developing T790 M mutations was 15.7 months. Patients harboring *HER2* amplification or SCLC transformation seemed to require a longer time to develop TKI resistance, while those with *PIK3CA* mutations developed resistance rapidly (Fig. 4).

Table 2
Distribution of *EGFR* mutations after resistance to first-generation EGFR-TKIs.

Mutations	Mutation rate, % (n/n)	Co-mutations	Reference
p.T790 M	76.9% (50/65)		[46]
p.C797S	3.1% (2/65)	p.T790 M	[47]
p.V834L	3.1% (2/65)	p.T790 M/None	[48]
p.E709G	1.5% (1/65)	p.T790 M	[49]
p.A755G	1.5% (1/65)	HER2 amplification	/
p.V843I	1.5% (1/65)	SCLC transformation	[50]
p.H870R	1.5% (1/65)	p.T790 M	[51]
p.A871G	1.5% (1/65)	p.T790 M	[52]
p.P1128S	1.5% (1/65)	PIK3CA	/
Total	95.4% (62/65)		

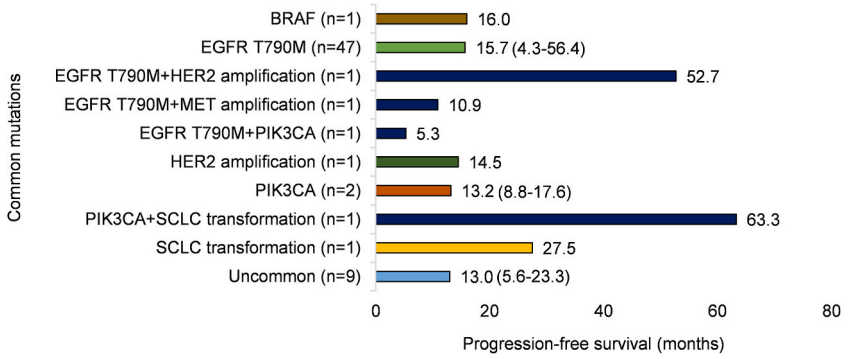

Fig. 4. Common resistance mutations and mPFS in first-generation EGFR-TKIs therapy. The ranges of PFS for these patients harboring common resistance mutations are displayed in the figure. mPFS, median PFS.

3.3. Mutation profiling predicts the efficacy of second-line EGFR-TKI therapy

Patients who received EGFR-TKIs as second-line treatment seemed to have a relatively better prognosis than those who received chemotherapy (mOS: 28.0 months vs. 16.1 months, HR: 0.54, 95% CI: 0.271–1.07, $P = 0.074$; Supplementary Fig. 3). In the comparison of mutation profiles between the group with better PFS (mPFS >12 months) and the group with poorer PFS (mPFS ≤12 months), certain genes exhibited higher mutation frequencies in the latter group (Fig. 5A and B). These genes included tumor protein p53 (*TP53*), DNA methyltransferase 1 (*DNMT1*), glutamate ionotropic receptor NMDA type subunit 2A (*GRIN2A*), hepatocyte growth factor (*HGF*), axin 1 (*AXIN1*), cyclin dependent kinase 6 (*CDK6*), EPH receptor A3 (*EPHA3*), fibroblast growth factor receptor 3 (*FGFR3*), GNAS complex locus (*GNAS*), IKAROS family zinc finger 1 (*IKZF1*), p21 (RAC1) activated kinase 5 (*PAK5*), DNA polymerase epsilon, catalytic subunit (*POLE*), RAD21 cohesin complex component (*RAD21*), RB transcriptional corepressor 1 (*RB1*) and RNA binding motif protein 10 (*RBM10*) as indicated in Fig. 5C. Moreover, the Kaplan–Meier survival analysis demonstrated that patients with mutations in *EPHA3*, *IKZF1*, *PAK5*, *POLE*, *RAD21* and *RBM10* experienced significantly worse PFS compared to those without

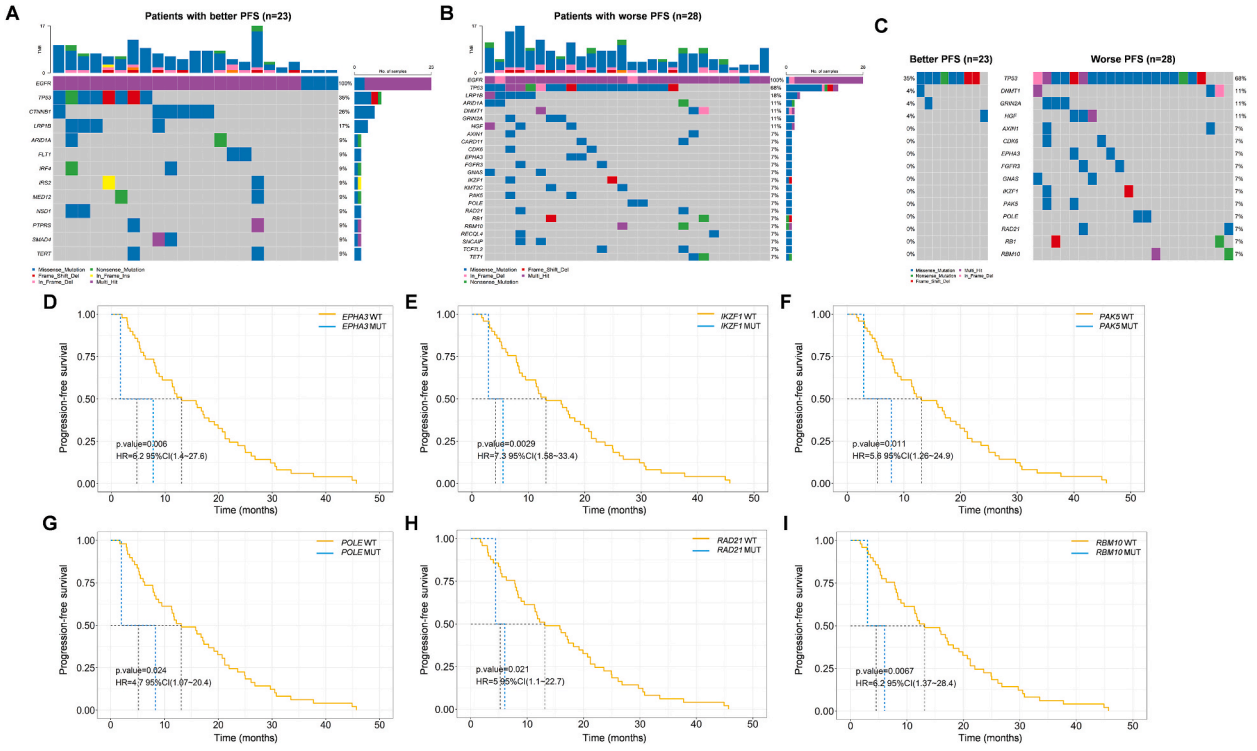

Fig. 5. Correlation analysis between gene mutations and PFS in response to second-line targeted therapies. (A) Mutation analysis in the group with better PFS. (B) Mutation analysis in the group with worse PFS. (C) Display of gene mutation frequency differences between the two groups with better and worse PFS. Kaplan–Meier survival analysis of *EPHA3* (D), *IKZF1* (E), *PAK5* (F), *POLE* (G), *RAD21* (H) and *RBM10* (I) mutations. A log-rank test was used to determine the difference between the groups. *$P < 0.05$. WT, wild type. MUT, mutation.

such mutations (HR: 6.2, 95% CI: 1.40–27.6, $P = 0.006$; 7.3, 1.58–33.4, $P = 0.003$; 5.6, 1.26–24.9, $P = 0.011$; 4.7, 1.07–20.4, $P = 0.024$; 5, 1.1–22.7, $P = 0.021$; 6.2, 1.37–28.4, $P = 0.007$; Fig. 5D–I). Multi-variable logistic regression analysis provided confirmation that *EPHA3*, *IKZF1* and *RBM10* served as independent predictors for poorer PFS in second-line EGFR-TKIs therapy (Supplementary Fig. 4). However, we found that patients with single T790 M mutations had worse PFS on osimertinib compared to those with T790 M combined with other *EGFR* mutations (11.7 months vs. 25.5 months, $P = 0.022$; Fig. 6A). Specifically, it appears that patients with T790 M combined with A871G, H870R, V834L, and C797S mutations tend to have better PFS (Fig. 6B).

3.4. Non-EGFR-dependent osimertinib resistance mechanisms are more predominant

In post-osimertinib resistance biopsy, we observed that five patients carried secondary *EGFR* mutations at residue C797. Four cases harbored *EGFR* amplification, and one patient with an *EGFR* L718 mutation did not have a coexisting C797 mutation. One patient had cyclin D1 (*CCND1*) amplification, one had *MET* amplification, and another had *HER2* amplification. Additionally, one patient presented with *PIK3CA* gene amplification together with *PIK3CA* mutation (Fig. 7A). In patients without *EGFR*-dependent mutations, other genetic alterations may be associated with resistance to osimertinib. Among these, *TP53* gene had the highest mutation frequency, followed by *CTNNB1* and *NF1*. However, these three genes were also present in the pre-osimertinib treatment biopsy of the patients. *ETV1* and *H2BC5* mutations were not detected in the pre-osimertinib treatment population, suggesting the possibility of new resistance mechanisms (Fig. 7B).

3.5. Clinical efficacy of combining osimertinib with anlotinib as a third-line treatment

The follow-up information for patients who received third-line treatments was shown in Table 3. The mOS of patients treated with the combination of osimertinib and anlotinib was 13.2 months, which was longer compared to patients who received anlotinib alone (13.2 months vs. 4.8 months, $P = 0.044$), other TKIs (13.2 months vs. 5.5 months, $P = 0.048$) or chemotherapy (13.2 months vs. 4.7 months, $P = 0.001$) (Fig. 8A). The mPFS of patients treated with the combination of osimertinib and anlotinib was longer than that of single TKI-treated patients (3 patients, 2 treated with anlotinib, and 1 with afatinib) (5.5 months vs. 2.0 months, $P = 0.125$; Fig. 8B).

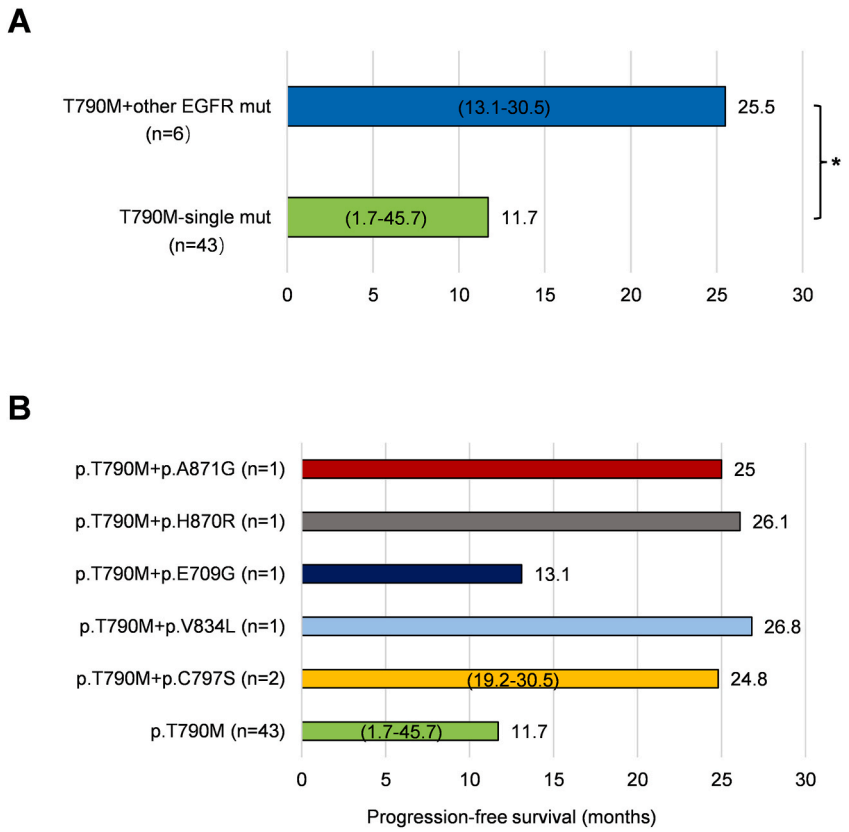

Fig. 6. Correlation analysis between *EGFR* mutations and PFS of osimertinib. (A) Influence of T790 M as a single mutation and T790 M combined with other *EGFR* mutations on PFS. (B) Presentation of mPFS in cases with T790 M combined with other *EGFR* mutations. The ranges of PFS are displayed in the figure. mPFS, median PFS. *$P < 0.05$.

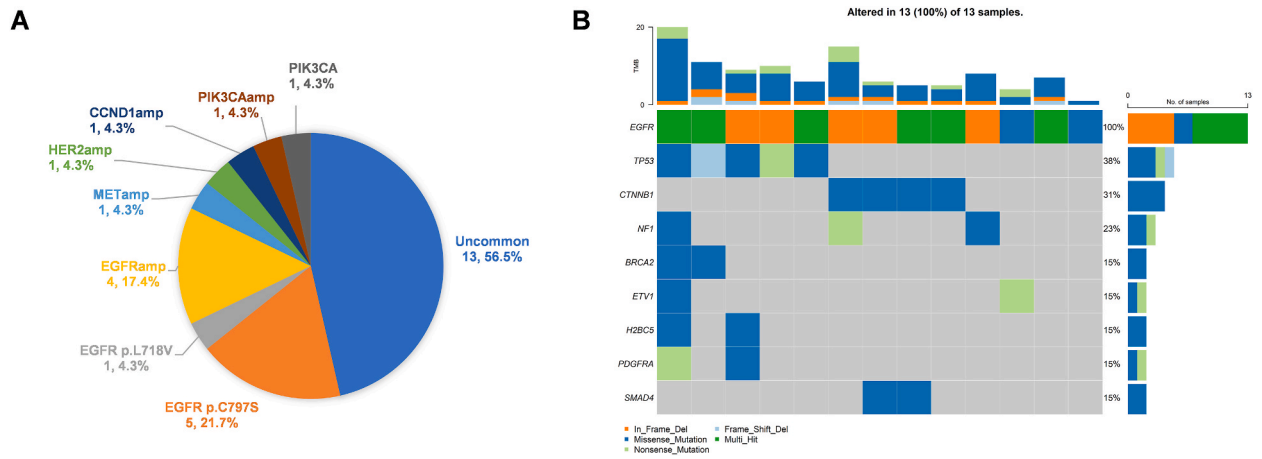

Fig. 7. Resistance mechanisms to osimertinib. (A) Common resistance mutations to osimertinib. (B) Mutational profiles in tissues obtained after resistance to osimertinib in patients without common resistance mutations.

3.6. GRIN2A is an independent predictor of shorter OS in third-line treatment

When comparing the mutation spectrum between the group with longer OS (>5.5 months) and the group with shorter OS (≤5.5 months) among patients who received third-line treatments (Fig. 9A), *GRIN2A* and *PIK3CA* mutations were more prevalent in patients with shorter OS (Fig. 9B). Kaplan–Meier survival analysis revealed that only *GRIN2A* mutations had significantly shorter OS compared to those without mutations (HR: 5.6, 95% CI: 1.31–23.8, $P = 0.009$; Fig. 9C and D). Multivariable logistic regression confirmed that *GRIN2A* mutation was an independent indicator of poorer OS in patients receiving third-line treatments after resistance to osimertinib (Supplementary Fig. 5).

4. Discussion

Tumor clonal changes can predict the efficacy of EGFR-TKIs and whether patients will develop drug resistance [29–31]. These genetic clonal changes may include *EGFR* mutations, mutations in other signaling pathway genes, alterations in cell cycle regulatory genes, and so on. These changes can influence the therapeutic effects of the EGFR-TKIs, making them ineffective against tumor cells, ultimately leading to drug resistance. Therefore, understanding a tumor clonal change is crucial for selecting appropriate treatment and monitoring treatment effectiveness. This personalized approach to medicine helps improve treatment outcomes and reduce unnecessary drug side effects.

Among first-generation EGFR-TKIs, gefitinib demonstrated superior efficacy compared to icotinib and erlotinib. Particularly among patients with the *EGFR* L858R mutations, gefitinib exhibited a longer PFS than the other two TKIs, although the difference was not statistically significant, aligning with previous research findings [32]. Gefitinib showed a trend of longer mPFS in *EGFR* L858R cohort than in 19Del cohort. A significant proportion of advanced NSCLC patients with *EGFR* mutations initially respond well to first-generation TKIs. However, the majority of these patients eventually develop resistance, due to various alterations such as *EGFR* T790 M mutation, *EGFR* amplification, *MET* and *HER2* amplification, *PIK3CA* mutation and SCLC transformation [33,34]. Acquired resistance is the primary challenge that hinders the clinical effectiveness of EGFR-TKIs. In this study, we observed differences in the resistance mechanisms among different *EGFR* mutation groups. The *EGFR* 19Del cohort exhibited a more diverse range of resistance mechanisms, with copy number alterations (CNAs) being more frequently observed in this mutation group. Conversely, SCLC transformation was predominantly observed in L858R cohort. The occurrence rate of the *EGFR* T790 M was higher in 19Del cohort compared to L858 cohort, which was consistent with previous reports [35,36]. However, other *EGFR* mutations were more prevalent in L858R group. Additionally, different first-generations of TKIs exhibited slightly distinct resistance mechanisms. CNAs were observed in patients treated with icotinib, and SCLC transformation was seen in patients treated with gefitinib, while erlotinib mainly encountered T790 M resistance form. This reminds us that for different treatment approaches, we should pay attention to the potential emergence of different resistance mechanisms and select appropriate detection methods.

Compared to patients receiving chemotherapy, those who continued to receive EGFR-TKIs treatment after first-generation TKIs resistance exhibited longer OS. Within the EGFR-TKIs group, patients with T790 M mutation who received osimertinib demonstrated a better prognosis. To comprehensively explore the predictive impact of gene alterations on the effectiveness and prognosis of second-line EGFR-TKIs, we conducted investigations into mutation patterns among patients with both shorter and longer PFS. The findings demonstrated that mutations in *EPHA3*, *IKZF1* and *RBM10* were markedly linked to poorer PFS. The identification of *EPHA3*, *IKZF1* and *RBM10* as predictive biomarkers for the efficacy of second-line EGFR-TKI treatment is a novel proposition. Further mechanistic studies are needed to confirm their impact in EGFR-TKI drug therapy. Osimertinib has been approved for treating NSCLC patients who have experienced disease progression after EGFR-TKIs therapy and carry the T790 M resistance mutation. Various resistance

Table 3
Third-line treatment and survival after osimertinib resistance.

Sample No.	Age at diagnosis	Sex	Histology	*EGFR* mutations in the first biopsy	First-line treatment	Second-line treatment	Third-line treatment	Death	Progression-free survival following third-line treatment (months)	Overall survival following third-line treatment (months)
shxk-1	53	Male	Adenocarcinoma	*EGFR* 19Del	Icotinib	Osimertinib	Osimertinib + Anlotinib	No	10	30.5
shxk-2	58	Male	Adenocarcinoma	*EGFR* 19Del	Icotinib	Osimertinib	Chemotherapy	Yes	/	3.5
shxk-3	47	Female	Adenocarcinoma	*EGFR* L858R	Gefitinib	Osimertinib	Osimertinib + Anlotinib	No	6	23.5
shxk-4	67	Female	Adenocarcinoma	*EGFR* 19Del	Icotinib	Osimertinib	Chemotherapy	Yes	/	4.5
shxk-5	55	Male	Adenocarcinoma	*EGFR* L858R	Icotinib	Osimertinib	Chemotherapy	Yes	/	5.0
shxk-6	56	Female	Adenocarcinoma	*EGFR* 19Del	Icotinib	Osimertinib	Osimertinib + Anlotinib	Yes	/	9.5
shxk-7	43	Male	Adenocarcinoma	*EGFR* 19Del	Erlotinib	Osimertinib	Osimertinib	Yes	/	5.5
shxk-8	74	Male	Adenocarcinoma	*EGFR* 19Del	Icotinib	Osimertinib	Anlotinib	Yes	1	4.5
shxk-9	54	Female	Adenocarcinoma	*EGFR* 19Del	Gefitinib	Osimertinib	Almonertinib	Yes	/	3.5
shxk-10	59	Female	Squamous cell carcinoma	*EGFR* 19Del	Icotinib	Osimertinib	Osimertinib	Yes	/	12.1
shxk-11	51	Male	Adenocarcinoma	*EGFR* 19Del	Icotinib	Osimertinib	Chemotherapy	Yes	/	11.5
shxk-12	54	Male	Adenocarcinoma	*EGFR* 19Del	Icotinib	Osimertinib	Osimertinib + Anlotinib	Yes	5.5	12.5
shxk-13	60	Male	Adenocarcinoma	*EGFR* 19Del	Gefitinib	Osimertinib	Anlotinib	Yes	3.8	5.1
shxk-14	67	Female	Adenocarcinoma	*EGFR* L858R	Icotinib	Osimertinib	Osimertinib + Anlotinib	Yes	/	5.5
shxk-15	64	Female	Adenocarcinoma	*EGFR* 19Del	Icotinib	Osimertinib	Osimertinib + Anlotinib	Yes	/	13.4
shxk-16	72	Male	Adenocarcinoma	*EGFR* 19Del	Icotinib	Osimertinib	Chemotherapy	Yes	/	4.2
shxk-17	63	Female	Adenocarcinoma	*EGFR* 19Del	Erlotinib	Osimertinib	Chemotherapy	No	/	8.5
shxk-22	58	Male	Adenocarcinoma	*EGFR* 19Del	Gefitinib	Osimertinib	Chemotherapy	Yes	/	3.1
shxk-25	68	Male	Adenocarcinoma	*EGFR* L858R	Icotinib	Osimertinib	Chemotherapy	Yes	/	5.3
shxk-45	70	Male	Adenocarcinoma	*EGFR* L858R	Gefitinib	Osimertinib	Afatinib	Yes	2	13.1
shxk-48	60	Male	Adenocarcinoma	*EGFR* 19Del	Icotinib	Osimertinib	Osimertinib + Anlotinib	Yes	3	30.0
shxk-58	68	Female	Adenocarcinoma	*EGFR* 19Del	Icotinib	Osimertinib	Osimertinib + Anlotinib	Yes	3	12.9
shxk-59	46	Male	Adenocarcinoma	*EGFR* 19Del	Erlotinib	Osimertinib	Crizotinib	Yes	/	4.9

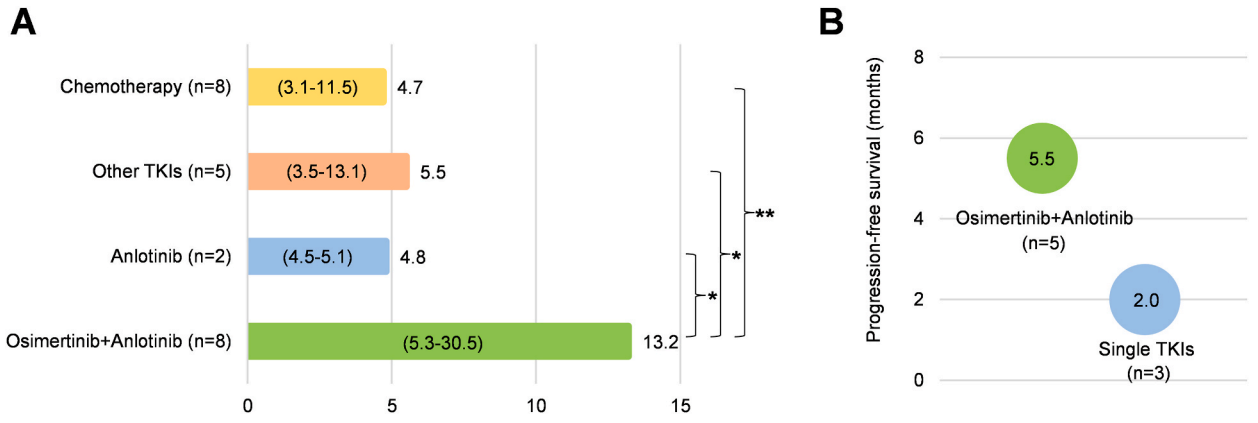

Fig. 8. Efficacy evaluation of third-line treatment regimens. Comparison of OS (A) and PFS (B) among different third-line treatment regimens after osimertinib resistance. *$P < 0.05$. **$P < 0.01$.

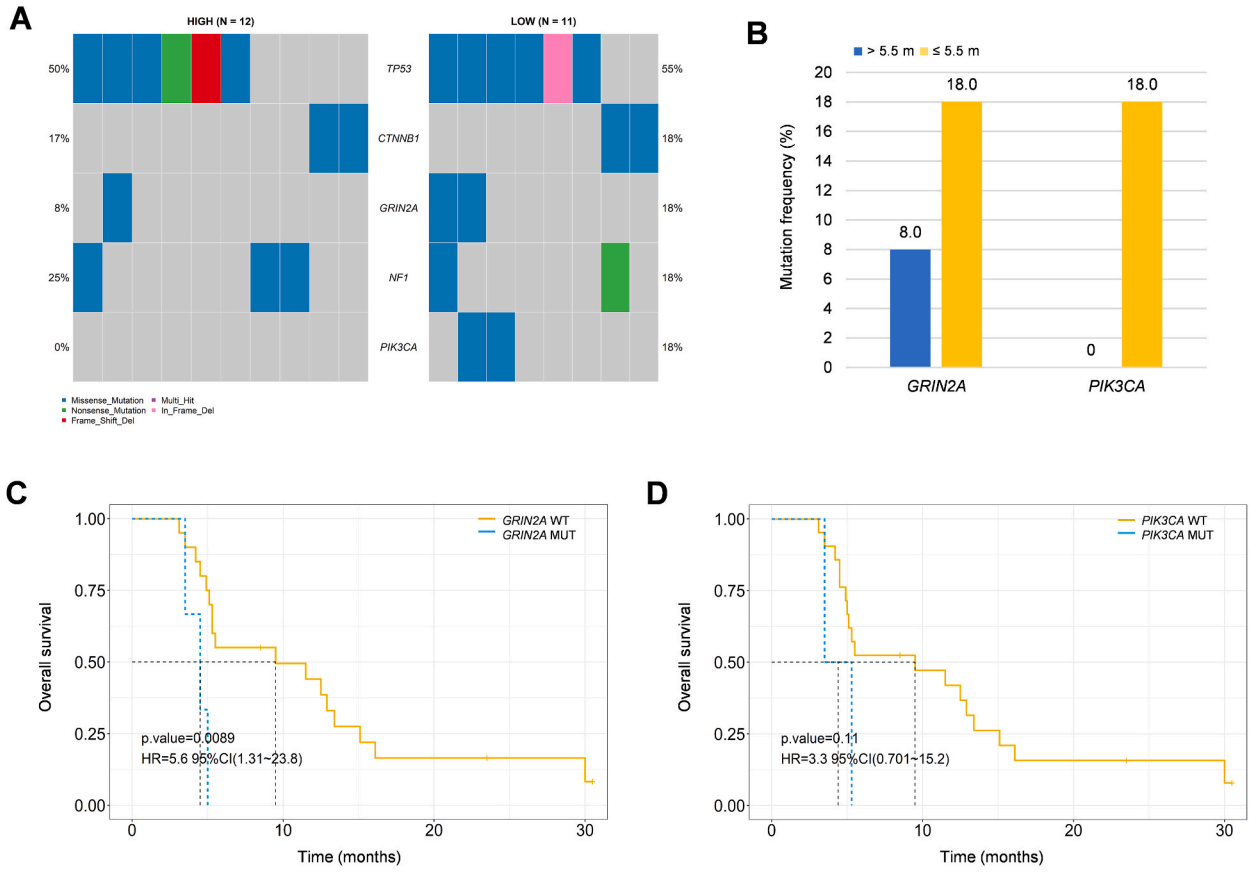

Fig. 9. Correlation analysis between gene mutations and OS in response to third-line treatment regimens. (A) Mutation analysis in the group with better OS and the group with shorter OS. (B) Genes with significant differences in mutation frequencies between the group with longer OS and the group with shorter OS. Kaplan–Meier survival analysis of *GRIN2A* (C) and *PIK3CA* (D) mutations. A log-rank test was used to determine the difference between the groups. *$P < 0.05$. **$P < 0.01$.

mechanisms to osimertinib, including *EGFR* mutations, alternative pathway activation, or histological transformations have been widely studied [37–40]. However, the literature has not yet extensively documented the prognostic significance of uncommon resistance mechanisms. Our study has found that non-*EGFR*-dependent acquired resistance mutations are the primary resistance mechanism to osimertinib, among which *ETV1* has been reported as a novel resistance gene [41] and further research on the relationship between *H2BC5* and clinical resistance to osimertinib requires a significant number of samples for validation.

In third-line treatments, the combination of osimetinib with anlotinib initially demonstrated encouraging clinical results, consistent with previous studies [42,43]. The mOS was 13.2 months, which was significantly higher than the survival observed in patients who received anlotinib alone, other TKIs therapy or chemotherapy after resistance to osimertinib. *GRIN2A* has been reported as a resistant gene to osimertinib [44]. *GRIN2A* mutations were associated with high tumor mutation burden (TMB) in patients with NSCLC [45]. However, fewer research reports have associated *GRIN2A* with poor prognosis in NSCLC. The current study identified that *GRIN2A* mutation acted as an independent prognostic biomarker for shorter OS in third-line treatment which underscores its importance in predicting patient outcomes following the development of resistance to osimertinib.

The limitation of this study lies in the small sample size of enrolled patients. In the future, we aim to collect a larger number of samples to further clarify the potential applications of NGS detection in assessing the efficacy and predicting the prognosis of targeted therapy. Moreover, novel mutated genes identified in the panel have shown close associations with the efficacy and prognosis of targeted therapy. Further research is warranted to delve into the mechanisms of these mutations contribute to variations in the efficacy of targeted therapy in NSCLC.

5. Conclusion

This study recognizes the dynamic tumor evolution in activating *EGFR*-mutant NSCLC, which can profoundly influence the efficacy and prognosis of targeted therapy. The efficacy and resistance mechanisms to first-generation EGFR-TKIs are intricately associated with *EGFR* mutations. In second-line EGFR-TKIs treatment, *EPHA3*, *IKZF1* and *RBM10* are independent predictors of PFS. For third-line treatments, *GRIN2A* mutation emerges as an independent risk indicator of OS. Our findings contribute to a more personalized approach in lung cancer treatment, ultimately leading to improved patient outcomes.

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Data availability statement

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics statement

This study was approved by the Ethics Committee of Shanghai Chest Hospital (KS [Y] 19101). All individuals signed informed consent.

CRediT authorship contribution statement

Hao Bai: Writing – original draft, Project administration, Methodology, Conceptualization. **Yan Zhou:** Project administration, Investigation, Data curation. **Wanting Liu:** Project administration, Formal analysis, Data curation. **Wang-yang Xu:** Writing – original draft, Supervision, Project administration, Methodology, Data curation. **Lei Cheng:** Supervision, Software, Methodology. **Yingying Huo:** Visualization, Validation, Methodology. **Hao Ji:** Writing – review & editing, Project administration, Investigation, Conceptualization. **Liwen Xiong:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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