

## Short-Term Responders of Non-Small Cell Lung Cancer Patients to EGFR Tyrosine Kinase Inhibitors Display High Prevalence of TP53 Mutations and Primary Resistance Mechanisms<sup>1,2</sup>



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### Abstract

Non-small cell lung cancer (NSCLC) with activating *EGFR* mutations in exon 19 and 21 typically responds to EGFR tyrosine kinase inhibitors (TKI); however, for some patients, responses last only a few months. The underlying mechanisms of such short responses have not been fully elucidated. Here, we sequenced the genomes of 16 short-term responders (SR) that had progression-free survival (PFS) of less than 6 months on the first-generation EGFR TKI and compared them to 12 long-term responders (LR) that had more than 24 months of PFS. All patients were diagnosed with advanced lung adenocarcinoma and harbored *EGFR* 19del or L858R mutations before treatment. Paired tumor samples collected before treatment and after relapse (or at the last follow-up) were subjected to targeted next-generation sequencing of 416 cancer-related genes. SR patients were significantly younger than LR patients ( $P < .001$ ). Collectively, 88% of SR patients had *TP53* variations compared to 13% of LR patients ( $P < .001$ ). Additionally, 37.5% of SR patients carried *EGFR* amplifications compared to 8% of LR patients. Other potential primary resistance factors were also identified in the pretreatment samples of 12 SR patients (75%), including *PTEN* loss; *BIM* deletion polymorphism; and amplifications of *EGFR*, *ERBB2*, *MET*, *HRAS*, and *AKT2*. Comparatively, only three LR patients (25%) were detected with *EGFR* or *AKT1* amplifications that could possibly exert resistance. The diverse preexisting resistance mechanisms in SR patients revealed the complexity of defining treatment strategies even for EGFR-sensitive mutations.

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### Introduction

*EGFR* activating mutations, which are widely known for their prominent role in tumorigenesis and progression, are found in ~20% of patients with non-small cell lung cancer (NSCLC) in Western countries and ~50% in Asia [1]. The two most common mutations, the *EGFR* exon 19 deletion and the exon 21 L858R substitution, account for >90% of known *EGFR* activating mutations [2,3] and are typically treated with first-generation (1st-gen) EGFR tyrosine kinase inhibitors (TKI), such as gefitinib, erlotinib, and icotinib [4,5]. The overall response rate to these small molecular drugs is ~75%, and the median progression-free survival time (PFS) is ~10 months [6–8]. However, in clinical practice, patients who have the indications for 1st-gen TKI treatment demonstrated significant heterogeneity in treatment responses with a PFS ranging from a few months to several years [9,10]. Recent studies have identified multiple

factors that are associated with poor responses or resistance to 1st-gen TKIs, including the primary existence or acquisition of the *EGFR* T790 M

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mutation, *BIM* polymorphisms, inactivation of *TP53*, the activation of alternative signaling pathways (e.g., MET, ERBB2, FLT4), and downstream effectors (e.g., *AKT* amplifications, *PTEN* loss, PIK3CA activation) [11–13]. Tumor cells can also experience histology transformation, such as epithelial-mesenchymal transition (EMT) or the transformation to small cell lung cancer, which reduces treatment sensitivity [12].

Given the complexity of tumor heterogeneity, using *EGFR*-sensitizing mutations as the sole indicator for TKI treatment decision-making without considering the concurrent mutations in the tumor may be insufficient to yield optimal outcomes. To address this issue and identify biomarkers for more precise prognoses, we selected two groups of NSCLC patients who carried *EGFR* 19del or L858R mutations and demonstrated extremely short ( $\leq 6$  months) or long ( $\geq 24$  months) response periods following 1st-gen TKI treatments. Tumor samples prior and posttreatment were collected for tumor genomic profiling by pan-cancer gene next-generation sequencing. It was observed that *TP53* inactivation mutations significantly accumulated in the short-term response group. Additionally, almost all patients in the short-term response group harbored multiple resistance factors that may have compromised TKI efficiency.

## Material and Methods

### Patient Enrollment and Sample Collection

Twenty-eight patients were enrolled from Zhejiang Cancer Hospital between 2011 and 2017, and the last follow-up was conducted in December 2017. Each patient provided written consent to contribute their clinical information and genetic test results for research and publication. The study was approved by the Ethics Committee of Zhejiang Cancer Hospital.

For each patient, formalin-fixed paraffin-embedded (FFPE) tissue sections or slides that were collected before TKI treatment were retrieved from the archive. Following TKI treatment, FFPE tissues or peripheral blood was collected again from patients, if clinical conditions permitted. All FFPE specimens were histologically examined by pathologists to ensure the tumor content was above 10% before proceeding to DNA extraction and sequencing.

### DNA extraction, library preparation, and targeted enrichment

FFPE samples were deparaffinized with xylene, and genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen) following manufacturer's instructions. For some patients, cell-free DNA (cfDNA) was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen). Genomic DNA of whole blood was extracted using the DNeasy Tissue and Blood Kit (Qiagen) and used as the normal control to remove germline variations. DNA was quantified on a Qubit 3.0 Fluorometer with the Qubit dsDNA HS Assay kit (Thermo Fisher), and the quality was evaluated by a Nanodrop 2000 (Thermo Fisher). The size distribution of cfDNA was analyzed using a Bioanalyzer 2100 with High Sensitivity DNA kit (Agilent Technologies).

Library construction was performed as previously described [14]. Briefly, 1–2  $\mu\text{g}$  of genomic DNA was sheared into ~350 bp fragments using a Covaris M220 instrument. For cfDNA, 2–200 ng was used as the input for library preparation, without DNA shearing. End repair, A-tailing, and adaptor ligation of fragmented DNA were performed using the KAPA Hyper DNA Library Prep kit (Roche Diagnostics), followed by size selection with Agencourt AMPure XP beads (Beckman Coulter). The ligated products were PCR amplified and purified by Agencourt AMPure XP beads.

Different DNA libraries with unique indexes were pooled and subjected to targeted enrichment using customized xGen lockdown probes (Integrated DNA Technologies) that were designed to capture 416 cancer-related genes and 16 frequently rearranged genes. Human cot-1 DNA (Life Technologies) and xGen Universal Blocking Oligos (Integrated DNA Technologies) were added as blocking reagents. The capture reaction was performed with Dynabeads M-270 (Life Technologies) and the xGen Lockdown Hybridization and Wash kit (Integrated DNA Technologies), according to manufacturers' protocols. Captured libraries were subjected to PCR amplification with KAPA HiFi HotStart ReadyMix (KAPA Biosystems). The purified library was quantified using the KAPA Library Quantification kit (KAPA Biosystems), and its fragment size distribution was analyzed using a Bioanalyzer 2100.

### Sequencing and Bioinformatics Analysis

DNA libraries were sequenced on an Illumina HiSeq4000 platform (Illumina). Sequencing data were processed and genomic alterations were analyzed according to a previous report with minor modifications [14]. Briefly, Trimmomatic was used for FASTQ file quality control (QC) to remove leading/trailing low quality (quality reading below 15) or N bases before mapping to the reference genome. Only qualified reads were mapped to the reference human genome, hg19, using the Burrows-Wheller Aligner (BWA-mem, v0.7.12) with default parameters. Single nucleotide variants (SNVs) and indels were detected by VarScan. Single nucleotide polymorphisms (SNPs) with mutation allele frequencies (MAFs)  $>30\%$  were filtered using the 1000 Genomes Project or 65,000 exomes project (ExAC) and were removed from the final reports if present in  $>1\%$  population frequency in the databases. ADTEX (<http://adtex.sourceforge.net>) was used to identify copy number variants (CNVs) using a normal human HapMap DNA sample, NA18535, as the reference. The relative depth ratios were smoothed by discrete wavelet transformation techniques prior to applying the Hidden Markov Model to estimate ploidy, normal contamination ratio, and absolute CNVs. Tumor mutation burden (TMB) was defined as the number of somatic mutations with synonymous somatic mutations included. Allele-specific copy number alterations and loss of heterozygosity (LOH) were analyzed by FACETS algorithms according to the instructions [15].

### Drug Resistance Mechanism Analysis

Thirteen SR patients had tumor tissue or liquid biopsy samples sequenced following resistance to 1st-gen TKIs. Acquired mutations were defined as newly observed alterations following the development of resistance. For the analysis of relative MAFs for epigenetic modifiers, *TP53* mutations or *EGFR*-activating mutations (only used in *TP53* mutant-free samples) were considered clonal and used for MAF correction for other mutations [16]. The method was based on the theory that drug resistance clones in tumors demonstrate a growth advantage under selective pressure.

## Results

### Clinical Characteristics of the Study Cohort

In this study, 28 patients with advanced lung adenocarcinoma were retrospectively included based on their responses to 1st-gen *EGFR* TKI treatments. Twenty-six patients were at stage IV disease with either distant or in-chest metastasis, while the remaining two patients

**Table 1.** Summary of Clinical Characteristics of the SR and LR Groups

		SR (n = 16)	LR (n = 12)	P Value
Age	Median (range)	52 (34-71)	66 (53-75)	P = .001
Sex	Female	9 (56.3%)	10 (83.3%)	NS
	Male	7 (43.7%)	2 (16.7%)	
Smoking	Yes	6 (37.5%)	2 (16.7%)	NS
	No	10 (62.5%)	10 (83.3%)	
Lines of 1st-gen TKI treatment	1st line	10 (62.5%)	7 (58.3%)	NS
	2nd line	6 (37.5%)	5 (41.7%)	
1st-gen TKI	Gefitinib	7 (43.75%)	4 (33.3%)	NS
	Icotinib	8 (50%)	8 (66.7%)	
	Erlotinib	1 (6.26%)	0	
Metastasis status	No	1 (6.3%)	1 (8.3%)	NS
	Yes	15 (93.7%)	11 (91.7%)	
EGFR mutations	19del	7 (43.8%)	6 (50%)	NS
	L858R	9 (56.2%)	6 (50%)	

NS, not significant.

were stage III disease (Supplementary Table 1). All patients harbored either EGFR exon19 deletions (19del, including two exon19 insertion/deletion alterations) or the L858R mutation, and had

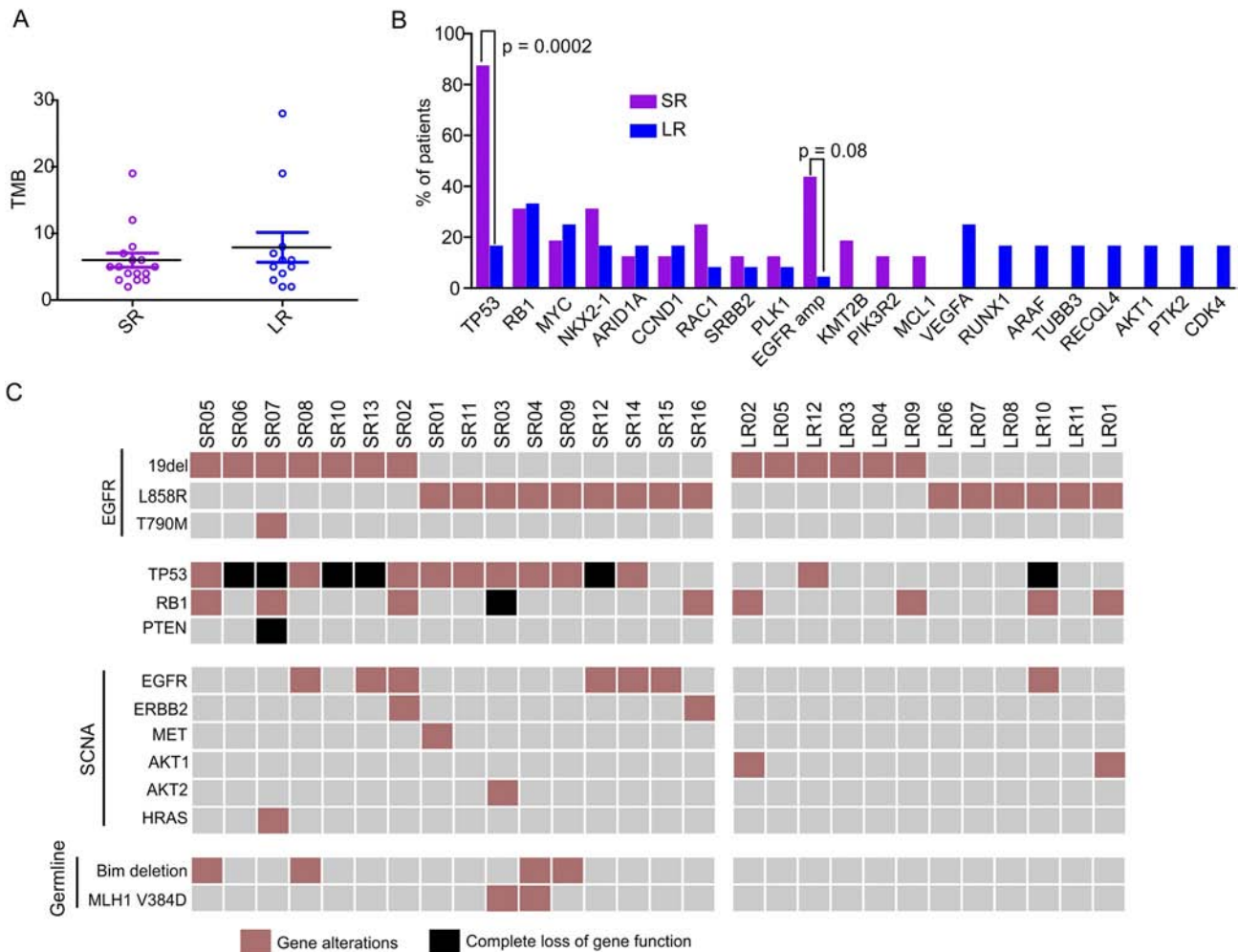
received the 1st-gen EGFR TKI as the first- or second-line treatment (Supplementary Table 1). Gefitinib was administered in 11 patients, while icotinib was administered in 16 patients (Table 1).

Patients were divided into two groups: the short-term responders (SR, n = 16) with a progression-free survival (PFS) of less than 6 months on 1st-gen TKI (median: 3 months, range: 1-6 months) and the long-term responders (LR, n = 12) with extremely long PFS of over 24 months (median: 36.5 months, range: 24-60 months) (Supplementary Table 1).

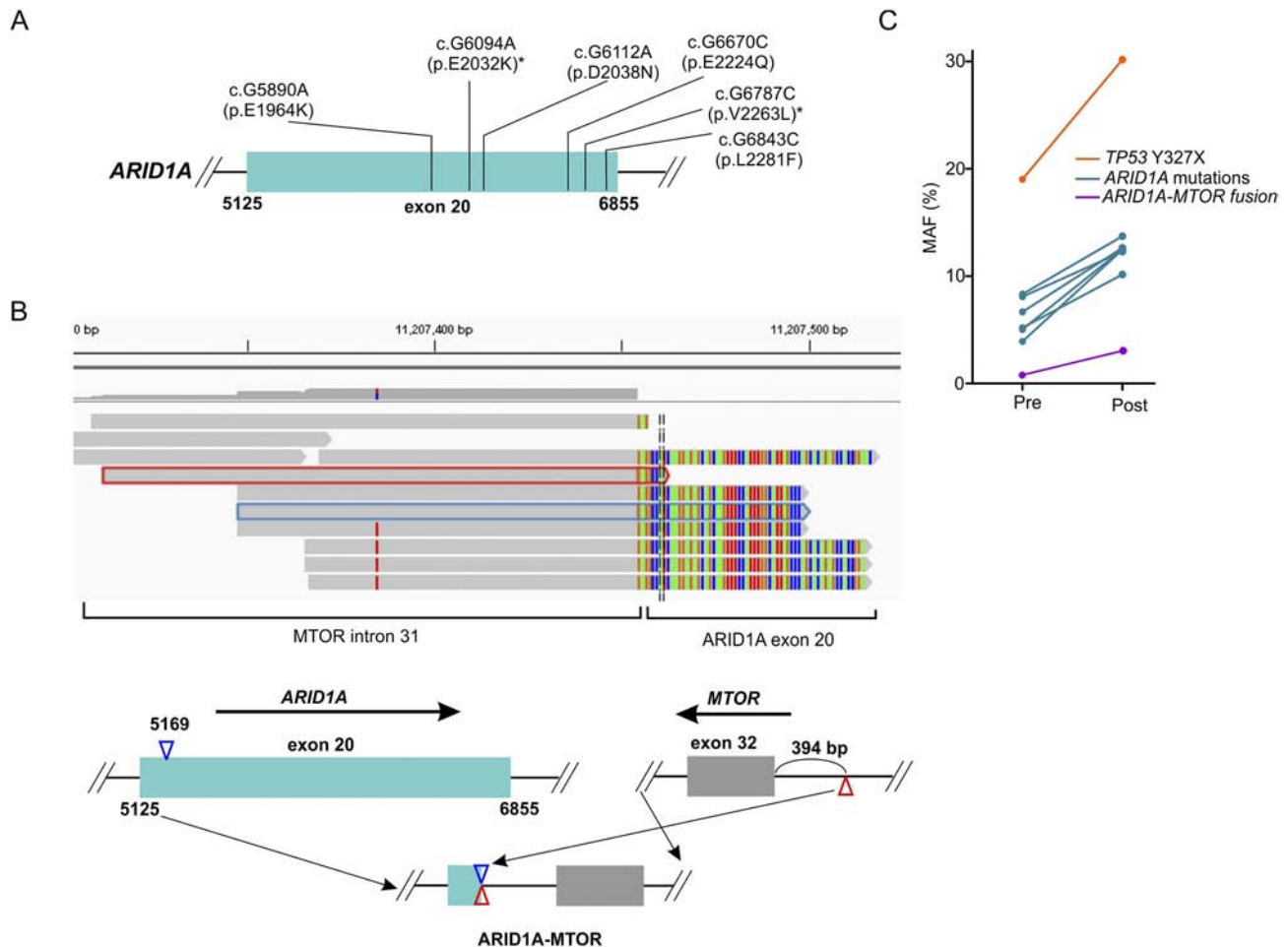
Demographic and clinical characteristics were compared between the SR and LR groups and revealed that SR patients (median: 52 years old, range 34-71) were significantly younger than LR patients (median: 66 years old, range 53-75) (P = .001) (Table 1). Other features, such as sex, smoking history, the treatment lines of TKI, the administered TKI, metastasis, and EGFR mutation status, were not significantly different between the two groups.

**High Prevalence of TP53 Alterations and EGFR Amplification in the SR Group Prior to TKI Treatment**

To explore the innate genomic alterations that might compromise 1st-gen TKI treatments, the tumor genomic backgrounds of all



**Figure 1.** Genetic profiling of tumor samples from SR and LR groups. (A) Tumor mutation burden of each patient in SR and LR groups. Each circle represents one patient. The Mann-Whitney test was used to assess statistical differences between the two groups. (B) Comparison of the most frequently mutated genes in SR and LR groups. The chi-squared test was used to compare the proportions of each group that contained the mutations listed. (C) Mutation plot of potential resistance mechanisms in each patient (each column represents one patient). Complete loss of gene function (black block) was due to the double strikes on two gene alleles.



**Figure 2.** Gene alterations of *ARID1A* in patient #SR01. (A) A schematic map indicating multiple missense mutations identified in exon20 of the *ARID1A* gene in patient #SR01. Asterisks (\*) indicate mutations present in the COSMIC database. (B) An integrative genomic viewer (IGV) visualization of the sequencing reads of the *ARID1A-MTOR* fusion gene (top panel). The gray portion indicates sequencing reads of *MTOR* intron 31, while the colored portion represents the sequencing reads of *ARID1A* exon 20. The schematic map (bottom panel) shows the structure of the *ARID1A-MTOR* fusion locus. Exons 1-20 of *ARID1A* (light green) were fused to intron 31 of *MTOR* (gray). Blue and red triangles indicate the broken positions of two genes. (C) Mutation allele frequency changes for genes before and after TKI treatment. *Pre*, pretreatment; *Post*, posttreatment.

patients prior to TKI treatment were profiled using targeted next-generation sequencing (NGS) covering >400 cancer-related genes. The prevalence of each mutation is displayed in Supplementary Figure 1. Despite the substantial difference in PFS between the two groups, the tumor mutation burden (TMB) in the pretreatment samples of the SR and LR patients were not significantly different (Figure 1A,  $P = .67$ ). However, *TP53* was mutated in 87.5% (14/16) of SR patients, which was significantly higher than 16.7% (2/12) in LR patients (Figure 1B) ( $P = .0002$ ). The *TP53* mutations in SR patients were dispersed throughout exons 4-8, 10, and 11, with exon 5 mutations accounting for 33%. Mutations in LR patients were in exons 6-8 only (Supplementary Table 2).

Complete loss of *TP53* function was observed in five SR patients (31.3%) due to the loss-of-function mutation on one allele and LOH of the other wild-type allele. Only one LR patient (8.3%) exhibited such a phenomenon (Figure 1C). In addition, *EGFR* copy number gain was more common in SR patients (6/16, 37.5%) than in LR patients (1/12, 8%) ( $P = .08$ , Figure 1, B and C). Allele-specific amplification of *EGFR* was analyzed by FACETS [15], which confirmed that seven *EGFR*-amplified samples (6 SR and 1 LR

patients) exhibited imbalanced amplification of the mutated *EGFR* allele (Supplementary Figure 2). As a result, the MAFs of the *EGFR* L858R or 19del mutations in those samples were all above 50% (Supplementary Figure 2A). One SR patient (#SR07) carried the *EGFR* T790 M mutation before TKI treatment, which caused intrinsic resistance to icotinib (Figure 1C).

#### Other Potential Factors Associated with the Short-Term Response to TKI Treatment

We summarized all known and putative factors that could shorten the response to TKIs, including the functional loss of *TP53* and *RB1*, the *EGFR* T790 M mutation, alternative pathway activation via other tyrosine kinase receptors (e.g., *ERBB2*, *MET*, *FLT4*), downstream factors (e.g., *PI3K/AKT* and *MEK/ERK*), and germline variations (e.g., *BIM* deletion polymorphism and *MLH1* V384D) [12,17]. It was observed that 93.8% (15/16) of SR patients harbored at least one intrinsic factor that could decrease TKI efficacy, while 75% (12/16) of patients had more than one factor (Figure 1C). One patient (#SR15) had no resistance factors identified prior to treatment, except for *EGFR* amplification, but she acquired the *EGFR* T790 M

**Table 2.** Alterations in Epigenetic Modifiers Identified in Pre/Posttreatment Samples

Patient ID	Gene	Alteration	Pretreatment MAF	Posttreatment MAF	Fold Change	Alteration Type
SR01	<i>ARID1A</i>	p.E2032K (c.G6094A)	6.68%	1.19		Missense
	<i>ARID1A</i>	p.E2224Q (c.G6670C)	8.33%	1.04		Missense
	<i>ARID1A</i>	p.V2263 L (c.G6787C)	5.04%	1.56		Missense
	<i>ARID1A</i>	p.D2038N (c.G6112A)	8.09%	0.96		Missense
	<i>ARID1A</i>	p.E1964K (c.G5890A)	5.20%	1.23		Missense
	<i>ARID1A</i>	p.L2281F (c.G6843C)	3.91%	2.01		Missense
	<i>ARID1A</i>	ARID1A-MTOR	1.00%	2.08		Fusion
SR02	<i>KMT2B</i>	p.L23351 (c.C7003A)	44.40%	0.72		Missense
	<i>KMT2B</i>	p.S2135X (c.C6404G)	40.06%	0.72		Stop gained
SR03	<i>KMT2B</i>	KMT2B:exon2-ZNF254&cLINC00662	5.63%	0.28		Fusion
SR04	<i>TET2</i>	p.V1064 fs (c.3191_3197delTTTGTGAC)	4.31%	1.19		Frameshift
	<i>DAXX</i>	p.P540fs (c.1618_1637delCCCTCCAGCATAGATGCTGA)	2.43%	1.47		Frameshift
SR08	<i>DAXX</i>	p.S564F (c.C1691T)	4.88%	1.22		Missense
	<i>ARID1A</i>	p.Q1519X (c.C4555T)	13.25%	0.77		Stop gained
	<i>SETD2</i>	p.E2528K (c.G7582A)	7.37%	0.69		Missense
	<i>EP300</i>	p.M2278 V (c.A6832G)	5.59%	N/A		Missense
	<i>KMT2B</i>	p.G2286 V (c.G6857 T)	7.09%	N/A		Missense
LR08	<i>BRD4</i>	p.D650N (c.G1948A)	6.63%	N/A		Missense
	<i>SETD2</i>	p.L483R (c.T1448G)	22.01%	N/A		Missense
	<i>KMT2A</i>	c.G503-1A	7.11%	N/A		Splicing variant
	<i>CREBBP</i>	p.L151 V (c.C451G)	2.53%	N/A		Missense
LR09	<i>ARID1A</i>	p.Y311N (c.T931A)	5.24%	N/A		Missense
	<i>ARID1A</i>	p.312_322del (c.936_965delCGGGGGCGACTACAGTGGCGGGCCCCAGGA)	4.72%	N/A		In frame deletion

mutation after 6 months of gefitinib treatment. Two other patients also acquired the *EGFR* T790 M mutation (patient #SR13, SR14), while one patient obtained *EGFR* amplification (#SR01) after resistance developed (Supplementary Table 3).

Aside from *TP53* and *EGFR* variations, other recurrent resistance factors in SR patients included *RBI* loss (31.3%,  $n = 5$ ), germline BIM deletion (25%,  $n = 4$ ), and the *MLH* V384D variant (12.5%,  $n = 2$ ). Other genes that recurrently mutated in SR patients but not LR patients included *KMT2B* (20%,  $n = 3$ ), *PIK3R2* (13%,  $n = 2$ ), and *MCL1* (13%,  $n = 2$ ) (Figure 1B) mutations, but the significance of those mutations requires further investigation in a larger patient cohort. Somatic copy number alterations (SCNA) were also more common in the SR group than in LR patients. In addition to *EGFR* amplification, five SR patients harbored SCNA for *ERBB2*, *MET*, *AKT2*, or *HRAS* (Figure 1C).

The mutation status of epigenetic regulators was also assessed since their abnormal activities are putative resistance mechanisms to TKIs [18]. Five SR patients and two LR patients had at least one genetic alteration in genes related to epigenetic regulation [19] (Table 2). In patient SR01, six *ARID1A* missense mutations were identified, as well as a fusion between *ARID1A* exon 20 and *MTOR* intron 31 that removed the glucocorticoid receptor-binding domain of *ARID1A* and impaired the chromatin-remodeling function of the SWI/SNF complex [20] (Figure 2, A and B). Following 3 months of gefitinib treatment, resequencing of the resistant tumor revealed that all *ARID1A* mutations were relatively enriched (the calculation method is described in Methods) during the course of treatment (Table 2, Figure 2C), suggesting that the functional loss of *ARID1A* might be associated with the TKI resistance. Moreover, patient #SR04 experienced *TET2* V1064 fs and *DAXX* P540fs enrichment following disease progression, implicating those factors in TKI resistance as well (Supplementary Table 3).

## Discussion

Previous reports indicated that 50%–60% of advanced *EGFR*-mutant lung cancers harbor *TP53* mutations [10,21,22]. The concomitant loss of heterozygosity of *TP53* also frequently occurs, resulting in a gain of function (GOF) of *TP53* and more aggressive tumorigenesis [23,24]. Among SR patients in this study, 88% (14 out of 16) carried

loss of function alterations in *TP53*, with five having concurrent LOH. The LOH frequency might be underestimated in targeted NGS since the SNPs in a chromosomal segment that can be used by FACETS for heterozygosity analyses are limited. Therefore, we suspect that the *TP53* GOF might be a more common phenomenon in advanced *EGFR*-sensitizing NSCLC patients that demonstrate poor responses to TKI treatments.

On the other hand, whether *EGFR* amplification can confer TKI resistance remains unknown. Shan et al. found that the concurrence of *EGFR* amplification and TKI-sensitive *EGFR* mutations in lung cancer patients was correlated with a longer PFS of TKI treatment [25]. However, another study found that *EGFR* amplification was more prevalent in patients with innate resistance to the third-generation TKI rociletinib than patients with acquired resistance, suggesting that *EGFR* amplification might be a resistance mechanism to TKIs. A third study by Shigenari stated that amplification of *EGFR* wild-type alleles conveyed resistance to *EGFR* TKIs [26]; however, in our study, allele-specific analysis of *EGFR* in six patients (6 SR and 1 LR patients) found uneven amplification of the mutant allele rather than the wild-type allele. Thus, it is more likely that *EGFR* amplification—particularly on the mutant allele—is a resistance mechanism in *EGFR*-sensitizing TKI treatments.

The resistance-conveying role of epigenetic modifiers in treatment is garnering attention due to their dynamic features compared to genetic heterogeneity, as well as the potential for reversing resistance or extending the treatment benefits by targeting such modifiers [18]. *ARID1A* is one of the most frequently mutated genes in human cancers and is present in 7% of lung adenocarcinomas [27]. Previous studies observed that loss of *ARID1A* was correlated with resistance to the ERBB2-targeting antibody trastuzumab by activating the AKT pathway [28]. In this study, we observed the outbreak of multiple missense mutations and one function-impairing fusion in *ARID1A* in patient #SR01. All mutation clones were enriched postprogression on gefitinib, indicating a possible correlation to resistance.

In patient #SR8, a frame-shift mutation of the *DAXX* gene, which encodes a chromatin-remodeling factor that regulates gene transcription, was also dramatically enriched following treatment. It has been

suggested that the Daxx protein is involved in the suppression of the EMT of tumor cells and its functional loss facilitates tumor invasion [29]. Since EMT is another resistance mechanism in TKI treatment, the functional damage of Daxx might also accelerate resistance.

By investigating the genetic profiles of two groups of patients with different response periods to 1st-gen TKI treatments (SR vs LR), we observed a significant accumulation of *TP53* alterations and more frequent *EGFR* amplifications in patients with short-term responses (SR), thus indicating their possible roles in delivering the poor outcomes. Additionally, mutations in epigenetic modifiers such as *ARID1A* and *DAXX* were more common in short-term responders, and their enrichment in the resistance samples suggests their possible involvement in TKI resistance. The validation of those findings in a large cohort will be useful in identifying molecular biomarkers for patient selection prior to TKI treatment.

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