Next-generation sequencing reveals genetic heterogeneity and resistance mechanisms in patients with *EGFR*-mutated non-small cell lung cancer treated with afatinib

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Co-occurring mutations do not hinder afatinib's effectiveness in *EGFR*-mutated NSCLC. *EGFR* p.T790M predicts better outcomes, while *MET* amplification and *TP53* mutations indicate poorer OS. https://bit.ly/3vydDiy

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Abstract

Background Afatinib, an irreversible ErbB family inhibitor, is widely used as first-line treatment in advanced lung adenocarcinoma patients harbouring mutant epidermal growth factor receptor (EGFR). With the advancements in next-generation sequencing (NGS), comprehensive research into the clinical impact of co-occurring genetic mutations and the molecular mechanisms of acquired resistance is required for afatinib users.

Materials From January 2010 to December 2019, we enrolled patients with advanced lung adenocarcinoma with *EGFR* mutations using afatinib as first-line treatment, and we retrospectively collected pre- and post-afatinib treatment specimens from these patients for NGS testing.

Results Of the 362 enrolled patients, 73 samples (68.9%) from 56 patients successfully returned complete NGS reports. In pre-afatinib treatment specimens, the most frequent co-occurring alterations were *TP53*, *MUC16*, *USH2A*, *SNYE1*, *RECQL4* and *FAT1*; however, they were not related to progression-free survival. Small cell lung cancer transformation, *EGFR* p.T790M, amplification of *MET*, *ERBB2*, *KRAS*, *EGFR*, cell cycle-regulated genes and *MDM2*, and *PTEN* alterations were identified as acquired resistance mechanisms. *EGFR* p.T790M (p=0.0304) and *APC* alterations (p=0.0311) in post-afatinib specimens were significantly associated with longer overall survival, while *MET* amplification was significantly associated with poor overall survival (p=0.0324). The co-occurrence of *TP53* alterations was significantly associated with shorter overall survival (p=0.0298).

Conclusions Our results show that the frequent co-occurring alterations in advanced *EGFR*-mutated lung adenocarcinoma did not influence the effectiveness of afatinib. *EGFR* p.T790M is not only the major resistance mechanism to afatinib but also related to favourable survival outcomes. *MET* amplification and *TP53* mutations were associated with poorer overall survival.

Introduction

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are effective in treating advanced non-small cell lung cancer (NSCLC) with mutant *EGFR* [1–4]. However, *de novo* resistance can occur in approximately 20–30% of patients, and other patients who initially respond well to TKIs may develop resistance later [4–6]. The *EGFR* p.T790M mutation remains the main acquired resistance mechanism after first-generation TKI treatment [7, 8]. Although osimertinib was developed to treat p.T790M, it has emerged as the first-line treatment for patients with common *EGFR* mutations [4, 9]. It is

crucial that the more diverse and complex mechanisms of resistance to EGFR TKIs, including osimertinib and other TKIs, are identified for the benefit of these patients.

Next-generation sequencing (NGS) can more accurately detect *de novo* p.T790M mutations at low allele frequencies, as well as other *EGFR* mutations like p.L747S and p.L747P, which are responsible for intrinsic resistance to first-generation TKIs, than commercial real-time PCR kits for *EGFR* detection [10, 11]. Therefore, NGS is a powerful tool to obtain detailed genetic information and help us understand cancer evolution through pre- and post-treatment assessments, which could provide answers about intrinsic and acquired resistance to EGFR TKIs [12].

Aberrations in cell-surface tyrosine kinase receptors and their downstream signalling pathways contribute to carcinogenesis, proliferation and drug resistance in NSCLC [13–16]. Co-mutations in *EGFR*-mutated lung tumours can lead to heterogeneity in TKI responses and survival outcomes [15–17]. Common genetic alterations, including *TP53*, *RB1*, *PIK3CA*, *ERBB2*, *CDK4/6* and *CCNE1*, co-occur in *EGFR*-mutated NSCLC and can affect therapeutic response and small cell transformation risk [6, 15, 17–19]. Amplification of *ERBB2*, *MET*, *CDK4/6* and *CCNE1* is associated with shorter progression-free survival (PFS) after TKI therapy [6, 20]. However, the impact of co-occurring mutations on TKI treatment effectiveness in *EGFR*-mutated NSCLC patients remains inconclusive.

Despite extensive research on resistance mechanisms associated with first- and third-generation TKIs, studies on afatinib are lacking [21, 22]. Afatinib, a second-generation EGFR TKI and ErbB family blocker, could effectively treat common, major uncommon and compound *EGFR* mutations [23]. Unlike other TKIs, afatinib irreversibly inhibits the tyrosine kinase domain of EGFR, and its biological and clinical functions differ from first- and third-generation TKIs [24]. Therefore, our study aims to determine co-occurring genetic alterations and molecular changes in pre- and post-afatinib-treated specimens of *EGFR*-mutated lung adenocarcinoma patients.

Materials and methods

Patients and data collection

We retrospectively obtained the electronic medical record data of patients with advanced *EGFR*-mutated lung adenocarcinoma who received first-line afatinib treatment at National Taiwan University Hospital (NTUH) between January 2010 and December 2019. The remaining formalin-fixed paraffin-embedded (FFPE) tissues were reassessed for NGS analysis. The study was approved by the Research Ethics Committee of NTUH (no. 202010107RINA) and conducted according to the Declaration of Helsinki principles and the International Conference on Harmonization Good Clinical Practice Guideline.

Patients' *EGFR* mutation status was determined using cobas EGFR Mutation Test v2 (Roche Diagnositics), Sequenom MassARRAY genotyping (Agena Bioscience) or direct DNA sequencing [11, 25]. Exon 19 deletion and p.L858R were stratified as common mutations; other mutations, such as p.G719X, p.L861Q and p.T790M, were classified as uncommon mutations.

Response to afatinib was evaluated using the Response Evaluation Criteria in Solid Tumours (RECIST, version 1.1) [26]. PFS was defined as the time from treatment initiation to detection of disease progression or death. Post-progression survival (PPS) was calculated as the time from tumour progression in patients treated with afatinib to death. Overall survival (OS) was calculated as the time from confirmation of advanced-stage (stage IIIB–IVB) lung adenocarcinoma or recurrence after curative treatment to death.

Tissue sample preparation, NGS and genomic analysis

The genomic DNA was extracted from FFPE samples and sequenced using NGS-based targeted sequencing with ACTOnco (ACT Genomics) and an Ion Torrent sequencer (Thermo Fisher Scientific), targeting 440 cancer-related genes (supplementary table S1). The mean coverage was >×500 and the target base coverage was ×100 ⩾85%.

Raw reads were mapped to the human reference genome (hg19) using the Ion Torrent Suite. Single nucleotide variants and short insertions/deletions (INDELs) were identified using the Torrent Variant Caller plug-in from the Clinvar, COSMIC and Genome Aggregation Databases and subsequently annotated using the Ensembl Variant Effect Predictor. Variants with coverage ⩾20, allele frequency ⩾5% and actionable hotspot variants with allele frequencies ⩾2% were reported. Variants with >1% minor allele frequency in the Genome Aggregation database were considered single nucleotide polymorphisms (SNPs). Tumour mutation burden was defined as the total number of nonsynonymous mutations per megabase

within tumour genes. Copy number alterations (CNAs) were analysed using ONCOCNV [27]. A CNA ⩾6 was defined as an amplification and a CNA=0 was defined as a homozygous deletion.

Statistical analysis

Categorical and continuous variables are summarised as percentages and medians. Survival analyses were performed using the Kaplan–Meier method, and log-rank tests were used to compare PFS, PPS and OS among the patient subgroups. PFS, PPS and OS are presented as medians with 95% confidence intervals. Statistical significance was defined as a two-sided p-value <0.05. SPSS version 22.0 (SPSS Inc.) and GraphPad Prism version 8 (GraphPad Software Inc.) were used for statistical analyses and illustrations.

Results

Patient recruitment

A total of 448 patients with advanced NSCLC who were treated with afatinib were recruited; 86 patients were excluded due to wild-type or unknown *EGFR* (n=18), concurrent use of other drugs (n=5), switching to another TKI before disease progression (n=43), receiving afatinib as the second-line or adjuvant therapy (n=16) or using afatinib for <7 days (n=4) (supplementary figure S1). Therefore, 362 patients with advanced *EGFR*-mutated lung adenocarcinoma treated with afatinib as the first-line therapy were enrolled and followed up until December 2021. At the cut-off date, 62 patients had been effectively treated with afatinib and 300 patients had experienced disease progression due to afatinib failure (supplementary figure S1).

Of the 300 patients with afatinib failure, 149 (49.7% of the total population) underwent tissue re-biopsy to examine the resistance mechanisms associated with afatinib. Only 106 tissue samples from 68 patients were available for NGS analysis. 88 tissue samples (83.0%) passed quality control, and 73 specimens (68.9%) from 56 patients passed DNA and sequencing quality control (supplementary figure S1).

Specimen acquisition for the successful NGS

In relation to identifying crucial factors for obtaining successful results in NGS, the preservation time of tissue and the timepoint of acquisition did not have an impact on NGS outcomes. Acquiring specimens *via* bronchoscopic biopsy was significantly associated with failed NGS results (p=0.031) (table 1). Cell blocks obtained from pleural effusion (four out of four specimens, 100%) successfully produced NGS results; specimens from bone tissues (four out of five specimens, 80%) did not.

Characteristics of the enrolled patients

The median age of the 362 enrolled afatinib users was 61.8 years. 213 patients (58.8%) were women, and 257 (71.0%) had never smoked (table 2). 332 patients (91.7%) had a good performance status (Eastern Cooperative Oncology Group performance score of 0–1) and 22 (6.1%) had other malignant diseases. Bone (40.1%) was the most frequent metastatic site. Out of the total patients, 281 (77.6%) had a common *EGFR* mutation, 16 (4.4%) had both common and uncommon mutations, and 65 (18.0%) had uncommon mutations (table 2).

Treatment responses were assessed based on imaging studies and a review of patients' medical records within the *EGFR* mutation subgroups of common mutation, co-occurring common and uncommon mutations (common/uncommon mutations) and uncommon mutations. In our cohort, the partial response rate was 73.2% (table 2). Specifically, partial response rates were 77.2% (217 of 281 patients) for the common mutation group, 68.8% (11 of 16 patients) for the common/uncommon mutations group and 56.9% (37 of 65 patients) for the uncommon mutations group (table 2).

EGFR *status identified* via *non-NGS and NGS methods*

Initially, five patients had common *EGFR* mutations in clinical practice, but NGS revealed that they also had other co-occurring *EGFR* mutations: two had exon 19 deletion and p.T790M, one had exon 19 deletion and p.A566T, one had p.L858R and p.E709G, and one had p.L858R and p.G810D (supplementary tables S2 and S3).

The median PFS, PPS and OS of the 56 patients in the NGS subgroup were 12.26 months, 25.54 months and 43.93 months, respectively (supplementary figure S2A–C). The median PFS of patients with common, common/uncommon and uncommon *EGFR* mutations was 14.62 months (95% CI 10.91–18.34 months), 10.82 months (95% CI 7.59–14.09 months) and 8.95 months (95% CI 0.21–17.69 months), respectively (p=0.299) (supplementary figure S2D). The median PPS of the three subgroups was 25.54 months (95% CI 15.73–35.36 months), 76.39 months (95% CI 0–166.76 months) and 16.53 months (95% CI 8.26–24.79 months), respectively (p=0.259) (supplementary figure S2E). The median OS of the three subgroups

TABLE 1 Factors associated with the successful obtainment of NGS from FFPE specimens

	Total specimens	Success for NGS analysis	Failure for NGS analysis	p-value
Specimens, n	106	73	33	
Timepoint of taking tissues				0.470
Pre-treatment specimen	44 (41.5)	32 (43.8)	12 (36.4)	
Post-treatment specimen	62 (58.5)	41 (56.2)	21 (63.6)	
Biopsy sites				0.274
Lung	52 (49.1)	37 (50.7)	15 (45.5)	
Pleura	10 (9.4)	6 (8.2)	4 (12.1)	
Mediastinal lymph node	8 (7.5)	4 (5.5)	4 (12.1)	
Neck lymph node	5 (4.7)	4 (5.5)	1 (3.0)	
Brain	8 (7.5)	6 (8.2)	2 (6.1)	
Soft tissue	7 (6.6)	6 (8.2)	1 (3.0)	
Liver	6 (5.7)	4 (5.5)	2 (6.1)	
Bone	5 (4.7)	1 (1.4)	4 (12.1)	
Pleural effusion (cell block)	4 (3.8)	4 (5.5)	0	
Adrenal gland	1 (0.9)	1 (1.4)	0	
Modality for taking tissues				0.031*
Surgery (VATS or excisional biopsy)	51 (48.1)	39 (53.4)	12 (36.4)	
CT-guided biopsy	21 (19.8)	15 (20.5)	6 (18.2)	
Echo-guided biopsy	18 (17.0)	13 (17.8)	5 (15.2)	
Bronchoscopy (including EBUS-TBNA)	16 (15.1)	6 (8.2)	10 (30.3)	
Tissue preservation time				0.845
⩽2 years	40 (37.7)	28 (38.4)	12 (36.4)	
>2 years	66 (62.3)	45 (61.6)	21 (63.6)	

Data presented as n (%), unless otherwise indicated. NGS: next-generation sequencing; FFPE: formalin-fixed paraffin-embedded; VATS: video-assisted thoracic surgery; CT: computed tomography; EBUS-TBNA: endobronchial ultrasound-guided transbronchial needle aspiration. *: p<0.05.

was 43.93 months (95% CI 30.12–57.75 months), 76.39 months (95% CI 2.57–150.21 months) and 24.00 months (95% CI 14.70–33.30 months), respectively (p=0.277) (supplementary figure S2F).

Tumour heterogeneity and response to afatinib

The most frequent co-occurring alterations in the 32 pre-afatinib treatment specimens were *TP53* (n=21, 65.6%), *MUC16* (n=14, 43.8%), *USH2A* (n=11, 34.4%), *SYNE1* (n=11, 34.4%), *RECQL4* (n=10, 31.3%) and *FAT1* (n=9, 28.1%); other co-occurring alterations included *EGFR* amplification (n=6, 18.8%), *ROS1* alterations (n=6, 18.8%), *MDM2* amplification/mutations (n=4, 12.5%), *MYC* amplification (n=4, 12.5%) and *RB1* alterations (n=3, 9.4%) (figure 1a and supplementary table S3). There was no correlation between *EGFR* mutation type and any co-occurring genetic alterations among the 32 patients (figure 1a). Although the co-occurrence of *EGFR* amplification was not significantly associated with poor PFS (p=0.0825), the Kaplan–Meier plot for afatinib PFS showed the two curves were separated without any crossover (supplementary figure S3A). Meanwhile, the post-afatinib therapy PFS was not associated with other frequently co-occurring genes such as *TP53*, *FAT1* and *SYNE1* (supplementary figure S3B–D).

Tumour evolution and resistance to afatinib

Compared to pre-afatinib NGS results, patients who failed afatinib treatment showed genetic alterations in p.T790M, *EGFR* amplification, tumour suppressor and associated genes, proto-oncogenes, receptor tyrosine kinase, EGFR regulatory pathways and cell cycle-regulated genes. However, DNA repair, DNA damage response, angiogenesis, apoptosis, transcription factors and their regulation and immune-related mechanisms were not significantly affected by afatinib failure (figure 1a, b and supplementary table S3).

EGFR p.T790M (n=11, 26.8%); amplification of *EGFR* (n=8, 19.5%), *MET* (n=5, 12.2%), *MDM2* (n=3, 7.3%), *ERBB2* (n=1, 2.4%) and *KRAS* (n=1, 2.4%); *PTEN* mutations (n=3, 7.3%); small cell lung cancer

TABLE 2 Characteristics of the enrolled patients

	Total population	NGS subgroup
Patients, n	362	56
Median age (range), years	61.8 (28–89)	59.5 (28–89)
Female	213 (58.8)	33 (58.9)
Never-smokers	257 (71.0)	40 (71.4)
ECOG 0–1	332 (91.7)	53 (94.6)
Co-existence with other malignancies	22 (6.1)	1 (1.8)
Tumour relapse (previous stage ⩽IIIA)	70 (19.3)	12 (23.2)
Metastatic sites		
Bone	145 (40.1)	20 (35.7)
Lung	144 (39.8)	17 (30.4)
Brain	110 (30.4)	16 (28.6)
Pleural seeding or effusion	101 (27.9)	15 (26.8)
Liver	26 (7.2)	2 (3.6)
Adrenal gland	16 (4.4)	3 (5.4)
***EGFR* mutation type**		
Common mutations	281 (77.6)	42 (75.0)
Exon 19 deletion	194 (53.6)	26 (46.4)
p.L858R	85 (23.5)	16 (28.6)
p.L858R+exon 19 deletion	3 (0.8)	0
Common/uncommon mutations	16 (4.4)	4 (7.1)
Uncommon mutations	65 (18.0)	10 (17.9)
Tumour response to afatinib		
Partial response:	265 (73.2)	44 (78.6)
for common mutations	217/281 (77.2)	35/42 (83.3)
for common/uncommon mutations	11/16 (68.8)	3/4 (75.0)
for uncommon mutations	37/65 (56.9)	6/10 (60.0)
Stable disease	64 (17.7)	10 (17.9)
Progressive disease	33 (9.1)	2(3.6)

Data presented as n (%) or n/N (%), unless otherwise indicated. NGS: next-generation sequencing; ECOG: Eastern Cooperative Oncology Group; EGFR: epidermal growth factor receptor.

(SCLC) transformation (n=2, 4.9%); and cell cycle-regulated gene amplification, including that of *CCNE1/2*, *CDK4/6* and *CCND1* (n=11, 26.8%), were identified in the post-afatinib treatment group (n=41) (figure 2 and supplementary table S3). The median tumour mutation burden was low (2.2 mutations·Mb^{-1}) in the post-afatinib group, except for one patient (74.9 mutations·Mb^{-1}) with the longest PFS among all enrolled patients. Among the 11 patients with *EGFR* p.T790M (26.8% of 41 patients), five (12.2%) had p.T790M alone and six (14.6%) had p.T790M co-occurring with other mechanisms. Finally, no resistance mechanisms were identified in 13 specimens (31.7%) from patients in whom afatinib therapy failed (figure 2 and supplementary figure S4).

Post-afatinib failure-induced co-occurring genetic alterations associated with survival outcomes

EGFR p.T790M (p=0.0275) and *APC* alterations (p=0.0304) were associated with a longer PPS (figure 3a, b). Conversely, *ALK* mutations (p=0.0386), *MET* amplification (p=0.0124) and *TP53* mutations (p=0.0189) were associated with significantly shorter PPS (figure 3c–e), but *EGFR* amplification was not (p=0.9499) (figure 3f).

Patients with *EGFR* p.T790M (p=0.0304) and *APC* alterations (p=0.0311) in the post-afatinib specimens had significantly longer OS than those without p.T790M or *APC* alterations (figure 4a, b). *ALK* mutations were associated with poor OS, but this association was not statistically significant (p=0.1044) (figure 4c). *MET* amplification after afatinib failure was significantly associated with poor OS (p=0.0324) (figure 4d). Patients with *TP53* mutations had significantly shorter OS than those without (p=0.0498) (figure 4e), but *EGFR* amplification was not associated with OS (p=0.7957) (figure 4f).

In the 41 post-afatinib specimens, the presence of any resistance mechanism (including *EGFR* p.T790M; amplification of *EGFR*, *MET*, *KRAS* and cell cycle regulatory genes; or *PTEN* alteration) was associated with a better median PFS (p=0.002) and OS (p=0.047) (supplementary figure S5A, B).

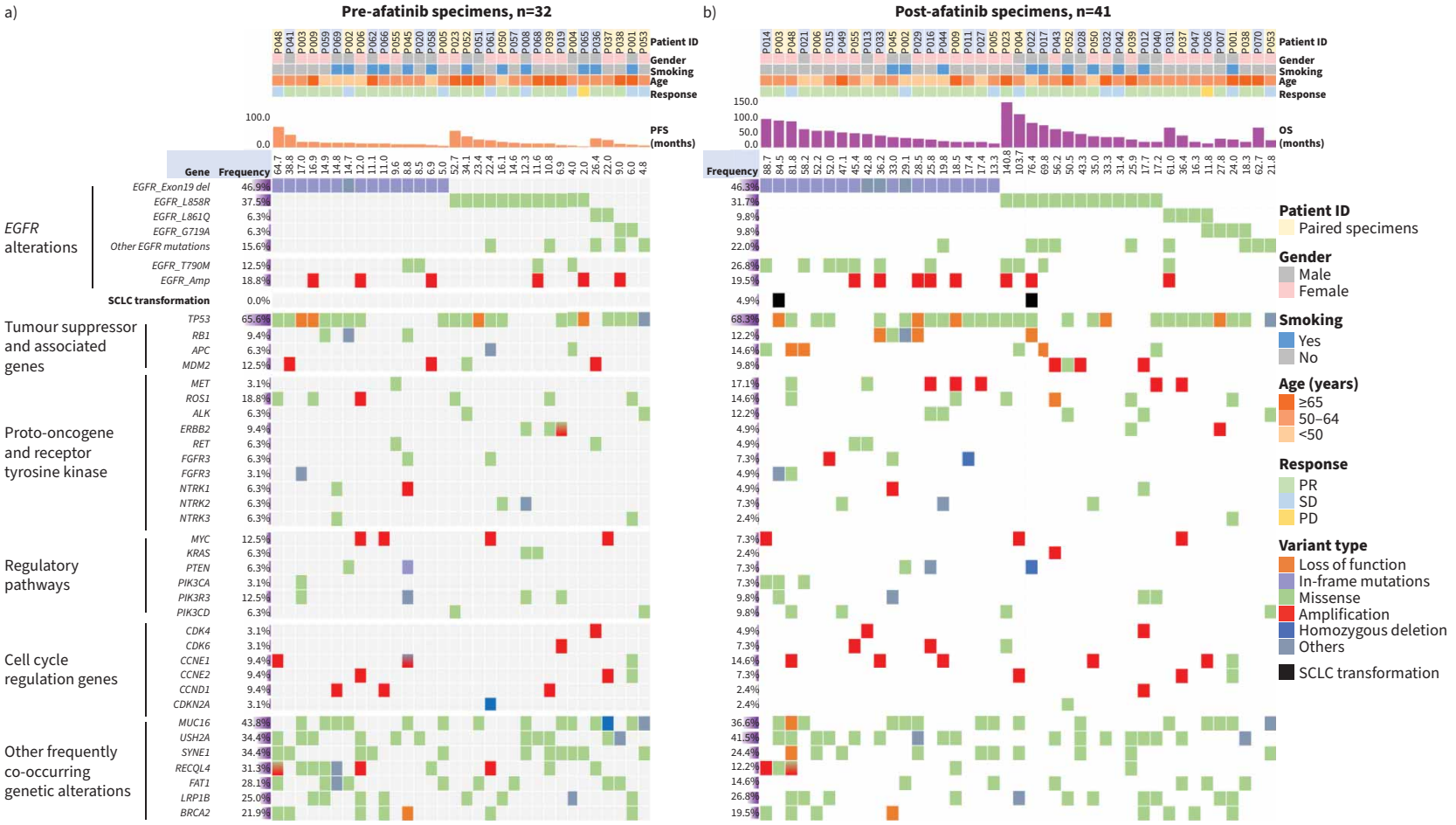

FIGURE 1 Oncoprints of concurrent genetic alterations detected in a) pre-afatinib treatment patients (n=32) and b) post-afatinib treatment patients (n=41). SCLC: small cell lung cancer; PFS: progression-free survival; OS: overall survival; PR: partial response; SD: stable disease; PD: progressive disease.

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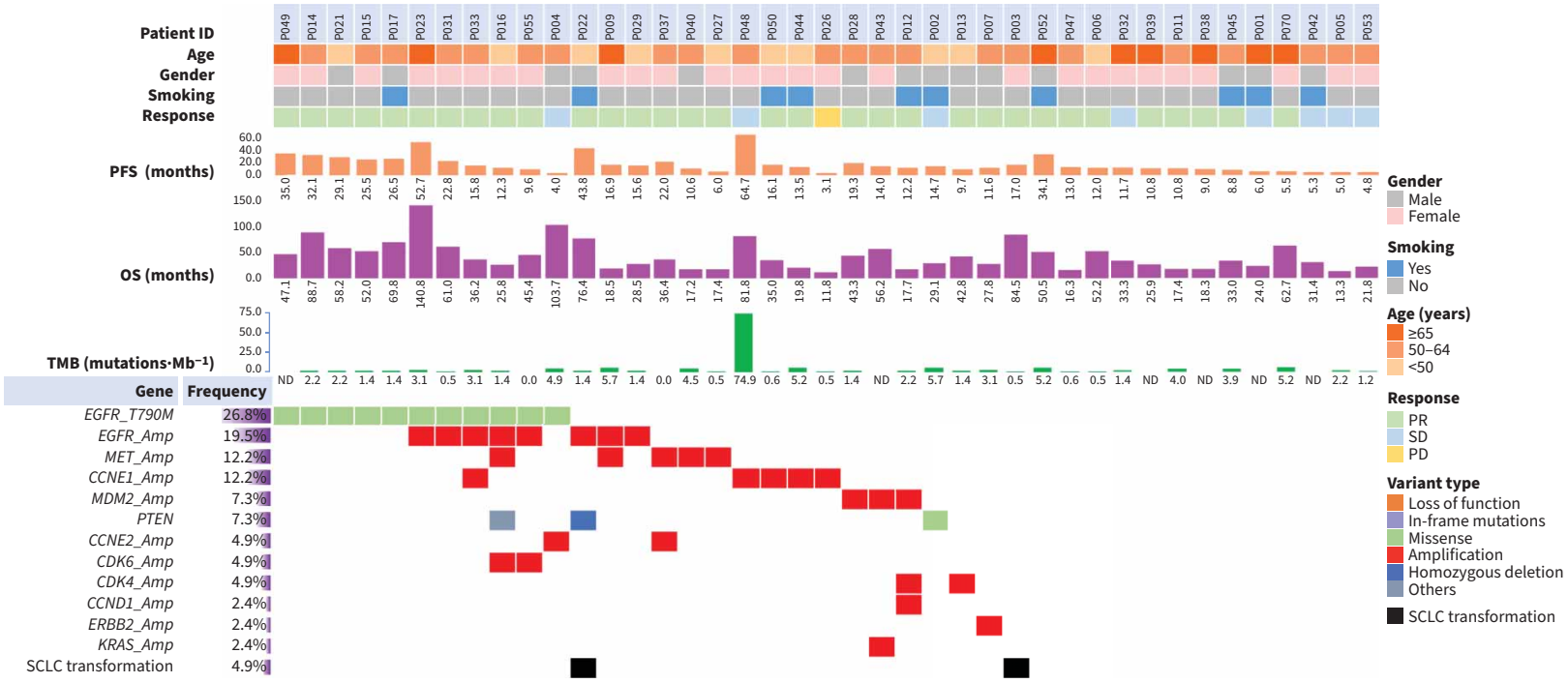

FIGURE 2 Oncoprints for resistance mechanisms in 41 patients after afatinib treatment. PFS: progression-free survival; OS: overall survival; TMB: tumour mutation burden; SCLC: small cell lung cancer; PR: partial response; SD: stable disease; PD: progressive disease; ND: not detected.

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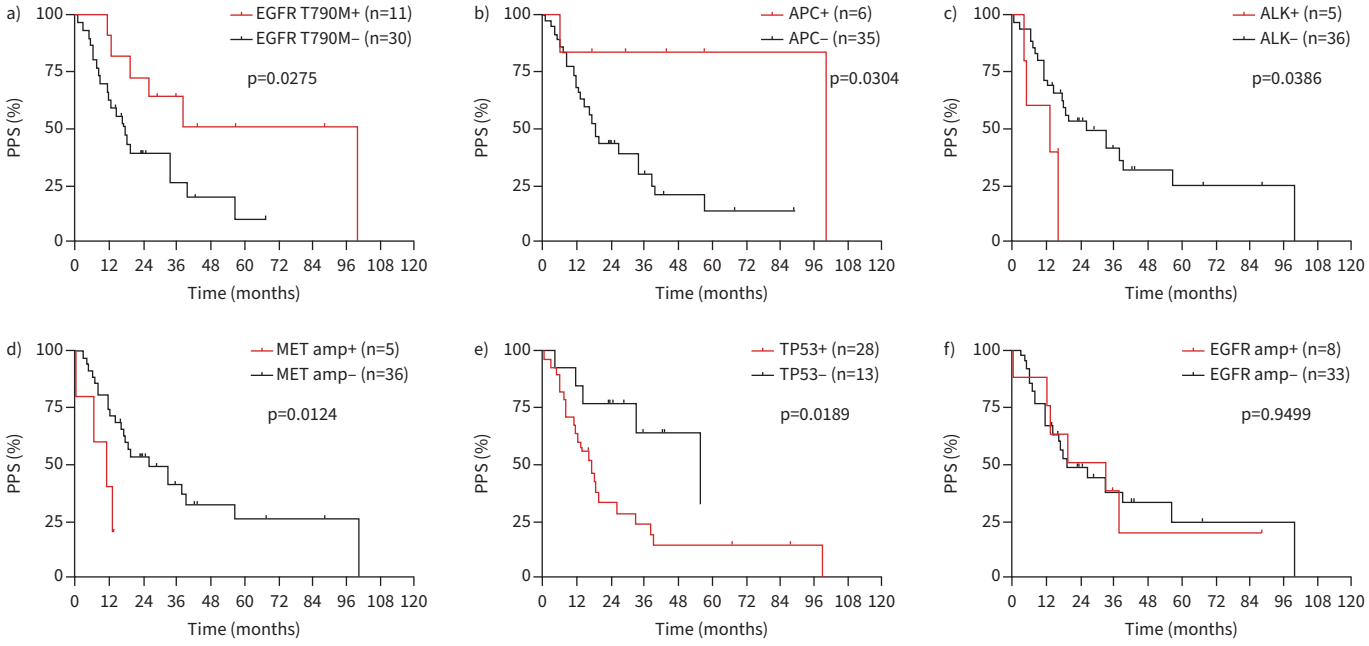

FIGURE 3 Kaplan–Meier curves of post-progression survival (PPS) in 41 patients with (+) or without (–) a) *EGFR* p.T790M, b) *APC* alterations, c) *ALK* mutations, d) *MET* amplification, e) *TP53* mutations and f) *EGFR* amplification.

Discussion

Targeted NGS can comprehensively explore genetic alterations in lung cancer and improve understanding of their interactions with driver genes. Our study found that afatinib could provide a consistent PFS in patients with advanced lung adenocarcinoma, regardless of the presence of uncommon *EGFR* mutations or co-occurring alterations by NGS results. Genomic profiling of post-treatment specimens revealed that *EGFR* p.T790M and *APC* alterations were associated with longer PPS and OS, while *MET* amplification and *TP53* mutations were associated with shorter PPS and OS.

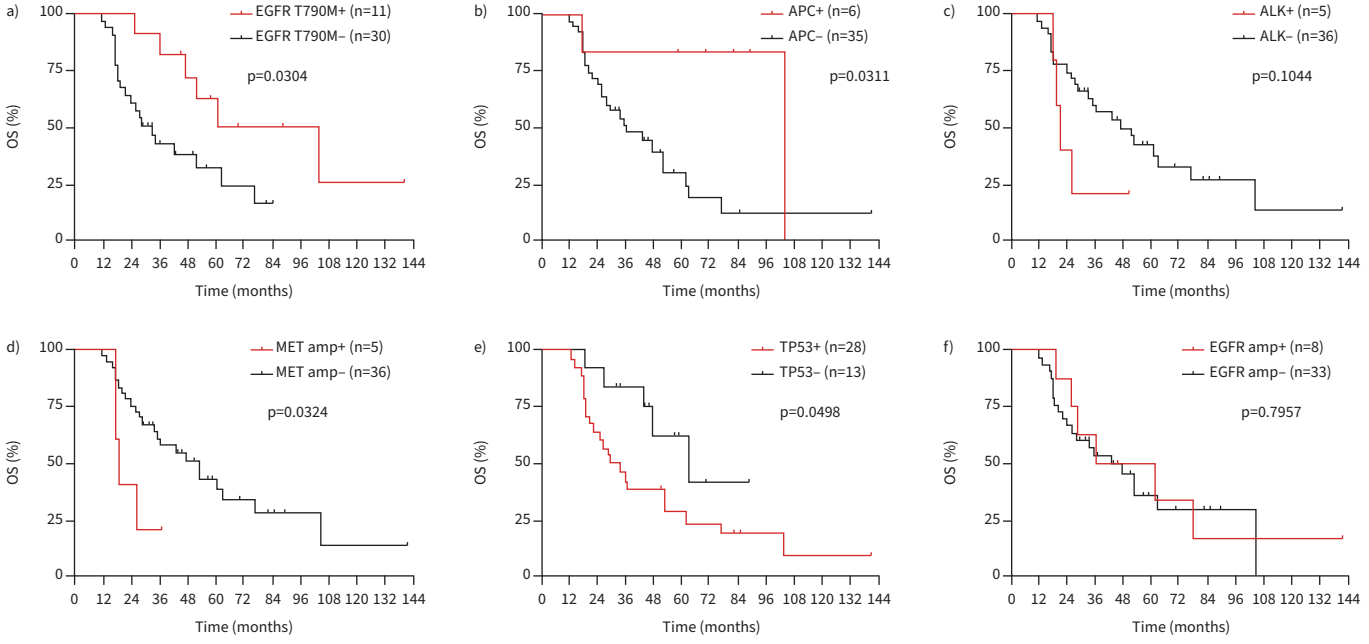

FIGURE 4 Kaplan–Meier curves of overall survival (OS) in 41 patients with or without a) *EGFR* p.T790M, b) *APC* alterations, c) *ALK* mutations, d) *MET* amplification, e) *TP53* mutations and f) *EGFR* amplification.

Some patients diagnosed with common *EGFR* mutations only during routine analysis were found to have uncommon *EGFR* mutations when evaluated by NGS. Afatinib is effective in treating patients with advanced NSCLC with both common and uncommon *EGFR* mutations [11, 23, 28], but first- and third-generation TKIs may not consistently be effective against co-occurring uncommon *EGFR* mutations or may be influenced by co-occurring mutations [11, 29, 30]. Co-occurring alterations, such as *TP53*, *PIK3CA* and *PTEN*, are reportedly associated with poorer outcomes, such as faster resistance to EGFR TKIs and shorter OS [18, 20, 31, 32]. In our study, the most frequent co-occurring alterations in the 32 pre-afatinib treatment specimens were *TP53*, *MUC16*, *USH2A*, *SYNE1*, *RECQL4*, *FAT1* and *EGFR* amplification; however, they were not significantly associated with any *EGFR* type or PFS outcome in afatinib users.

TP53 alterations are the most frequently co-occurring alteration in *EGFR*-mutated NSCLC. Their impact on clinical outcomes may vary depending on cohort size, TKIs and NGS panels used [18, 33, 34]. Nearly 70% of patients with *EGFR* mutation-related lung adenocarcinoma in our study had co-occurring *TP53* alterations. In this study, *TP53* mutations in pre-afatinib treatment specimens were not related to PFS with afatinib, while mutations in post-afatinib treatment specimens were associated with shorter PPS and OS. Five of the six patients with *MDM2* gene amplification did not have *TP53* mutations, which is consistent with a previous study's finding that *MDM2* overexpression and *TP53* mutations are mutually exclusive in some human tumours [18, 35]. The tumour suppressor protein p53 can be degraded by E3 ligase mouse double minute 2 homolog (MDM2), which can be upregulated by abnormal *MDM2* amplification, which could also lead to oncogenesis and primary resistance to TKI in wild-type *TP53* cancers [6, 36]. Meanwhile, *APC* inactivation involving the Wnt pathway promotes *EGFR*-driven *TP53*-deficient lung adenocarcinoma growth *in vivo* [37], but our study showed that the presence of *APC* alterations after afatinib treatment was associated with favourable survival outcomes. The role of *APC* in *EGFR*-mutated lung adenocarcinomas treated with TKI is seldom investigated and requires further study.

We discovered that afatinib-resistance mechanisms share some common mechanisms with resistance to first- and third-generation TKIs [22, 38]. Co-occurrence of *EGFR* amplification in pre-afatinib specimens was associated with poor PFS in our study. Several studies have reported that *EGFR* amplification may be a resistance mechanism to TKI [22, 39]. Among the 41 patients with post-afatinib treatment specimens, five patients (12.2%) were identified to have *MET* amplification as the major acquired resistance mechanism. *MET* amplification-dependent resistance is also recognised as an acquired mechanism in certain NSCLC cases with driver oncogene mutations, such as *EGFR* and *BRAF* mutations, and *ALK*, *RET*, *ROS1* and *NTRK* fusions [40]. Genetic alterations of cyclin-dependent kinases (CDKs) 4/6 and other cell cycle-regulated genes are involved in the development of TKI resistance [41]; CDK 4/6 inhibitors could overcome post-TKI acquired resistance [42]. DNA repair and damage response, transcription factors and their regulation, and immune-related mechanisms did not show molecular changes in our post-afatinib treatment specimens. Acquisition of the p.T790M mutation following EGFR TKI failure remains an important factor in extending patients' PPS and OS owing to the efficacy of osimertinib as a subsequent therapeutic option [9]. Notably, in clinical studies and in our patients, the presence of acquired *MET* amplification after afatinib failure was associated with poor survival outcomes [43]. Compared with first- and third-generation EGFR TKIs, afatinib could irreversibly bind the tyrosine kinase domain of pan-ErbB family members, and afatinib could simultaneously share some resistance mechanisms to first- and third-generation TKIs [22, 38, 44].

To successfully guide cancer treatment and predict patient response, high-quality DNA/RNA and adequate tissue specimens are essential for NGS studies. In our study, tissue re-biopsy was performed on approximately 50% of patients (149 out of 300) with afatinib failure, and 70% of stored tissue samples (73 samples) successfully yielded NGS results. Tissue acquisition is necessary for diagnosis and to ensure sufficient tumour DNA for NGS. The use of nitric acid-based agents to accelerate decalcification and diagnose metastatic bone specimens may affect molecular studies due to poor DNA quality [45]. Although EDTA is less destructive, it is not routinely used in our hospital, and the choice of decalcification agent could have contributed to the lower NGS success rate. Our study also found that a cell block specimen from pleural effusion can be a reliable alternative for NGS, which is consistent with a previous study [46].

Although our study identified important findings, it had limitations, including a small sample size, potential sampling bias and reliance on a commercialised targeted NGS panel. During the study period, gefitinib, erlotinib and afatinib were all reimbursed by the National Health Insurance and prescribed as first-line EGFR TKIs in Taiwan. The drug was chosen according to the physician's preference or experience as discussed in our previous studies [47]. Patients were retrospectively selected based on having received afatinib and undergone biopsies before and after treatment, which may have resulted in a biased

sample. Additionally, the use of the ACTOnco panel for targeted DNA-based amplicon sequencing of 440 genes may have limited the detection of non-targeted genes, fusion genes and low-frequency somatic variations of SNPs and INDELs.

Conclusions

Afatinib as a first-line therapy could provide a consistent PFS outcome in patients with advanced *EGFR*-mutated lung adenocarcinoma, regardless of the presence of uncommon *EGFR* mutations or other co-occurring alterations. NGS of post-afatinib therapy specimens revealed that the mechanisms of resistance to afatinib are complex and heterogeneous. Acquired *EGFR* p.T790M mutation was the main resistance mechanism to afatinib and was associated with better survival outcomes. However, *MET* amplification and *TP53* mutations poorly affected PPS and OS.

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