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Original Article

Genetic alterations predict poor efficacy, outcomes and resistance to second-line osimertinib treatment in non-small cell lung cancer

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Abstract: The genetic heterogeneity of non-small cell lung cancer (NSCLC) may impact clinical response and outcomes to targeted therapies. In second-line osimertinib treatment for NSCLC, real-world data on genetic biomarkers for treatment efficacy and prognosis remain incomplete. This real-world study involved 68 NSCLC patients receiving first-generation epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs). All of these patients developed resistance, and 49 of them subsequently underwent second-line osimertinib treatment. A 639-gene DNA panel was employed to assess the impact of molecular alterations on treatment efficacy, clinical outcomes and resistance. The findings showed that the median progression-free survival (PFS) for second-line osimertinib therapy was 13.3 months. Genes alterations such as P21 (RAC1) activated kinase 5 (*PAK5*), RNA binding motif protein 10 (*RBM10*), and EPH receptor A3 (*EPHA3*) mutations were associated with significantly shorter PFS in osimertinib therapy. At multivariate analysis, they were all independent risk predictors of shorter PFS. Additionally, the median overall survival (OS) for osimertinib was 26.2 months. Glutamate ionotropic receptor NMDA type subunit 2A (*GRIN2A*), hepatocyte growth factor (*HGF*), and *RBM10* mutations were significantly associated with poorer OS in osimertinib treatment. The multivariate analysis demonstrated that only *RBM10* mutation emerged as an independent risk predictor of shorter OS. *In vitro* experiments showed that *RBM10* mutations could promote the proliferation and migration ability of NSCLC cells and reduced cell apoptosis. The resistance mechanisms to osimertinib were heterogeneous. Histone cluster 1 H2B family member D (*HIST1H2BD*) acted as a novel resistance mechanism to osimertinib. Previously unreported *HIST1H2BD* mutations (p.K25Q and p.E36D) were detected in the NSCLC tissues. *In vitro* experiments confirmed that *HIST1H2BD* mutations led to resistance to osimertinib. In summary, we demonstrate that genetic biomarkers, such as *PAK5*, *RBM10*, and *EPHA3*, are independent predictors of PFS in second-line osimertinib treatment, with *RBM10* emerging as an independent predictor of OS. Additionally, *HIST1H2BD* represents a novel resistance mutation to osimertinib. All of these findings offer valuable insights for making personalized treatment strategies for NSCLC patients.

Keywords: Clinical outcome, efficacy, genetic alteration, non-small cell lung cancer, resistance

Introduction

Non-small-cell lung cancer (NSCLC) is the predominant histological subtype, accounting for more than 85% of all lung cancers [1]. For *EGFR* mutation-positive NSCLC, EGFR tyrosine kinase inhibitors (EGFR-TKIs) are recommended as first-line treatment [2]. While gefitinib, icotinib, or erlotinib have shown improved outcomes compared to chemotherapy in several prospective randomized clinical trials [3-5], acquired resistance inevitably develops [6]. The most common resistance mechanism of first-generation EGFR-TKIs is p.Thr790Met (T790M) mutation in *EGFR* exon 20 [7]. However, osimertinib, has proven successful in overcoming this limitation [8]. Osimertinib, a third-generation EGFR-TKI, has emerged as a therapeutic agent

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for blocking the growth of *EGFR* T790M-positive tumors [9]. In patients who have developed resistance to first- or second-generation EGFR-TKIs, osimertinib has demonstrated clinical efficacy in phase I/II trials [10-12]. For instance, in a phase I dose escalation study of AZD9291 (AURA), the objective response rate (ORR) reached 51% in the whole population. Among the subset of *EGFR* T790M-positive patients, the ORR was 67%. The median progression-free survival (PFS) was notably longer in *EGFR* T790M-positive patients (9.6 months) compared to *EGFR* T790M-negative patients (2.8 months) [10].

The genetic heterogeneity of NSCLC patients can influence clinical responses and treatment outcomes to EGFR-TKIs. Previous reports have suggested that mutations in tumor protein p53 (*TP53*), KRAS proto-oncogene, GTPase (*KRAS*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), and B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) can act as driver mutations, co-existing with *EGFR*, and impact the efficacy of EGFR-TKIs [13, 14]. The clinical outcomes of osimertinib exhibit significant diversity, and the co-mutation spectrum of NSCLC patients is associated with the response to osimertinib and the time to osimertinib treatment discontinuation [15, 16]. As a second-line therapy, real-world data on genetic biomarkers for osimertinib treatment efficacy and prognosis remain incomplete.

The NCCN guidelines emphasize that genetic testing for gene mutations in NSCLC specimens, especially tissue specimens, is crucial for identifying potentially effective targeted therapies and avoiding treatments that are unlikely to provide clinical benefits. The advent of targeted drugs has significantly transformed the treatment landscape for NSCLC [17]. The standard practice in NSCLC treatment now involves molecular profiling of tumor DNA and RNA mutations using next-generation sequencing (NGS) to identify genomic alterations targeted by clinically approved targeted drugs [18]. Current guidelines from international cancer organizations strongly advocate for molecular testing in NSCLC patients, especially for actionable mutations such as *EGFR*, *KRAS*, *BRAF*, erb-b2 receptor tyrosine kinase 2 (*ERBB2*), ALK receptor tyrosine kinase (*ALK*), ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*), ret proto-oncogene (*RET*), neurotrophic receptor kinase (*NTRK*) and MET proto-oncogene, receptor tyrosine kinase (*MET*) exon 14 skipping mutations [18, 19]. Moreover, comprehensive testing panels are readily available to identify patients eligible for participation in investigational clinical trials [20]. The study aims to investigate whether precision treatment guided by NGS gene sequencing can yield superior survival benefits for patients with advanced NSCLC in China.

This real-world study comprised 68 NSCLC patients. We illustrated the practicality of utilizing DNA sequencing results to explore the association between NGS based gene alterations and the efficacy and prognosis of second-line osimertinib therapy, offering valuable insights for making personalized treatment management for advanced NSCLC patients.

Methods and materials

Patients and samples collection

A real-world cohort of 68 patients diagnosed with NSCLC was recruited from Shanghai Chest Hospital between 2013 and 2019 and was diligently followed throughout the entire treatment course. None of these patients underwent surgical treatment, and the histological type of their biopsy tissues was determined by two pathologists following the Union for International Cancer Control (UICC) classification of tumor node metastasis (TNM) for disease staging. Patients with other malignant tumors were not included in this study. The collected basic clinical information encompasses age, sex, histology, TNM staging [21], and smoking history (Supplementary Table 1). This study was approved by the Ethics Committee of Shanghai Chest Hospital (LS1010 and KS [Y] 19101). All individuals signed informed consent.

NGS testing and genetic alterations analysis

OncoAim® Panoramic Detection Panel (Singlera Genomics (Shanghai) Ltd., China) was employed to detect genetic alterations in the second and third biopsy tissues. This panel references cancer databases and clinical guidelines, encompassing a spectrum of genes, including risk genes, those targeted by FDA-approved and clinical trial targeted drugs,

immunotherapy markers and radiotherapy and chemotherapy effect-related genes. The genes included in the panel are shown in Supplementary Table 2. According to the manufacturer's protocols, DNA was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). The quality of DNA was assessed by 1% agarose gel electrophoresis and the quantification was detected using the Qubit dsDNA HS Assay kit along with the Qubit 3.0 fluorimeter (Life Technologies, Eugene, Oregon, USA). Libraries construction adhered to Illumina's standard procedures (Illumina, Inc., California, USA), involving the preparation of 20 ng DNA with a KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, USA). The library products underwent sequencing through 75 bp paired-end runs on the Illumina MiSeq platform. The raw data containing sequence information and quality information were aligned to the University of California at Santa Cruz (UCSC) human reference genome (GRCh37/hg19). Genomic variants, including single-nucleotide variants (SNVs), insertions, deletions, copy number variations, and amino acid changes were identified using the SnpEff tool (http://snpeff.sourceforge.net/) [22]. Variations identified in tumor tissues but not in matched blood samples were considered somatic alterations.

Treatment response and survival analysis

Clinical responses were assessed using the Response Evaluation Criteria in Solid Tumors Version 1.1 (RECIST 1.1) [23]. Evaluations were conducted every 3 months, starting from 1 month after the initiation of treatment. PFS was defined as the duration from the start of treatment until disease progression or death. For patients without disease relapse by the cut-off date (June 30, 2023), their data were censored at the time of their last follow-up. Overall survival (OS) was defined as the time from the start of first-line treatment to death.

Cells, reagents and plasmids

Two human NSCLC tumor cell lines, NCI-H1975 cells (*EGFR*, L858R and T790M) (RRID: CVCL_1511) and NCL-H358 cells (RRID: CVCL_1559) were cultured in RPMI1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA). The cells were cultured at 37°C in a 5% CO_2-humidified atmosphere. Icotinib and osimertinib were purchased from Selleck Chemicals (Houston, TX, USA). Full-length cDNA fragments of human *EGFR* containing activating mutations (E746_A750del and p.T790M), human RNA binding motif protein 10 (*RBM10*) containing activating mutations and human Histone cluster 1 H2B family member D (*HIST1H2BD*) containing activating mutations were generated. These mutated full-length *EGFR*, *RBM10* or *HIST1H2BD* cDNAs were introduced into the PCDNA3.1 vector and confirmed by sequencing.

Cell proliferation assay

A total of 10^4 cells were seeded in each well of a 96-well plate and cultured in RPMI1640 medium supplemented with 10% FBS. The cell proliferation was assessed using the Cell Counting Kit-8 reagent (BBI Life Sciences, Shanghai, China) following the manufacturer's instructions. The count of viable cells was recorded, and the proliferation was graphed using GraphPad Prism 9.5.0 software (GraphPad Software Inc., San Diego, CA, USA). Each experiment was conducted in triplicate.

Cell migration assay

Cell migration was assessed using a 24-well Bio-Coat Cell Migration Chambers (BD Biosciences, Massachusetts, USA). In brief, 200 µl of cell suspension was seeded in the upper chamber, and after 24 hours of incubation, the upper surface of the membrane was swabbed to remove non-migrating cells. The migrating cells on the lower surface were fixed with methanol and stained with crystal violet solution. The average cell count was determined by counting cells in three random microscopic fields.

Cell apoptosis assay

Apoptotic cells were detected using an annexin V-FITC/PI apoptosis detection kit (BioVision, CA, USA) following the manufacturer's instruction. Briefly, cells were trypsinized, washed, and collected. Cells were then suspended in binding buffer stained with Annexin-FITC and PI solution for 40 min. The percentages of apoptotic cells were determined by a flow cytometer (FACS-Canto, BD Bioscience, USA).

Table 1. The clinical characteristics of 68 NSCLC patients

Characteristics	Cohort (n = 68)
Age (year)	
Median (range)	60 (39-80)
Sex, n (%)	
Male	34 (50.0)
Female	34 (50.0)
Smoking status, n (%)	
Never	35 (51.5)
Ever	33 (48.5)
Histology, n (%)	
Adenocarcinoma	63 (92.6)
Squamous cell carcinoma	2 (2.9)
Adenosquamous carcinoma	3 (4.4)
TNM stage, n (%)	
I	0 (0)
II	1 (1.5)
III	7 (10.3)
IV	60 (88.2)
First-line treatment, n (%)	
Icotinib	49 (72.1)
Gefitinib	10 (14.7)
Erlotinib	9 (13.2)
Second-line treatment, n (%)	
Osimertinib	51 (75.0)
Erlotinib + Chemotherapy	1 (1.5)
Icotinib + Chemotherapy	4 (5.9)
Crizotinib	2 (2.9)
Chemotherapy	10 (14.7)
Over survival (months)	
Median (range)	40.7 (10.6-98.7)

NSCLC, Non-small cell lung cancer. TNM, Tumor Node Metastasis classification. NA, not available value.

Cell growth inhibition assay

A total of 10^4 transfected cells were seeded in individual wells of a 96-well plate, cultivated in RPMI1640 medium. Subsequently, dimethyl sulfoxide (DMSO) or EGFR-TKIs was applied at indicated drug concentrations and cultured for 24 hours. The inhibitory effects of EGFR-TKIs on cell growth were assessed using the Cell Counting Kit-8 reagent (BBI Life Sciences in Shanghai, China). Each experiment was conducted in triplicate.

Statistical analysis

Mutational profiling was performed using MAF Visualization tools (maftools) in R (version 4.1.0) (http://www.r-project.org) [24]. To visualize the 3-D structures of proteins, the ITASSER server (http://zhanglab.ccmb.med.umich.edu/ITASSER) [25] was utilized. Categorical variables were analyzed using Chi-Squared Test, and continuous variables were assessed using the Mann-Whitney U test. Statistical significance was determined when the *P*-value < 0.05. PFS and OS were calculated by using the Kaplan-Meier survival analysis and a log-rank test was employed to compare the cumulative survival among different groups.

Results

Patients' baseline characteristics and mutation profiling

Herein, we enrolled 68 patients with *EGFR* mutant NSCLC. Among them, half were women (34/68), and the majority had adenocarcinoma histology (63/68) and advanced stage (67/68) (**Table 1**). Icotinib, gefitinib, and erlotinib were administered to 49 (72.1%), 10 (14.7%), and 9 (13.2%) patients, respectively. The median time to treatment discontinuation was 15.4 months. After developing resistance to the first-generation TKIs, 65 patients underwent a second biopsy (**Figure 1**). Cancer-related gene mutations were detected in all patients, with high mutation frequencies in genes such as *EGFR* (95%), *TP53* (52%), LDL receptor related protein 1B (*LRP1B*) (15%), catenin beta 1 (*CTNNB1*) (11%), glutamate ionotropic receptor NMDA type subunit 2A (*GRIN2A*) (9%) and lysine methyltransferase 2C (*KMT2C*) (9%) (**Figure 2A**). Among 62 cases with *EGFR* mutations, 52% (32/62) exhibited co-mutations with *TP53* mutations. Other co-mutations with high frequencies included *LRP1B* (16%), *CTNNB1* (11%), *GRIN2A* (10%), and *KMT2C* (10%) (**Figure 2B**). No significant connections were found between gene mutations and clinical factors like sex, age, and smoking status.

PAK5, RBM10, and EPHA3 mutations as independent biomarkers for poor PFS in second-line osimertinib treatment

A total of 49 T790M-positive patients receiving osimertinib, and 2 ALK-positive patients receiving crizotinib. The median PFS (mPFS) for these 51 patients receiving second-line targeted therapies was 12.2 months, for osimertinib, it was 13.3 months. Considering the impact of genet-

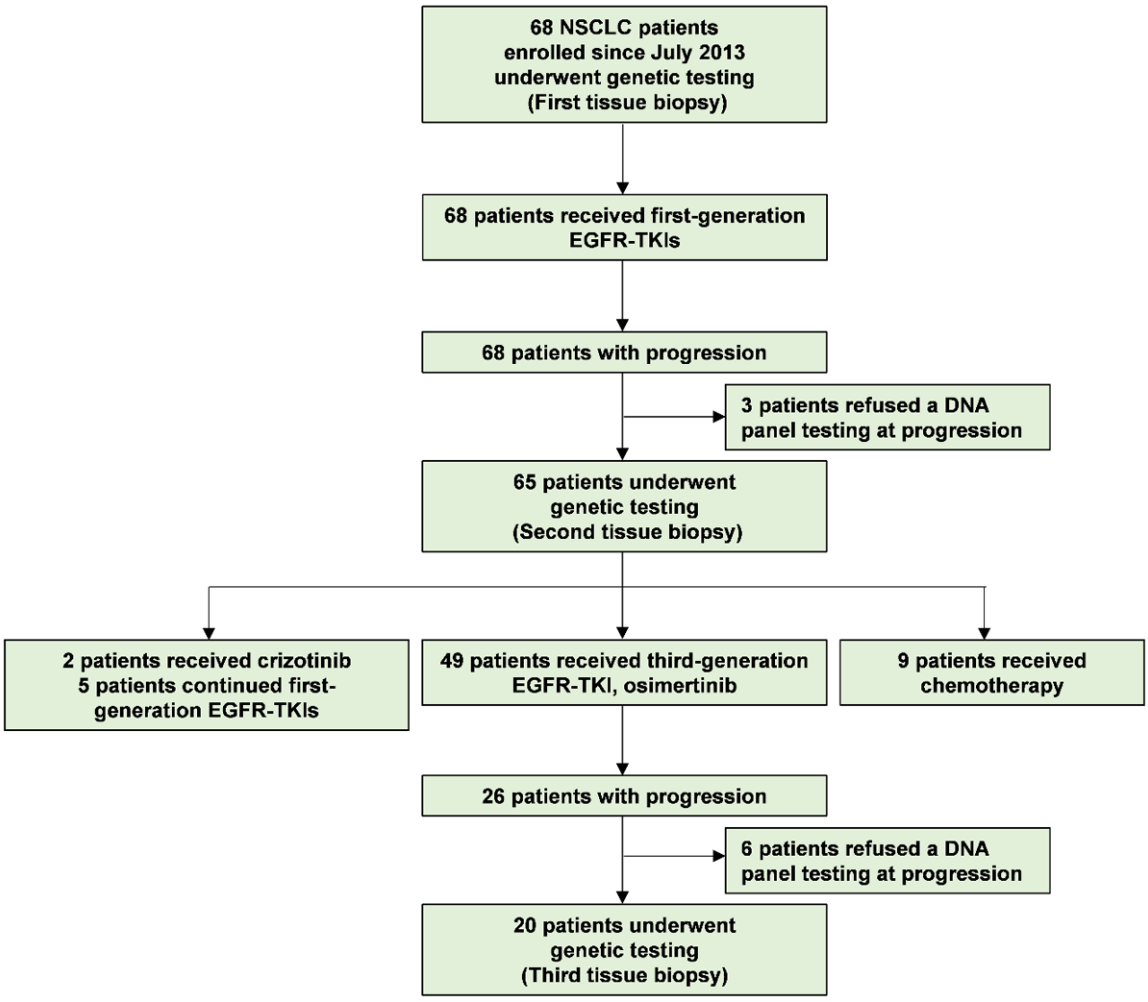


Figure 1. Study flow diagram. The end of the follow-up period was 30 June 2023. A total of 68 patients with *EGFR* mutant NSCLC were enrolled. All patients received first-generation EGFR-TKIs. Upon developing resistance to first-generation TKIs, 65 patients who accepted genetic testing were treated with second-line treatments. Among them, 51 patients received second-line EGFR-TKIs treatment, 5 patients continued first-generation EGFR-TKIs treatment, and 9 patients received chemotherapy. After resistance to second-line osimertinib, 20 patients underwent genetic testing. Molecular changes in each biopsy were detected using a customized DNA panel.

ic alterations on PFS specifically for osimertinib. We compared the mutation profiles of the group with better PFS (mPFS > 13 months) and the group with worse PFS (mPFS ≤ 13 months) in response to osimertinib. *TP53* mutations were more common in patients with worse PFS than in those with better PFS (65.4% vs. 34.8%, $P = 0.032$). Additionally, higher mutation frequencies of *GRIN2A*, hepatocyte growth factor (*HGF*), p21 (RAC1) activated kinase 5 (*PAK5*), RNA binding motif protein 10 (*RBM10*), and EPH receptor A3 (*EPHA3*) were observed in the worse PFS cohort (**Figure 3A** and Supplementary Figure 1A, 1B). Kaplan-Meier survival analysis revealed that patients with *GRIN2A*, *PAK5*, *RBM10*, and *EPHA3* mutations exhibited significantly worse PFS than those without mutations (HR: 7.1, 95% CI: 1.57-32.3, $P = 0.003$; 6.7, 1.48-30.4, $P = 0.004$; 7.7, 1.64-35.8, $P = 0.002$; 7.1, 1.57-32.3, $P = 0.003$; **Figure 3B-G**). Specifically, multivariable logistic regression confirmed that *PAK5*, *RBM10*, and *EPHA3* mutations as independent risk predictors of poor PFS in osimertinib treatment (**Figure 4**). No significant difference in mPFS was found between patients with *EGFR* single mutations

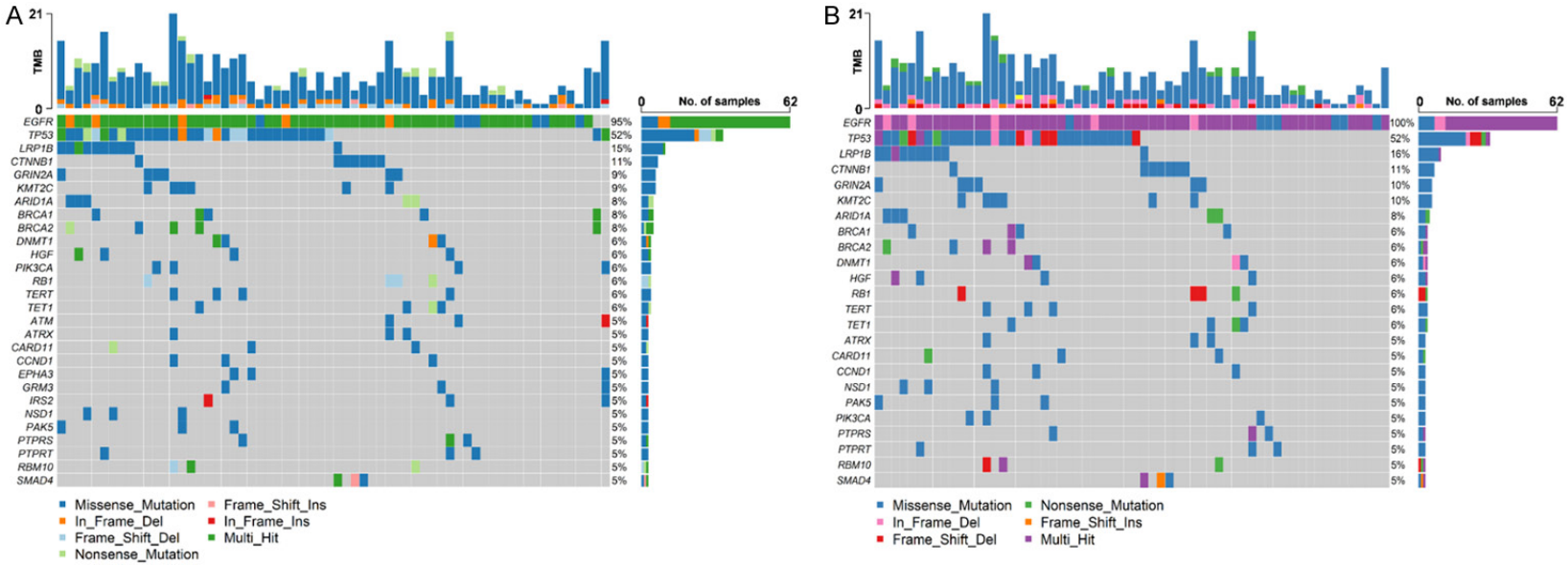


Figure 2. Analysis of the mutation profiling. A. Genetic profiling of 65 patients after developing resistance to first-generation EGFR-TKIs. B. Mutation spectrum of patients with *EGFR* mutations.

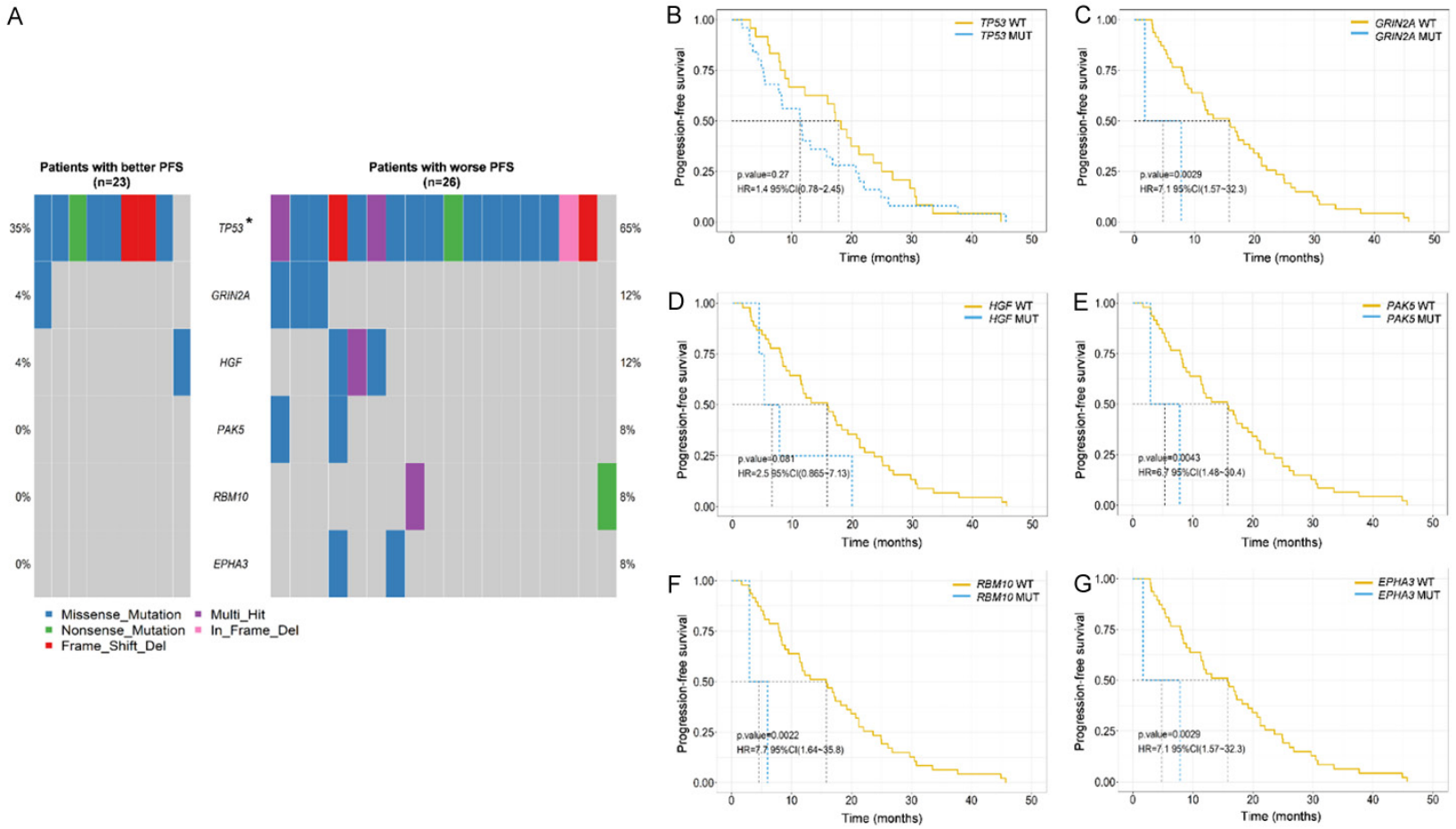


Figure 3. Correlation analysis between mutation profiling and PFS in response to second-line osimertinib. (A) Genes exhibiting significant differences in mutation frequencies between the group with better PFS and the group with worse PFS in response to osimertinib. Kaplan-Meier survival analysis of *TP53* (B), *GRIN2A* (C), *HGF* (D), *PAK5* (E), *RBM10* (F), and *EPHA3* (G) mutations. A log-rank test was used to determine the difference between the groups. *$P < 0.05$. WT, wild type. MUT, mutation.

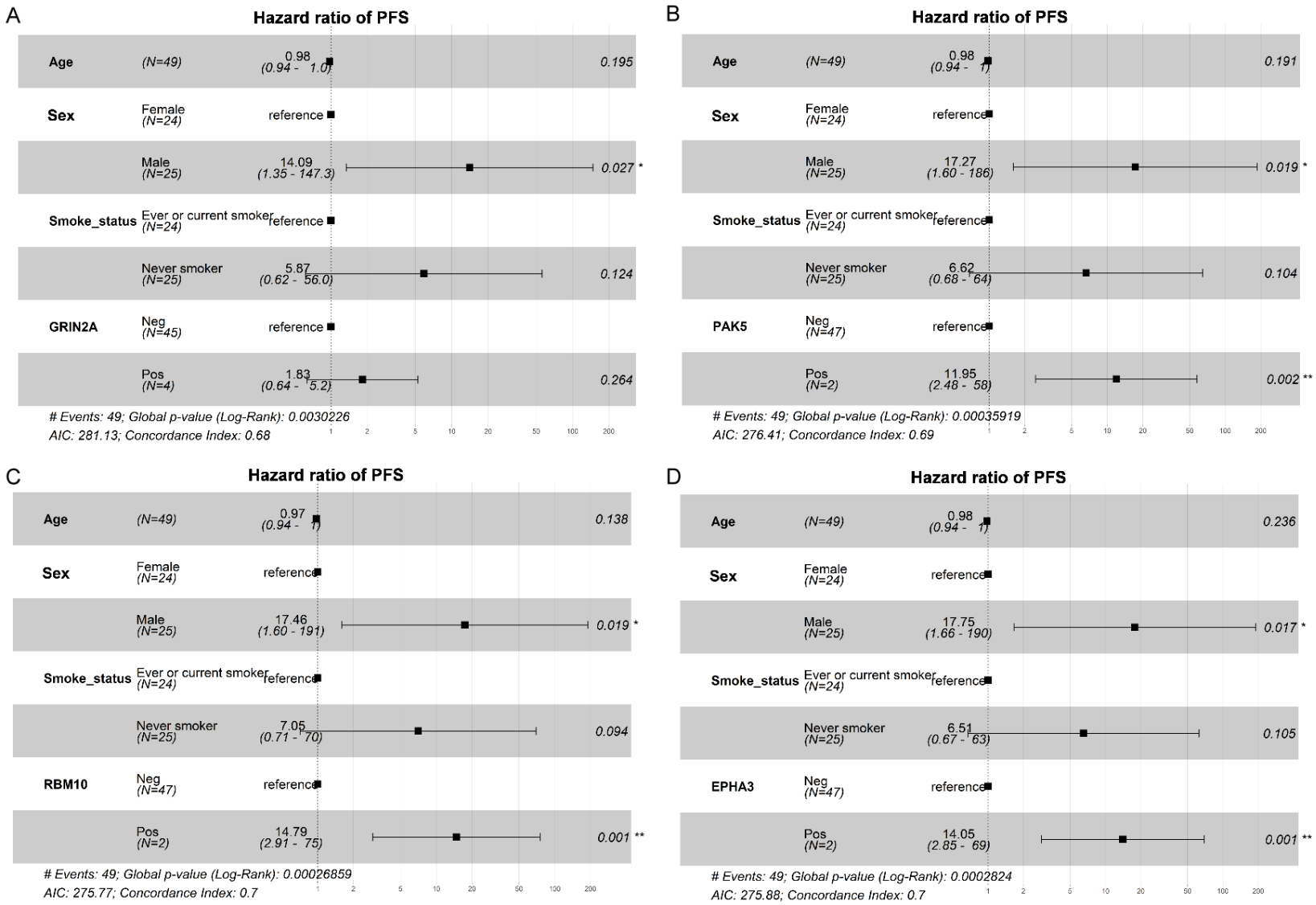


Figure 4. Multivariable analyses for PFS. Multivariable analyses for PFS including *GRIN2A* (A), *PAK5* (B), *RBM10* (C) and *EPHA3* (D) mutations. *$P < 0.05$, **$P < 0.01$.

and those with *EGFR* co-mutations (16.6 months vs. 12.2 months, *P* = 0.709; Supplementary Figure 2).

RBM10 as an independent predictor of short OS in second-line osimertinib treatment

Patients receiving EGFR-TKIs as second-line treatment had a relatively better prognosis compared to chemotherapy (mOS: 24.3 months vs. 14.3 months, HR: 0.58, 95% CI: 0.259-1.32, *P* = 0.190; Supplementary Figure 3A). Patients with T790M mutations treated with osimertinib showed better survival than T790M-negative patients receiving other TKIs (mOS: 26.2 months vs. 14.8 months, HR: 0.43, 95% CI: 0.161-1.13, *P* = 0.078; Supplementary Figure 3B). When comparing the mutation spectrum between the group with longer OS (mOS > 24 months) and the group with shorter OS (mOS ≤ 24 months) among patients receiving second-line therapies, significantly higher mutation frequencies of *TP53* (64.7% vs. 38.7%, *P* = 0.036) and *HGF* (12.1% vs. 0%, *P* = 0.048) in the shorter OS cohort were observed (**Figure 5A**). Kaplan-Meier survival analysis revealed that only *RBM10* mutations correlated with significantly shorter OS (HR: 5.8, 95% CI: 1.64-20.3, *P* = 0.002; **Figure 5B-G**).

Focusing on the mutation spectrum difference between the group with longer OS (mOS > 26 months) and the group with shorter OS (mOS ≤ 26 months) among patients receiving osimertinib, higher mutation frequencies of *LRP1B*, *GRIN2A*, *HGF*, *PAK5*, and *RBM10* were associated with shorter survival (Supplementary Figure 4A, 4B). The significantly higher mutation frequencies of *TP53*, *GRIN2A*, and *HGF* were detected in the shorter OS group compared to the longer OS group (68.2% vs. 37.0%, *P* = 0.030; 18.2% vs. 0%, *P* = 0.021; 18.2% vs. 0%, *P* = 0.022; **Figure 6A**). Kaplan-Meier survival analysis revealed that patients with *GRIN2A*, *HGF* and *RBM10* mutations exhibited significantly shorter OS than those without mutations (HR: 3.1, 95% CI: 1.01-9.19, *P* = 0.037; 2.9, 0.967-8.57, *P* = 0.047; 12.0, 2.25-68.3, *P* < 0.001; **Figure 6B-G**). Multivariable logistic regression confirmed that *RBM10* mutation as an independent risk predictor of OS in osimertinib treatment (**Figure 7**). *EGFR* mutations and *EGFR* co-mutations did not significantly affect OS in second-line treatments (Supplementary Figure 5).

RBM10 mutations promoted tumor cell proliferation and migration, while reducing cell apoptosis in vitro

The mutation sites of *RBM10* are shown in **Figure 8A** and have not been reported before. We transfected *RBM10* mutant plasmids into NCI-H1975 cells. No significant differences in the expression of *RBM10* mRNA were observed (**Figure 8B**). On the 2nd day after transfection, we observed that *RBM10* mutations markedly promoted the proliferation of tumor cells (**Figure 8C**). To assess the impact of *RBM10* mutations on the cell migration ability of human NSCLC cells, a transwell assay was conducted. The results revealed that *RBM10* mutant cells exhibited enhanced cell migration compared to *RBM10* wild-type cells (**Figure 8D**). In addition, *RBM10* mutant cells were associated with a decreased proportion of apoptotic cells (**Figure 8E**). When these cells were exposed to increased doses of osimertinib, those transfected with *RBM10* wild-type and mutant plasmids did not show significant difference in resistance to osimertinib (**Figure 8F**, **8G**).

Heterogeneity of resistance mechanisms to osimertinib

Among the 20 patients whose samples were paired before and after osimertinib resistance, we observed differences in the genetic profiles. Known *EGFR*-dependent resistant mutations and activation of alternative pathways were identified in 40% of all the patients, displaying great heterogeneity. C797S mutations were detected in 4 patients. All these 4 patients retained the T790M mutation, with 3 having both c.2389 T > A and 1 with c.2390 G > C for C797S. In addition to C797S, L718V was detected in 1 patient. *EGFR* amplifications were detected in 4 patients. Non-*EGRR* resistance mechanisms were identified in 3 patients with PI3K-AKT-mTOR signaling related genomic alterations occurring in 1 patient. Additionally, activation of *CCND1* was detected in 1 patient and *MET* amplification was identified in 1 patient. Remarkably, 3 patients harbored mutations in alternative pathways combined with *EGFR*-dependent mutations, including *EGFR/PIK3CA* mutation in 1 patient, *EGFR/CCND1* mutation in 1 patient and *EGFR/MET* mutation in 1 patient (**Table 2**). It is worth noting that among 60% (12/20) patients without common

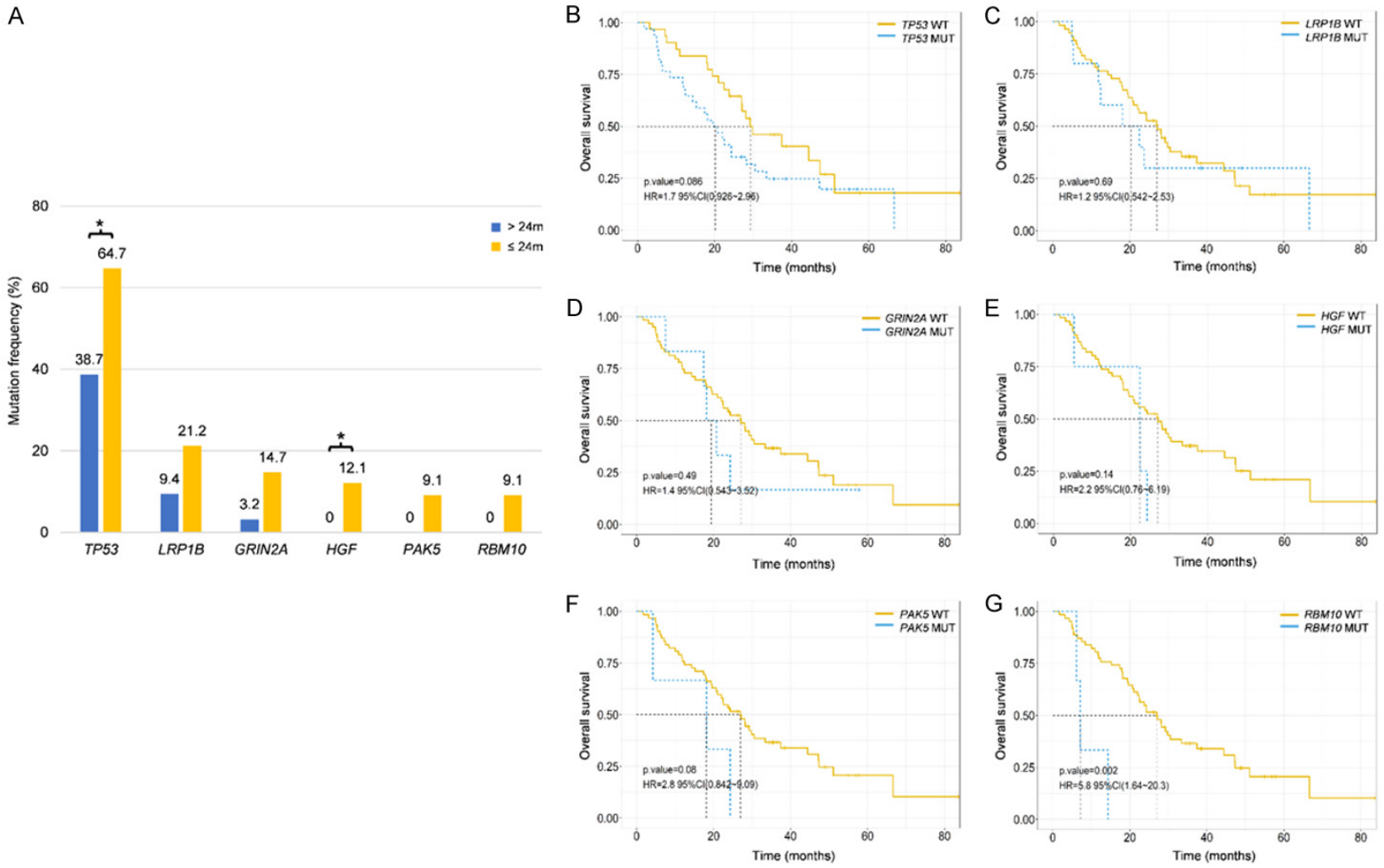


Figure 5. Correlation analysis between mutation profiling and OS in response to second-line therapy. (A) Genes with significant differences in mutation frequencies between the group with a longer OS and a shorter OS in response to second-line therapy. Kaplan-Meier survival analysis of *TP53* (B), *LRP1B* (C), *GRIN2A* (D), *HGF* (E), *PAK5* (F) and *RBM10* (G) genes. A log-rank test was used to determine the difference between the groups. *$P < 0.05$. WT, wild type. MUT, mutation.

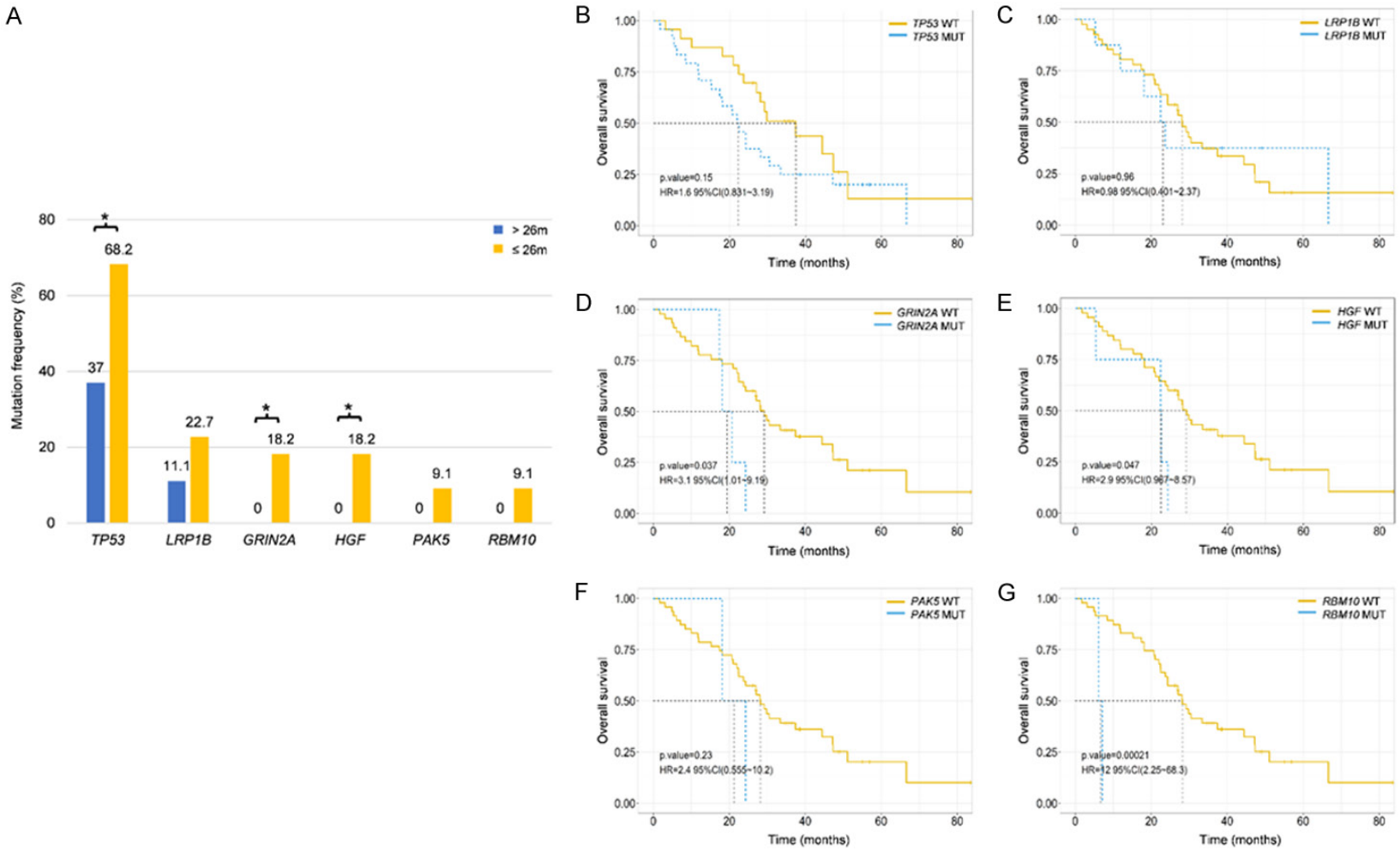


Figure 6. Correlation analysis between mutation profiling and OS in response to second-line osimertinib. (A) Genes with significant differences in mutation frequencies between the group a longer OS and the group with a shorter OS in response to osimertinib. Kaplan-Meier survival analysis of *TP53* (B), *LRP1B* (C), *GRIN2A* (D), *HGF* (E), *PAK5* (F) and *RBM10* (G) mutations. A log-rank test was used to determine the difference between the groups. **P* < 0.05. WT, wild type. MUT, mutation.

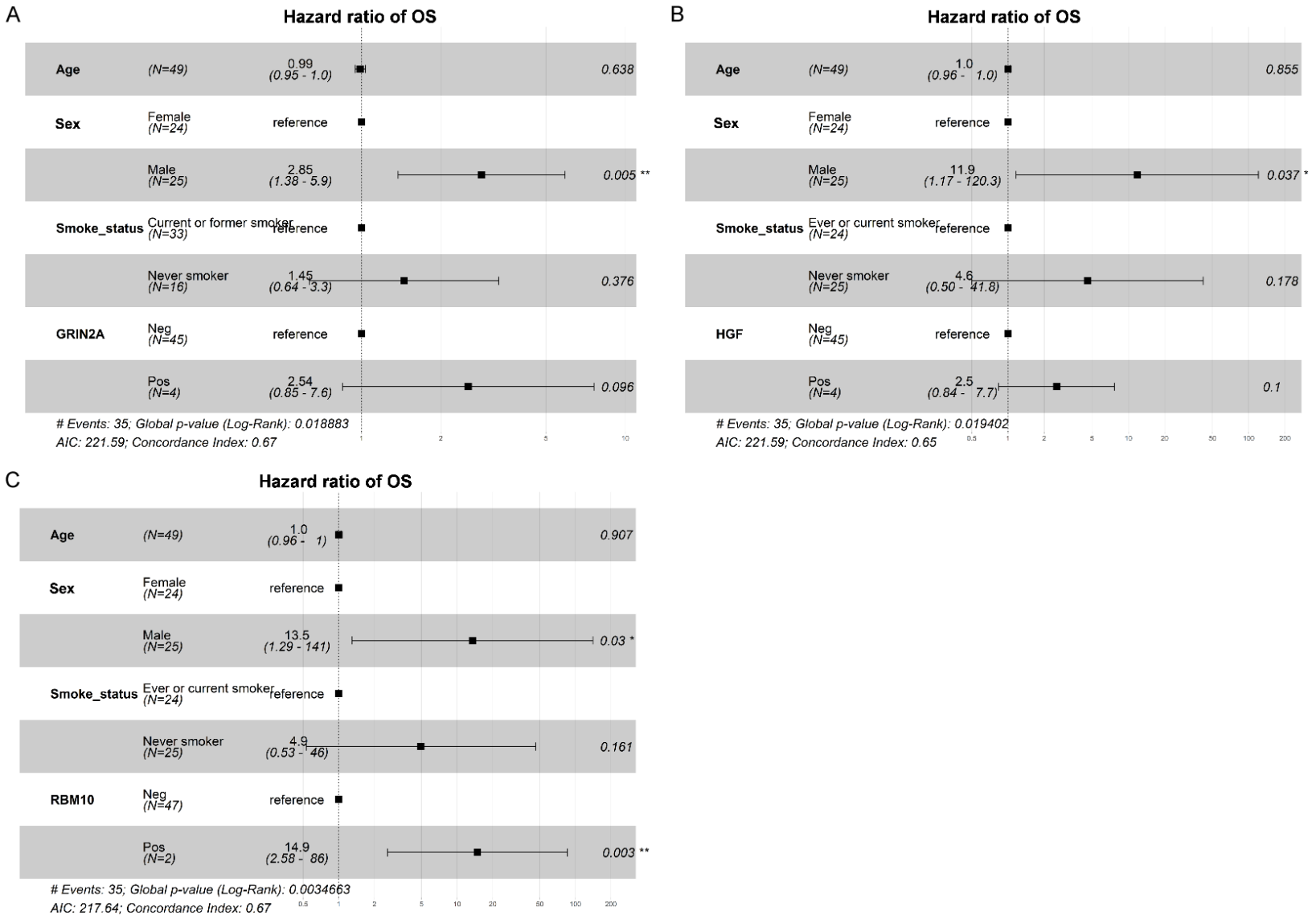


Figure 7. Multivariable analyses for OS. Multivariable analyses for OS including *GRIN2A* (A), *HGF* (B) and *RBM10* (C) mutations. *$P < 0.05$, **$P < 0.01$.

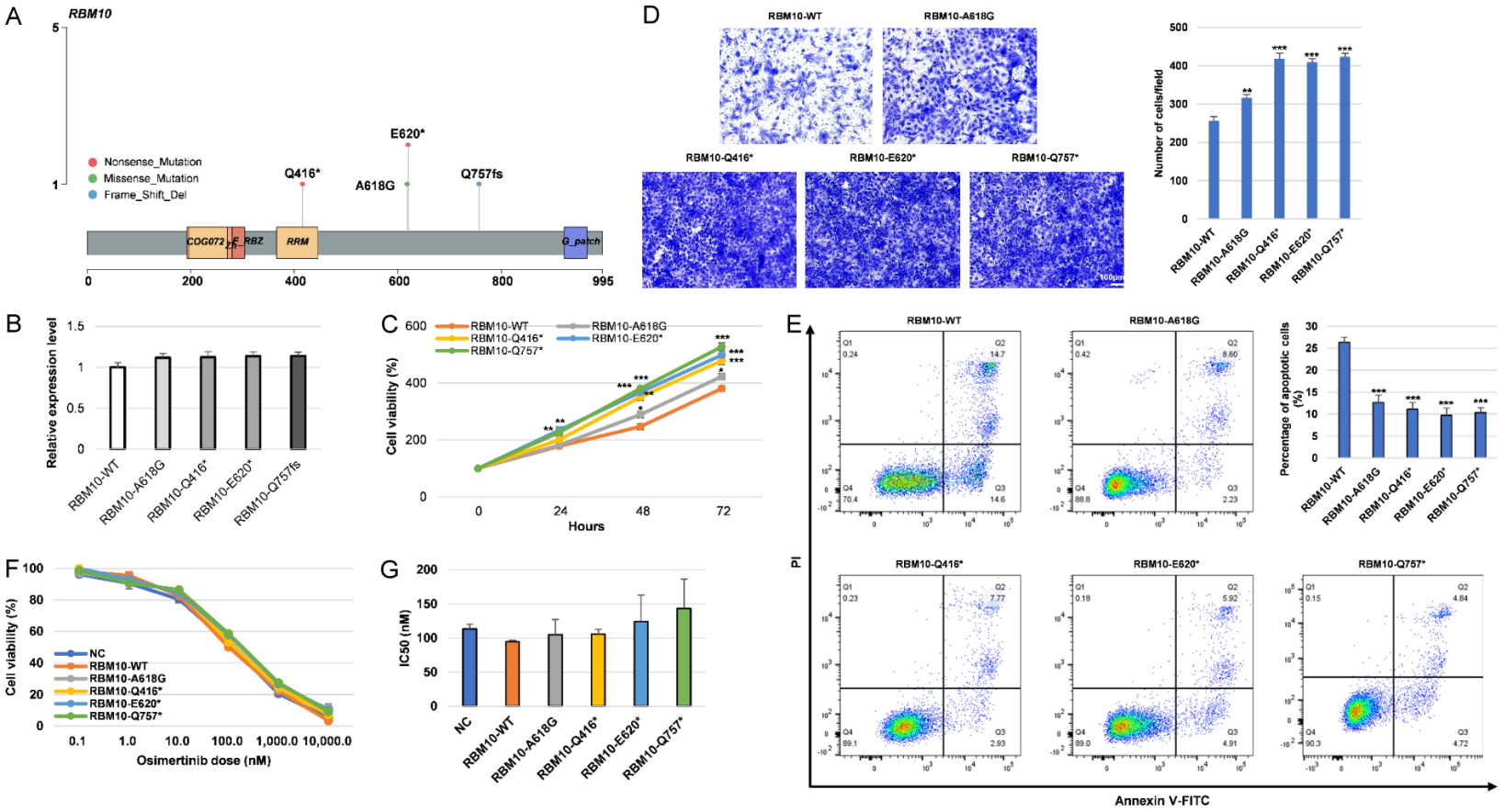


Figure 8. *RBM10* mutations promoted cell proliferation and migration and reduced apoptosis. A. Mutant sites of *RBM10* gene. B. Real-time PCR was employed to assess the mRNA expression levels of *RBM10* following transfection with the *RBM10* WT and mutant plasmids. C. Cell proliferation of NCI-H1975 cells after transfection with the *RBM10* WT and mutant plasmids. D. The migration of NCI-H1975 cells was assessed using a transwell assay. The migration ability of cells was determined by counting the number of cells that had migrated through the membrane, as indicated by crystal violet staining. The quantification results were obtained from three independent experiments. E. Cell apoptosis was assessed through flow cytometry analysis. F, G. NCI-H1975 cells were treated with indicated concentrations of osimertinib for 24 h. Cell viability and the IC50 values of osimertinib were displayed. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. NC, negative control. WT, wild type.

Table 2. Known resistance mechanisms to osimertinib in patients

Resistant mutations	(n = 20)
EGFR-dependent pathways	
C797S	4
L718V	1
EGFR amplification	4
Concurrent *EGFR* mutations	1
Alternative pathways activation	
Activating of PI3K-AKT-mTOR signaling	1
Activating mutation of *CCND1*	1
Amplification of *MET*	1
Concurrent mutations with *EGFR*	3

mutations, no mutations were found in the *BRCA2* and *HIST1H2BD* genes in the pre-treatment tissues of the paired samples (**Figure 9A**). Additionally, only *HIST1H2BD* was not detected in any of the 68 patients at the time of diagnosis, confirming its acquisition as a drug-resistance factor. In this study, two novel *HIST1H2BD* mutations, K25Q and E36D, were identified in two cases, and they were not located in a commonly known functional domain (**Figure 9B**). Both mutations altered the protein's structure according to computer simulations (**Figure 9C-F**).

HIST1H2BD p.K25Q and p.E36D mutations induced resistance to osimertinib

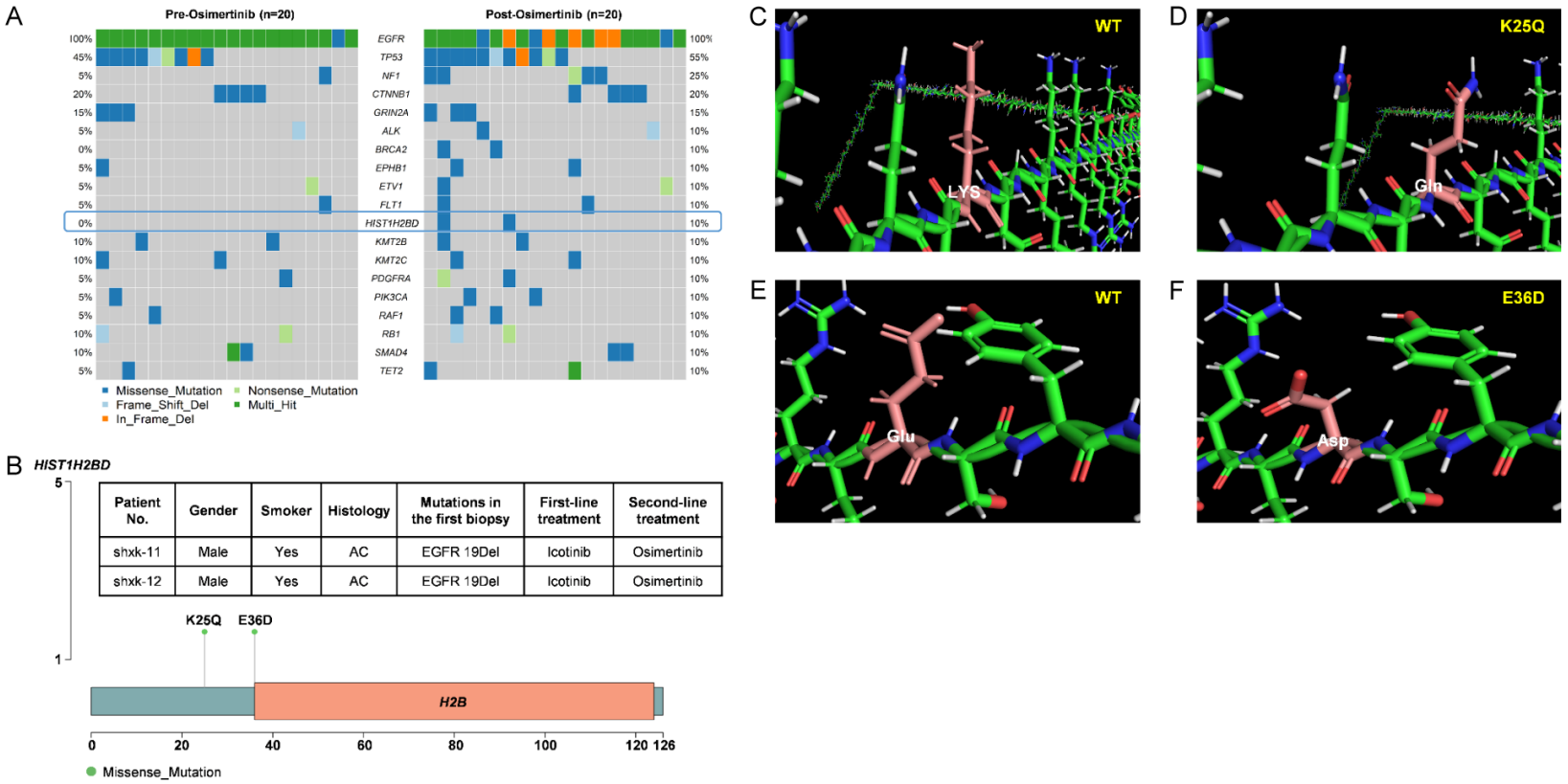
We transfected *HIST1H2BD* mutant plasmids into NCI-H358 cells. We found that there was no significant difference in the expression of *HIST1H2BD* mRNA (**Figure 10A**). We next determined whether *HIST1H2BD* p.K25Q and p.E36D mutations contributed to osimertinib resistance. As expected, cells expressing the *EGFR* 19del mutation were sensitive to icotinib and osimertinib (**Figure 10B** and **10C**), while cells containing *EGFR* T790M were sensitive to osimertinib. *EGFR* mutant cells transfected with the *HIST1H2BD* p.K25Q and p.E36D mutant variants exhibited strong resistance to osimertinib (**Figure 10D**). As illustrated in Supplementary Figure 6A, a higher number of *EGFR* mutant cells transfected with the *HIST1H2BD* p.K25Q and p.E36D mutant variants passed through the upper membrane of the transwell inserts compared to *HIST1H2BD* wild-type transfected cells, following treatment with 100 nM osimertinib. Moreover, in the presence of osimertinib, *HIST1H2BD* p.K25Q and p.E36D mutations were associated with a reduction in cell apoptosis (Supplementary Figure 6B).

Discussion

Patients with *EGFR* mutations can benefit from EGFR-TKIs treatment. However, the heterogeneity of lung cancer results in different responses and outcomes among patients possessing the same sensitive *EGFR* mutations. This study presents a comprehensive analysis of the mutation spectrum in NSCLC patients, emphasizing the necessity of heightened attention and collaborative efforts to unravel the biological and clinical implications of rare mutations for precise clinical treatment and prognostic monitoring [26, 27].

To simultaneously investigate the predictive impact of gene alterations on the efficacy and prognosis of osimertinib treatment, this study further examined the mutation patterns in patients with shorter PFS and those with longer PFS. The results revealed that mutations in *GRIN2A*, *PAK5*, *RBM10*, and *EPHA3* were significantly associated with shorter PFS. Among them, *PAK5*, *RBM10*, and *EPHA3* were identified as independent risk predictors of PFS. Previous studies have reported that *PAK5*, *RBM10*, and *EPHA3* were related to the proliferation and metastasis of lung cancer [28-30], but this study is the first to confirm that these three genes independently contribute to worse PFS in osimertinib treatment. Additionally, we observed significant associations between *GRIN2A*, *HGF*, and *RBM10* mutations and shorter OS. Notably, *RBM10* emerged not only as an independent risk predictor of PFS but also an independent risk factor for OS. Moreover, *BRCA1* and *BRCA2* mutations may be related to chemotherapy prognosis rather than TKIs treatment, although no statistical differences were observed. To validate these findings, larger sample sizes will be imperative in future research.

Previous research has shown that *RBM10* deficiency in *EGFR* mutant lung adenocarcinoma reduces the apoptotic response to EGFR-TKIs treatment, leading to tumor progression and poorer clinical outcomes. *RBM10* controls the alternative splicing of apoptosis regulatory factor Bcl-x to produce two subtypes: Bcl-xL (anti-



Patient No.	Gender	Smoker	Histology	Mutations in the first biopsy	First-line treatment	Second-line treatment
shxk-11	Male	Yes	AC	EGFR 19Del	Icotinib	Osimertinib
shxk-12	Male	Yes	AC	EGFR 19Del	Icotinib	Osimertinib

Figure 9. Novel resistance mechanisms to osimertinib. A. Comparison of gene mutation frequencies in tissues obtained from before osimertinib treatment and after the development of osimertinib resistance. B. Distribution of *HIST1H2BD* mutation sites. C-F. 3D protein structures of wild-type and mutant proteins of *HIST1H2BD*.

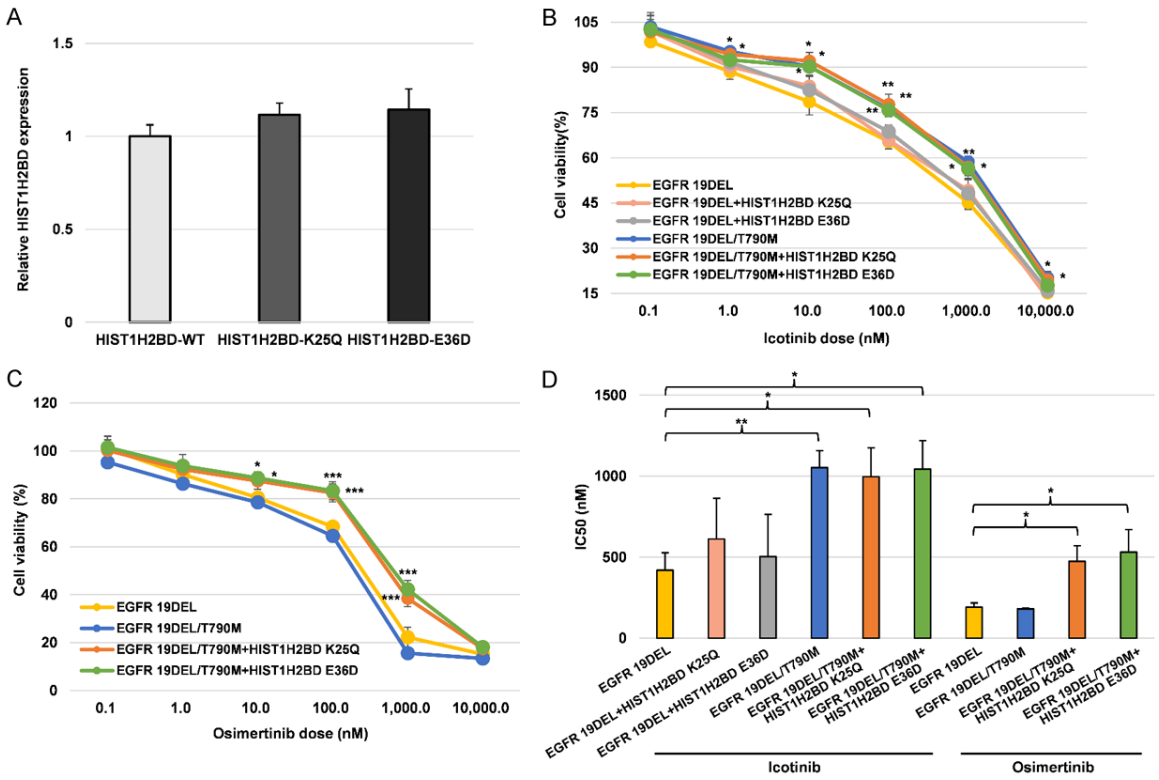


Figure 10. *HIST1H2BD* p.K25Q and p.E36D mutations induced resistance to osimertinib *in vitro*. (A) Real time-PCR was utilized to assess *HIST1H2BD* mRNA expression levels following transfection with *HIST1H2BD* wild-type and mutant plasmids. H358 cells carrying *EGFR* 19DEL and T790M mutations, along with the indicated mutations, were subjected to treatment with icotinib (B) and osimertinib (C) at specified concentrations. Cell viability was assessed following a 24-hour treatment and depicted for comparison with untreated control cells. (D) IC50 values of cells in response to osimertinib were depicted in bar graphs for comparison analysis. **P* < 0.05, ***P* < 0.01, ***, *P* < 0.001.

apoptosis) and Bcl-xS (pro-apoptosis) [31, 32]. Shigeki Nanjo et al reported that *RBM10* deficiency modifies Bcl-x splicing to increase Bcl-xL, thereby limiting cell apoptosis after EGFR-TKIs treatment [33]. In clinical trials, the mutation and/or expression of *RBM10* may be a promising biomarker for the response to osimertinib and navitoclax [34], and our study also confirms this, proving that *RBM10* as a biomarker for EGFR-TKI treatment response is a future research area that can help enhance patient treatment choices and improve clinical outcomes. Besides Bcl-x, will *RBM10* regulate its participation in treatment response through other pathways? This is the direction of our future mechanism research.

Previous studies have reported a detection rate of *EGFR*-dependent mutations in post-osimertinib treatment samples ranging from 70% to 100% [35]. Our findings, however, show a lower rate of 40%. This discrepancy indicates the importance and urgency of exploring non-*EGFR* pathway mechanisms of osimertinib resistance [36]. Among the 60% of patients without *EGFR* secondary mutations and other common resistance mutations, we identified mutations in the histone gene, *HIST1H2BD*, in two patients' post-resistance tissues simultaneously. Histone plays a crucial role in regulating gene expression and chromosomal structure within cells [37]. Some tumor cells may undergo histone modifications after receiving osimertinib treatment, leading to changes in gene expression patterns and affecting the drug's effectiveness [38]. Combining osimertinib with the Histone deacetylases (HDACs) inhibitor can effectively overcome acquired resistance of *EGFR* mutant NSCLC cells to osimertinib [39, 40]. Previous research has demonstrated that poly (ADP-ribose) glycohydrolase (*PARG*) silencing could inhibit *HIST1H2BD* expression during BaP-induced lung carcinogenesis [41], but

there is currently a lack of research on *HIST-1H2BD* in the context of osimertinib resistance. This provides us with a direction for our subsequent study on how *HIST1H2BD* mutations lead to resistance to osimertinib. The biggest limitation of this study is the sample size, although these are still differences between groups with a better prognosis and those with a worse prognosis.

Conclusion

This study underscores the heterogeneity of NSCLC, a factor that impacts the response and resistance to second-line osimertinib treatment. *RBM10* is not only an independent risk factor for osimertinib PFS, but also an independent risk factor for OS. Additionally, our findings indicate that resistance to osimertinib is highly heterogenous among individuals, warranting deeper investigation.

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Disclosure of conflict of interest

WYX, YYH and LD were employed by company Singlera Genomics (Shanghai) Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019; 69: 7-34.

[2] Greenhalgh J, Boland A, Bates V, Vecchio F, Dundar Y, Chaplin M and Green JA. First-line treatment of advanced epidermal growth factor receptor (EGFR) mutation positive non-squamous non-small cell lung cancer. Cochrane Database Syst Rev 2021; 3: CD010383.

[3] Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, Gemma A, Harada M, Yoshizawa H, Kinoshita I, Fujita Y, Okinaga S, Hirano H, Yoshimori K, Harada T, Ogura T, Ando M, Miyazawa H, Tanaka T, Saijo Y, Hagiwara K, Morita S and Nukiwa T; North-East Japan Study Group. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 2010; 362: 2380-2388.

[4] He J, Su C, Liang W, Xu S, Wu L, Fu X, Zhang X, Ge D, Chen Q, Mao W, Xu L, Chen C, Hu B, Shao G, Hu J, Zhao J, Liu X, Liu Z, Wang Z, Xiao Z, Gong T, Lin W, Li X, Ye F, Liu Y, Ma H, Huang Y, Zhou J, Wang Z, Fu J, Ding L, Mao L and Zhou C. Icotinib versus chemotherapy as adjuvant treatment for stage II-IIIA EGFR-mutant non-small-cell lung cancer (EVIDENCE): a randomised, open-label, phase 3 trial. Lancet Respir Med 2021; 9: 1021-1029.

[5] Yue D, Xu S, Wang Q, Li X, Shen Y, Zhao H, Chen C, Mao W, Liu W, Liu J, Zhang L, Ma H, Li Q, Yang Y, Liu Y, Chen H, Zhang Z, Zhang B and Wang C. Updated overall survival and exploratory analysis from randomized, phase II EVAN study of erlotinib versus vinorelbine plus cisplatin adjuvant therapy in stage IIIA epidermal growth factor receptor+ non-small-cell lung cancer. J Clin Oncol 2022; 40: 3912-3917.

[6] Recondo G, Facchinetti F, Olaussen KA, Besse B and Friboulet L. Making the first move in EGFR-driven or ALK-driven NSCLC: first-generation or next-generation TKI? Nat Rev Clin Oncol 2018; 15: 694-708.

[7] Wu S, Luo M, To KKW, Zhang J, Su C, Zhang H, An S, Wang F, Chen D and Fu L. Intercellular transfer of exosomal wild type EGFR triggers osimertinib resistance in non-small cell lung cancer. Mol Cancer 2021; 20: 17.

[8] Remon J, Steuer CE, Ramalingam SS and Felip E. Osimertinib and other third-generation EGFR TKI in EGFR-mutant NSCLC patients. Ann Oncol 2018; 29: i20-i27.

[9] Cross DA, Ashton SE, Ghiorghiu S, Eberlein C, Nebhan CA, Spitzler PJ, Orme JP, Finlay MR, Ward RA, Mellor MJ, Hughes G, Rahi A, Jacobs VN, Red Brewer M, Ichihara E, Sun J, Jin H, Ballard P, Al-Kadhimi K, Rowlinson R, Klinowska T, Richmond GH, Cantarini M, Kim DW, Ranson MR and Pao W. AZD9291, an irreversible EGFR TKI, overcomes T790M-mediated resistance to EGFR inhibitors in lung cancer. Cancer Discov 2014; 4: 1046-1061.

[10] Janne PA, Yang JC, Kim DW, Planchard D, Ohe Y, Ramalingam SS, Ahn MJ, Kim SW, Su WC, Horn L, Haggstrom D, Felip E, Kim JH, Frewer P, Cantarini M, Brown KH, Dickinson PA, Ghiorghiu S and Ranson M. AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. N Engl J Med 2015; 372: 1689-1699.

[11] Yang JC, Ahn MJ, Kim DW, Ramalingam SS, Sequist LV, Su WC, Kim SW, Kim JH, Planchard D, Felip E, Blackhall F, Haggstrom D, Yoh K, Novello S, Gold K, Hirashima T, Lin CC, Mann H, Cantarini M, Ghiorghiu S and Janne PA. Osimertinib in pretreated T790M-positive advanced non-small-cell lung cancer: AURA study phase II extension component. J Clin Oncol 2017; 35: 1288-1296.

[12] Goss G, Tsai CM, Shepherd FA, Bazhenova L, Lee JS, Chang GC, Crino L, Satouchi M, Chu Q, Hida T, Han JY, Juan O, Dunphy F, Nishio M, Kang JH, Majem M, Mann H, Cantarini M, Ghiorghiu S and Mitsudomi T. Osimertinib for pretreated EGFR Thr790Met-positive advanced non-small-cell lung cancer (AURA2): a multicentre, open-label, single-arm, phase 2 study. Lancet Oncol 2016; 17: 1643-1652.

[13] Deng LL, Gao G, Deng HB, Wang F, Wang ZH and Yang Y. Co-occurring genetic alterations predict distant metastasis and poor efficacy of first-line EGFR-TKIs in EGFR-mutant NSCLC. J Cancer Res Clin Oncol 2019; 145: 2613-2624.

[14] Jin Y, Shi X, Zhao J, He Q, Chen M, Yan J, Ou Q, Wu X, Shao YW and Yu X. Mechanisms of primary resistance to EGFR targeted therapy in advanced lung adenocarcinomas. Lung Cancer 2018; 124: 110-116.

[15] Zhang L, Yang X, Ming Z, Shi J, Lv X, Li W, Yuan B, Chen Y, Liu B, Qin K, Liu J, Wei Q, Gu D, Chen R, Yuan M, Cui J, Ou SI and Yang S. Molecular characteristics of the uncommon EGFR Exon 21 T854A mutation and response to osimertinib in patients with non-small cell lung cancer. Clin Lung Cancer 2022; 23: 311-319.

[16] Zhao J, Lin G, Zhuo M, Fan Z, Miao L, Chen L, Zeng A, Yin R, Ou Y, Shi Z, Yin J, Gao W, Chen J, Zhou X, Zeng Y, Liu X, Xu H, Chen R, Xia X and Carbone DP. Next-generation sequencing based mutation profiling reveals heterogeneity of clinical response and resistance to osimertinib. Lung Cancer 2020; 141: 114-118.

[17] Roberts TJ, Kehl KL, Brooks GA, Sholl L, Wright AA, Landrum MB and Keating NL. Practice-level variation in molecular testing and use of targeted therapy for patients with non-small cell lung cancer and colorectal cancer. JAMA Netw Open 2023; 6: e2310809.

[18] Hendriks LE, Kerr KM, Menis J, Mok TS, Nestle U, Passaro A, Peters S, Planchard D, Smit EF, Solomon BJ, Veronesi G and Reck M; ESMO Guidelines Committee. Electronic address: clinicalguidelines@esmo.org. Oncogene-addicted metastatic non-small-cell lung cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. Ann Oncol 2023; 34: 339-357.

[19] Planchard D, Popat S, Kerr K, Novello S, Smit EF, Faivre-Finn C, Mok TS, Reck M, Van Schil PE, Hellmann MD and Peters S; ESMO Guidelines Committee. Metastatic non-small cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2018; 29 Suppl 4: iv192-iv237.

[20] Vingiani A, Agnelli L, Duca M, Lorenzini D, Damian S, Proto C, Niger M, Nichetti F, Tamborini E, Perrone F, Piccolo A, Manoukian S, Azzollini J, Brambilla M, Colombo E, Lopez S, Vernieri C, Marra F, Conca E, Busico A, Capone I, Bozzi F, Angelini M, Devecchi A, Salvatori R, De Micheli V, Baggi A, Pasini S, Jommi C, Ladisa V, Apolone G, De Braud F and Pruneri G. Molecular tumor board as a clinical tool for converting molecular data into real-world patient care. JCO Precis Oncol 2023; 7: e2300067.

[21] Goldstraw P, Chansky K, Crowley J, Rami-Porta R, Asamura H, Eberhardt WE, Nicholson AG, Groome P, Mitchell A and Bolejack V; International Association for the Study of Lung Cancer Staging and Prognostic Factors Committee, Advisory Boards, and Participating Institutions; International Association for the Study of Lung Cancer Staging and Prognostic Factors Committee Advisory Boards and Participating Institutions. The IASLC lung cancer staging project: proposals for revision of the TNM stage Groupings in the forthcoming (eighth) edition of the TNM classification for lung cancer. J Thorac Oncol 2016; 11: 39-51.

[22] Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, Land SJ, Lu X and Ruden DM. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly (Austin) 2012; 6: 80-92.

[23] Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D and Verweij J. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 2009; 45: 228-247.

[24] Mayakonda A, Lin DC, Assenov Y, Plass C and Koeffler HP. Maftools: efficient and comprehensive analysis of somatic variants in cancer. Genome Res 2018; 28: 1747-1756.

[25] Yang J and Zhang Y. I-TASSER server: new development for protein structure and function predictions. Nucleic Acids Res 2015; 43: W174-W181.

[26] Blons H, Oudart JB, Merlio JP, Debieuvre D, de Fraipont F, Audigier-Valette C, Escande F, Hominal S, Bringuier PP, Fraboulet-Moreau S, Ouafik L, Moro-Sibilot D, Lemoine A, Langlais A, Missy P, Morin F, Souquet PJ, Barlesi F, Cadranel J and Beau-Faller M; French Cooperative Thoracic Intergroup (IFCT). PTEN, ATM, IDH1 mutations and MAPK pathway activation as modulators of PFS and OS in patients treated by first line EGFR TKI, an ancillary study of the French Cooperative Thoracic Intergroup (IFCT) Biomarkers France project. Lung Cancer 2021; 151: 69-75.

[27] Liu J, Xu WY, Ye M, Liu Z and Li C. Genetic alteration profiling of chinese lung adenocarcinoma and its effect on targeted therapy efficacy. Front Oncol 2021; 11: 726547.

[28] Bao Z, Ji W, Yang Y, Chen Z, Li Z, Wang K, Lu T, Yu Y, Xia W and Lu S. PAK5 promotes the cell stemness ability by phosphorylating SOX2 in lung squamous cell carcinomas. Exp Cell Res 2020; 395: 112187.

[29] Li Z, Xue Q, Xu J, Zhang P and Ding B. The role of RBM10 mutations in the development, treatment, and prognosis of lung adenocarcinoma. Cell Cycle 2020; 19: 2918-2926.

[30] Zhuang G, Song W, Amato K, Hwang Y, Lee K, Boothby M, Ye F, Guo Y, Shyr Y, Lin L, Carbone DP, Brantley-Sieders DM and Chen J. Effects of cancer-associated EPHA3 mutations on lung cancer. J Natl Cancer Inst 2012; 104: 1182-1197.

[31] Inoue A, Yamamoto N, Kimura M, Nishio K, Yamane H and Nakajima K. RBM10 regulates alternative splicing. FEBS Lett 2014; 588: 942-947.

[32] Hernandez J, Bechara E, Schlesinger D, Delgado J, Serrano L and Valcarcel J. Tumor suppressor properties of the splicing regulatory factor RBM10. RNA Biol 2016; 13: 466-472.

[33] Nanjo S, Wu W, Karachaliou N, Blakely CM, Suzuki J, Chou YT, Ali SM, Kerr DL, Olivas VR, Shue J, Rotow J, Mayekar MK, Haderk F, Chatterjee N, Urisman A, Yeo JC, Skanderup AJ, Tan AC, Tam WL, Arrieta O, Hosomichi K, Nishiyama A, Yano S, Kirichok Y, Tan DS, Rosell R, Okimoto RA and Bivona TG. Deficiency of the splicing factor RBM10 limits EGFR inhibitor response in EGFR-mutant lung cancer. J Clin Invest 2022; 132: e145099.

[34] Bertino EM, Gentzler RD, Clifford S, Kolesar J, Muzikansky A, Haura EB, Piotrowska Z, Camidge DR, Stinchcombe TE, Hann C, Malhotra J, Villaruz LC, Paweletz CP, Lau CL, Sholl L, Takebe N, Moscow JA, Shapiro GI, Janne PA and Oxnard GR. Phase IB study of osimertinib in combination with navitoclax in EGFR-mutant NSCLC following resistance to initial EGFR therapy (ETCTN 9903). Clin Cancer Res 2021; 27: 1604-1611.

[35] Kato R, Hayashi H, Sakai K, Suzuki S, Haratani K, Takahama T, Tanizaki J, Nonagase Y, Tanaka K, Yoshida T, Takeda M, Yonesaka K, Kaneda H, Nishio K and Nakagawa K. CAPP-seq analysis of circulating tumor DNA from patients with EGFR T790M-positive lung cancer after osimertinib. Int J Clin Oncol 2021; 26: 1628-1639.

[36] Oxnard GR, Hu Y, Mileham KF, Husain H, Costa DB, Tracy P, Feeney N, Sholl LM, Dahlberg SE, Redig AJ, Kwiatkowski DJ, Rabin MS, Paweletz CP, Thress KS and Janne PA. Assessment of resistance mechanisms and clinical implications in patients with EGFR T790M-positive lung cancer and acquired resistance to osimertinib. JAMA Oncol 2018; 4: 1527-1534.

[37] Jenuwein T and Allis CD. Translating the histone code. Science 2001; 293: 1074-1080.

[38] Tanimoto A, Takeuchi S, Arai S, Fukuda K, Yamada T, Roca X, Ong ST and Yano S. Histone deacetylase 3 inhibition overcomes BIM deletion polymorphism-mediated osimertinib resistance in EGFR-mutant lung cancer. Clin Cancer Res 2017; 23: 3139-3149.

[39] Zang H, Qian G, Zong D, Fan S, Owonikoko TK, Ramalingam SS and Sun SY. Overcoming acquired resistance of epidermal growth factor receptor-mutant non-small cell lung cancer cells to osimertinib by combining osimertinib with the histone deacetylase inhibitor panobinostat (LBH589). Cancer 2020; 126: 2024-2033.

[40] Dong H, Yin H, Zhao C, Cao J, Xu W and Zhang Y. Design, synthesis and biological evaluation of novel osimertinib-based HDAC and EGFR dual inhibitors. Molecules 2019; 24: 2407.

[41] Zeng Z, Lu J, Wu D, Zuo R, Li Y, Huang H, Yuan J and Hu Z. Poly(ADP-ribose) glycohydrolase silencing-mediated H2B expression inhibits benzo(a)pyrene-induced carcinogenesis. Environ Toxicol 2021; 36: 291-297.

Supplementary Table 1. Patient clinical information and treatment

Patient No.	Age at diagnosis (years)	Sex	Smoking Status	Pathological Histology	TNM stage	EGFR Mutations in the first biopsy	First-line EGFR-TKIs	Second-line treatment	Death	Overall survival (months)
shxk-1	53	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	No	89.5
shxk-2	58	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	40.9
shxk-3	47	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Gefitinib	Osimertinib	No	44.0
shxk-4	67	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	58.0
shxk-5	55	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Osimertinib	Yes	36.3
shxk-6	56	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	79.0
shxk-7	43	Male	Never smoker	Adenocarcinoma	III	EGFR 19Del	Erlotinib	Osimertinib	Yes	28.4
shxk-8	74	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	43.0
shxk-9	54	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Gefitinib	Osimertinib	Yes	23.7
shxk-10	59	Female	Never smoker	Squamous cell carcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	46.8
shxk-11	51	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	38.5
shxk-12	54	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	47.4
shxk-13	60	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Gefitinib	Osimertinib	Yes	41.8
shxk-14	67	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Osimertinib	Yes	34.9
shxk-15	64	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	73.0
shxk-16	72	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	40.8
shxk-17	63	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Erlotinib	Osimertinib	No	46.2
shxk-18	56	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Erlotinib	Erlotinib+Chemotherapy	Yes	40.1
shxk-19	70	Male	Ever or current smoker	Adenosquamous carcinoma	III	EGFR 19Del	Icotinib	Osimertinib	Yes	33.7
shxk-20	46	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Icotinib+Chemotherapy	Yes	35.1
shxk-21	53	Male	Ever or current smoker	Adenocarcinoma	III	EGFR 19Del	Icotinib	Icotinib+Chemotherapy	Yes	21.9
shxk-22	58	Male	Ever or current smoker	Adenocarcinoma	III	EGFR 19Del	Gefitinib	Osimertinib	Yes	29.0
shxk-23	44	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Osimertinib	No	70.2
shxk-24	53	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	58.7
shxk-25	68	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Osimertinib	Yes	10.6
shxk-26	55	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Chemotherapy	Yes	19.6
shxk-27	68	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Erlotinib	Osimertinib	Yes	21.1
shxk-28	55	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	15.5
shxk-29	68	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Gefitinib	Chemotherapy	Yes	36.0
shxk-30	65	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Chemotherapy	Yes	38.0
shxk-31	44	Female	Never smoker	Adenosquamous carcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	58.6
shxk-32	41	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Gefitinib	Crizotinib	Yes	29.3

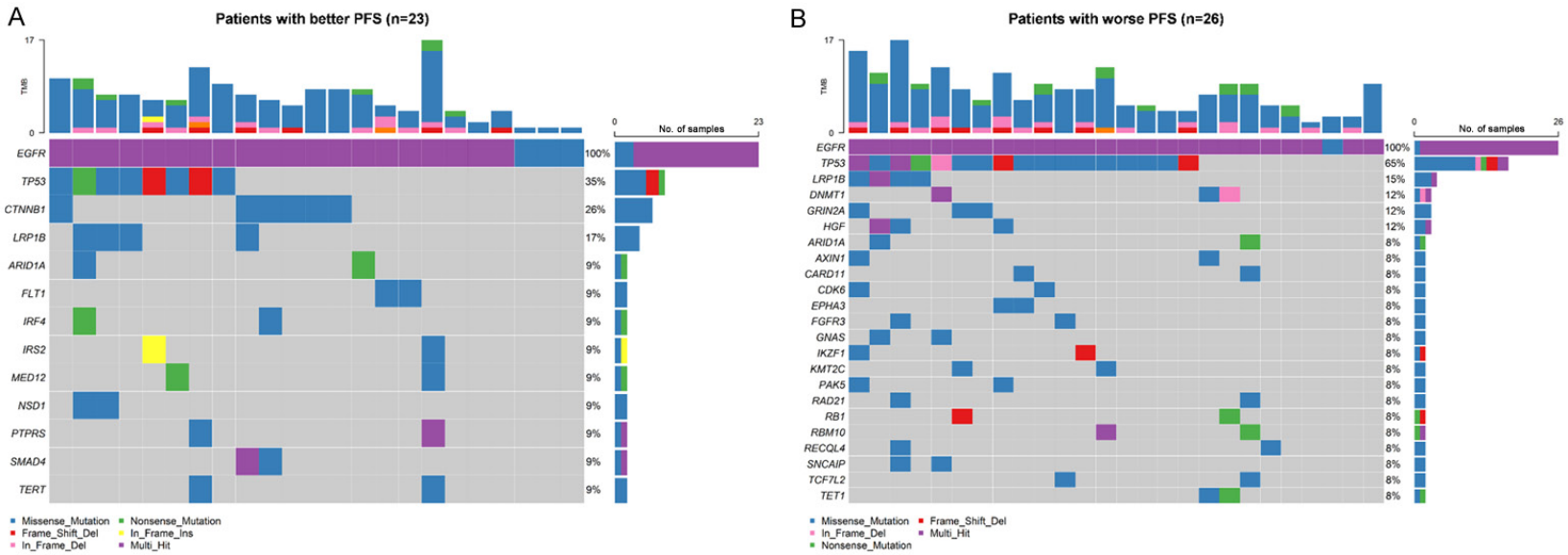
shxk-33	62	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Osimertinib	No	89.6
shxk-34	49	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Erlotinib	Osimertinib	Yes	24.0
shxk-35	60	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Osimertinib	Yes	12.7
shxk-36	66	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Osimertinib	Yes	42.6
shxk-37	62	Female	Never smoker	Adenocarcinoma	III	EGFR 19Del	Gefitinib	Crizotinib	Yes	21.7
shxk-38	56	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Chemotherapy	Yes	27.9
shxk-39	80	Female	Never smoker	Adenocarcinoma	III	EGFR L858R	Icotinib	Osimertinib	Yes	46.3
shxk-40	49	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR L858R	Erlotinib	Osimertinib	Yes	17.5
shxk-41	56	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	48.6
shxk-42	73	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Icotinib+Chemotherapy	Yes	29.5
shxk-43	72	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Osimertinib	No	98.5
shxk-44	66	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	34.0
shxk-45	70	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR L858R	Gefitinib	Osimertinib	Yes	59.4
shxk-46	62	Male	Ever or current smoker	Adenocarcinoma	III	EGFR 19Del	Icotinib	Osimertinib	Yes	30.3
shxk-47	73	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Erlotinib	Osimertinib	Yes	68.8
shxk-48	60	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	75.7
shxk-49	61	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	No	89.3
shxk-50	56	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Osimertinib	Yes	38.0
shxk-51	73	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	No	65.1
shxk-52	57	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Osimertinib	Yes	43.0
shxk-53	48	Male	Ever or current smoker	Squamous cell carcinoma	IV	EGFR L858R	Icotinib	Chemotherapy	Yes	17.6
shxk-54	55	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	50.9
shxk-55	77	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	No	44.7
shxk-56	62	Female	Never smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Osimertinib	No	79.7
shxk-57	73	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	No	70.7
shxk-58	68	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	48.8
shxk-59	46	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Erlotinib	Osimertinib	Yes	12.0
shxk-60	49	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Erlotinib	Osimertinib	No	45.0
shxk-61	74	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Chemotherapy	Yes	24.1
shxk-62	57	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR L858R	Gefitinib	Chemotherapy	No	98.7
shxk-63	74	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Chemotherapy	Yes	61.4
shxk-64	58	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR L858R	Icotinib	Icotinib+Chemotherapy	Yes	40.6
shxk-65	65	Male	Ever or current smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Chemotherapy	Yes	21.2
shxk-66	78	Female	Never smoker	Adenocarcinoma	IV	EGFR 19Del	Icotinib	Osimertinib	Yes	33.9
shxk-67	39	Male	Ever or current smoker	Adenosquamous carcinoma	IV	EGFR L858R	Icotinib	Chemotherapy	Yes	33.5
shxk-68	72	Female	Never smoker	Adenocarcinoma	II	EGFR L858R	Gefitinib	Osimertinib	Yes	81.8

Supplementary Table 2. Genes list of Singlera OncoAim® Panoramic Detection Panel

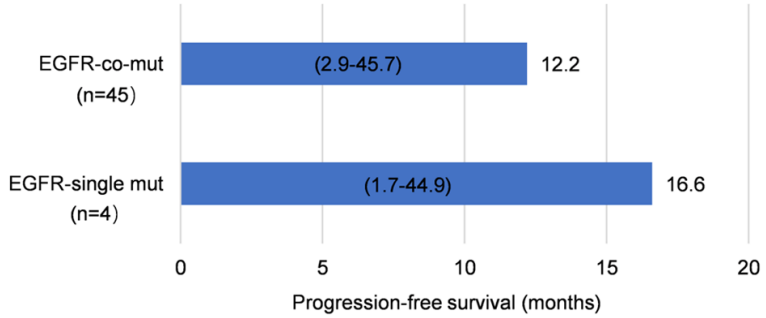
ABCB1	BRD4	COL5A1	EPCAM	FGFR3	HIST3H3	KMT2B	MSI2	PCNA	PTPRT	SETD8	TEK	ZFHX3
ABCB9	BRIP1	CREBBP	EPHA3	FGFR4	HLA-A	KMT2C	MST1	PDCD1	QKI	SF3B1	TERT	ZNF217
ABL1	BTG1	CRKL	EPHA5	FH	HLA-B	KMT2D	MST1R	PDCD1LG2	RAB35	SGK1	TET1	ZNF703
ABL2	BTG2	CRLF2	EPHA7	FLCN	HLA-C	KNSTRN	MTAP	PDGFRA	RAC1	SH2B3	TET2	
ACE2	BTK	CSDE1	EPHB1	FLT1	HLA-DRB1	KRAS	MTHFR	PDGFRB	RAC2	SH2D1A	TGFBR1	
ACVR1	C10orf54	CSF1R	EPHB4	FLT3	HMGB1	LATS1	MTOR	PDIA3	RAD21	SHOC2	TGFBR2	
ACVR1B	C11orf30	CSF3R	EPHX1	FLT4	HMGN1	LATS2	MTRR	PDK1	RAD50	SHQ1	TIPARP	
AGO2	C8orf34	CTCF	ERAP1	FOXA1	HNF1A	LGALS9	MUTYH	PDPK1	RAD51	SLC34A2	TMEM127	
AKT1	CALR	CTLA4	ERAP2	FOXL2	HOXB13	LGMN	MYB	PGR	RAD51B	SLCO1B1	TMPRSS2	
AKT2	CANX	CTNNA1	ERBB2	FOXO1	HRAS	LIG1	MYC	PHF6	RAD51C	SLIT2	TNF	
AKT3	CARD11	CTNNB1	ERBB3	FOXP1	HSD3B1	LIG3	MYCL	PHOX2B	RAD51D	SLX4	TNFAIP3	
ALK	CARM1	CTSB	ERBB4	FRS2	HSP90AA1	LMO1	MYCN	PIK3C2B	RAD52	SMAD2	TNFRSF14	
ALOX12B	CASP8	CTSL	ERCC1	FUBP1	ICOSLG	LNPEP	MYD88	PIK3C2G	RAD54L	SMAD3	TNFRSF9	
AMER1	CBFB	CTSS	ERCC2	FYN	ID3	LRP1B	MYOD1	PIK3C3	RAF1	SMAD4	TNFSF14	
ANKRD11	CBL	CUL3	ERCC3	GABRA6	IDE	LTK	NBN	PIK3CA	RANBP2	SMARCA4	TNFSF18	
APC	CBR3	CUL4A	ERCC4	GATA1	IDH1	LYN	NCOA3	PIK3CB	RARA	SMARCB1	TNFSF4	
AR	CCND1	CXCR4	ERCC5	GATA2	IDH2	LZTR1	NCOR1	PIK3CD	RASA1	SMARCD1	TNFSF9	
ARAF	CCND2	CYLD	ERF	GATA3	IFI30	MAF	NEGR1	PIK3CG	RB1	SMO	TOP1	
ARFRP1	CCND3	CYP17A1	ERG	GATA4	IFNGR1	MAGI2	NF1	PIK3R1	RBM10	SMYD3	TOP2A	
ARID1A	CCNE1	CYP19A1	ERRFI1	GATA6	IGF1	MALT1	NF2	PIK3R2	RECQL	SNCAIP	TP53	
ARID1B	CD200	CYP2C19	ESR1	GID4	IGF1R	MAP2K1	NFE2L2	PIK3R3	RECQL4	SOCS1	TP53BP1	
ARID2	CD22	CYP2C8	ETV1	GLI1	IGF2	MAP2K2	NFKBIA	PIM1	REL	SOD2	TP63	
ARID5B	CD274	CYP2C9	ETV4	GNA11	IKBKE	MAP2K4	NKX2-1	PLCG2	RET	SOS1	TP73	
ASXL1	CD276	CYP2D6	ETV5	GNA13	IKZF1	MAP3K1	NKX3-1	PLK2	RFWD2	SOX10	TPMT	
ASXL2	CD40	CYP3A4	ETV6	GNAQ	IL10	MAP3K13	NOTCH1	PMAIP1	RHEB	SOX17	TPP2	
ATM	CD40LG	CYSLTR2	EWSR1	GNAS	IL7R	MAP3K14	NOTCH2	PMS1	RHOA	SOX2	TRAF2	
ATR	CD48	DAXX	EXO1	GPR124	INHA	MAPK1	NOTCH3	PMS2	RICTOR	SOX9	TRAF7	
ATRX	CD70	DCUN1D1	EZH1	GPS2	INHBA	MAPK3	NOTCH4	PNRC1	RIT1	SPEN	TSC1	
AURKA	CD74	DDR1	EZH2	GREM1	INPP4A	MAPKAP1	NPEPPS	POLB	RNF43	SPOP	TSC2	
AURKB	CD79A	DDR2	EZR	GRIN2A	INPP4B	MAX	NPM1	POLD1	ROS1	SPRED1	TSHR	
AXIN1	CD79B	DHFR	FAM175A	GRM3	INPPL1	MCL1	NQO1	POLE	RPS6KA4	SPTA1	TYMS	
AXIN2	CD80	DICER1	FAM46C	GSK3B	INSR	MDC1	NRAS	PPARG	RPS6KB2	SRC	TYRO3	
AXL	CD86	DIS3	FAM58A	GSTP1	IRF2	MDM2	NRD1	PPM1D	RPTOR	SRSF2	U2AF1	
B2M	CDA	DMD	FANCA	H3F3A	IRF4	MDM4	NSD1	PPP2R1A	RRAGC	STAG2	UGT1A1	

BABAM1	CDC42	DNAJB1	FANCC	H3F3B	IRS1	MED12	NT5C2	PPP2R2A	RRAS	STAT3	UGT1A9
BAP1	CDC73	DNMT1	FANCD2	H3F3C	IRS2	MEF2B	NTHL1	PPP4R2	RRAS2	STAT4	UPF1
BARD1	CDH1	DNMT3A	FANCE	HDAC1	ITGAV	MEN1	NTRK1	PPP6C	RRM1	STAT5A	VEGFA
BBC3	CDK12	DNMT3B	FANCF	HERC1	ITGB3	MERTK	NTRK2	PRDM1	RSPO2	STAT5B	VHL
BCL10	CDK4	DOT1L	FANCG	HGF	JAK1	MET	NTRK3	PRDM14	RTEL1	STK11	VTCN1
BCL2	CDK6	DPYD	FANCL	HIST1H1C	JAK2	MGA	NUF2	PREX2	RUNX1	STK19	WHSC1
BCL2L1	CDK8	DROSHA	FAS	HIST1H2BD	JAK3	MICA	NUP93	PRKAR1A	RUNX1T1	STK40	WHSC1L1
BCL2L11	CDKN1A	DUSP4	FAT1	HIST1H3A	JUN	MICB	NUTM1	PRKCI	RXRA	SUFU	WISP3
BCL2L2	CDKN1B	DYNC2H1	FBXW7	HIST1H3B	KAT6A	MITF	P2RY8	PRKD1	RYBP	SUZ12	WT1
BCL6	CDKN2A	E2F3	FGF10	HIST1H3C	KDM5A	MKNK1	PAK1	PRKDC	SDC4	SYK	WWTR1
BCOR	CDKN2B	EED	FGF12	HIST1H3D	KDM5C	MLH1	PAK3	PRSS8	SDHA	TAF1	XIAP
BCORL1	CDKN2C	EGFL7	FGF14	HIST1H3E	KDM6A	MLH3	PAK7	PTCH1	SDHAF2	TAP1	XPC
BCR	CEBPA	EGFR	FGF19	HIST1H3F	KDR	MPL	PALB2	PTEN	SDHB	TAP2	XPO1
BIRC3	CENPA	EIF1AX	FGF23	HIST1H3G	KEAP1	MRE11	PARK2	PTGS2	SDHC	TAPBP	XRCC1
BLM	CHD2	EIF4A2	FGF3	HIST1H3H	KEL	MRE11A	PARP1	PTP4A1	SDHD	TAPBPL	XRCC2
BMPR1A	CHD4	EIF4E	FGF4	HIST1H3I	KIT	MSH2	PARP2	PTPN11	SESN1	TBX3	XRCC5
BRAF	CHEK1	ELF3	FGF6	HIST1H3J	KLF4	MSH3	PARP3	PTPRD	SESN2	TCEB1	YAP1
BRCA1	CHEK2	EP300	FGFR1	HIST2H3C	KLHL6	MSH6	PAX5	PTPRO	SESN3	TCF3	YES1
BRCA2	CIC	EPAS1	FGFR2	HIST2H3D	KMT2A	MFAT31	PBRM1	PTPRS	SETD2	TCF7L2	ZBTB2

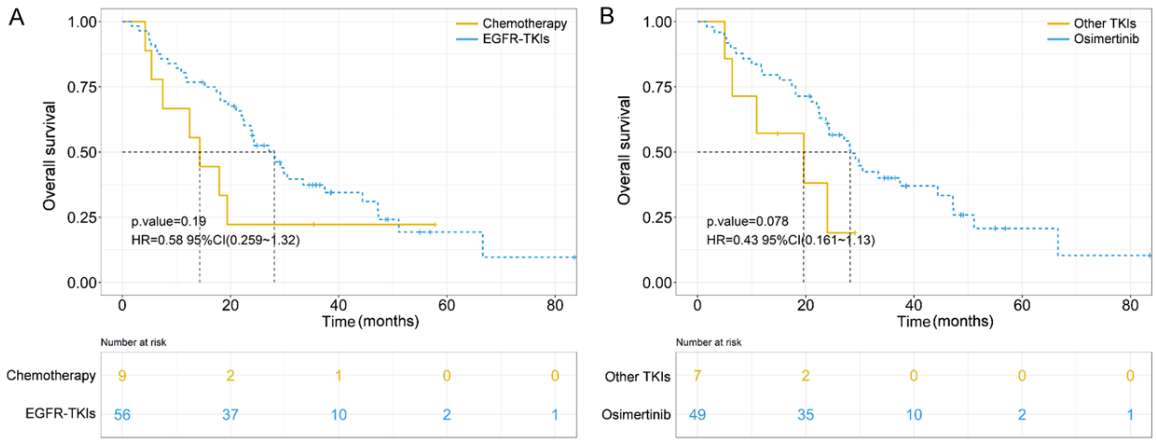
The DNA panel is a hybridization capture-based Next-Generation Sequencing (NGS) panel specifically designed to detect single nucleotide variants (SNVs), insertion and deletion alterations (InDels), and copy number alterations (CNAs) within 639 cancer-associated genes in tumor samples.



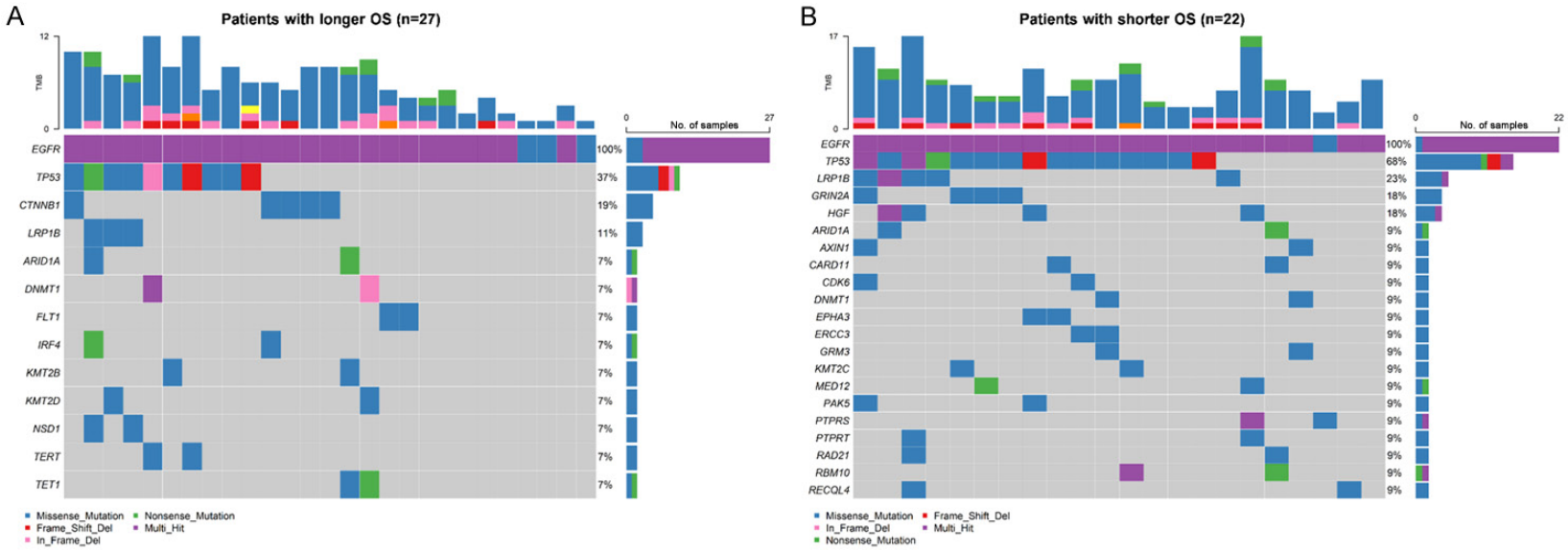
Supplementary Figure 1. The mutation profiles of the two groups with better and worse PFS in response to second-line targeted therapies. A. Mutation analysis in patients receiving second-line targeted therapies with a better PFS. B. Mutation analysis in patients receiving osimertinib with a worse PFS.



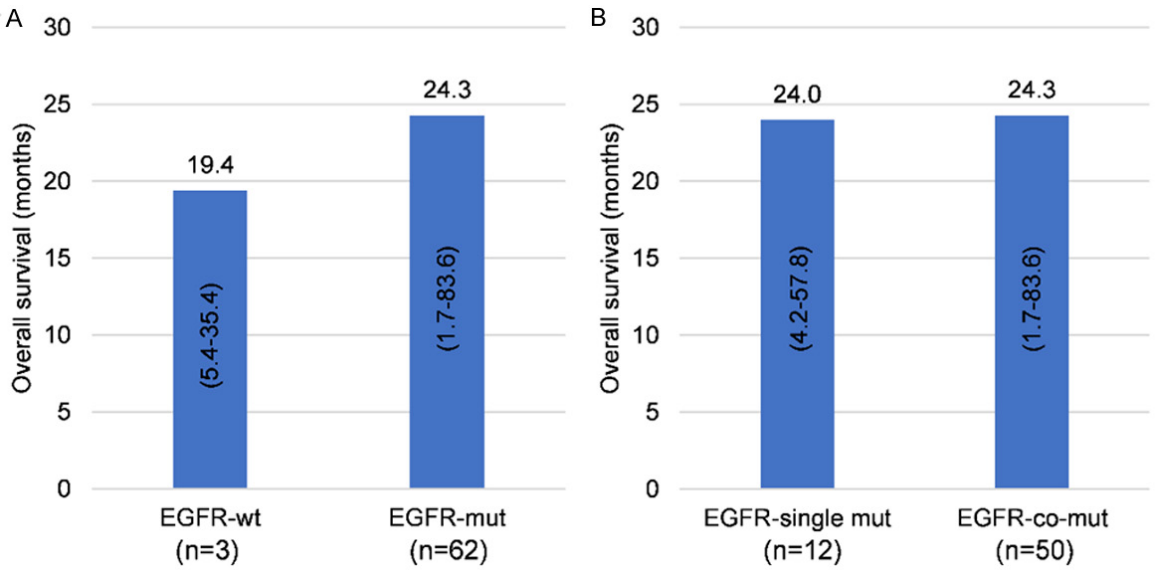
Supplementary Figure 2. Correlation analysis between *EGFR* mutations and PFS of osimertinib was shown. The ranges of PFS are displayed in the figure.



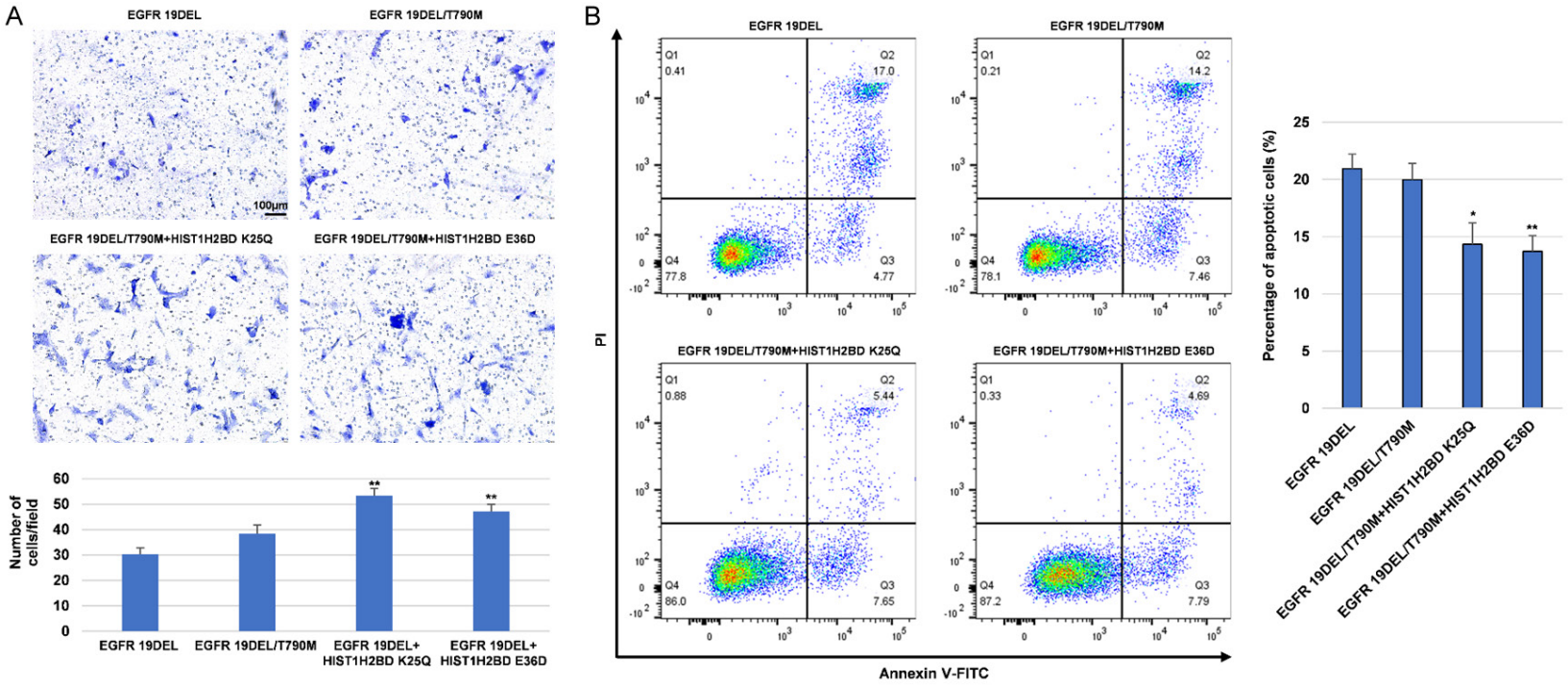
Supplementary Figure 3. The survival of different second-line treatment strategies. A. Comparison of survival between patients treated with EGFR-TKIs and chemotherapy. B. Comparison of survival between patients treated with osimertinib and other TKIs. A log-rank test was used to determine the difference between the groups.



Supplementary Figure 4. The mutation profiles of the group with longer OS and the group with shorter OS in response to second-line therapies. A. Mutation analysis in the patients receiving osimertinib with longer OS (> 26 months). B. Mutation analysis in the patients receiving osimertinib with shorter OS (≤ 26 months).



Supplementary Figure 5. The correlation between *EGFR* mutations and OS in second-line treatments. A. OS in the second-line treatments for patients with *EGFR* mutations and *EGFR* wild-types. B. OS in the second-line treatments for patients with *EGFR* single mutations and patients with *EGFR* co-mutations. The ranges of OS are displayed in the figure.



Supplementary Figure 6. Characterization of the migration ability and apoptosis of *HIST1H2BD* p.K25Q and p.E36D mutations. A. The migration of *HIST1H2BD* p.K25Q and p.E36D mutant cells treated with 100 nM osimertinib for 24 hours was assessed using a transwell assay. B. Cell apoptosis was evaluated through flow cytometry analysis. **P* < 0.05, ***P* < 0.01.