Acquired multiple *EGFR* mutations-mediated resistance to a third-generation tyrosine kinase inhibitor in a patient with lung adenocarcinoma who responded to afatinib: A case report and literature review

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Abstract. For patients with advanced non-small cell lung cancer (NSCLC) that have epidermal growth factor receptor (*EGFR*) mutations, EGFR tyrosine kinase inhibitors (TKIs) are the standard treatment and have significant clinical benefits. Third-generation TKIs, such as osimertinib, almonertinib and furmonertinib, are effective for the treatment of NSCLC that is EGFR-sensitizing mutation-positive and T790M-positive. Despite the efficacy of third-generation TKIs, patients inevitably develop resistance and the resistance mechanisms are heterogeneous. Second-generation inhibitors, such as afatinib, may be crucial in treating diseases that have developed resistance to first- or third-generation inhibitors. However, the clinical effect of afatinib in patients with acquired multiple *EGFR* mutations is not well defined. To the best of our knowledge, the present report describes the first case of a patient with lung adenocarcinoma who had multiple co-existing *EGFR* resistance mutations, including *EGFR* L718Q, *EGFR* C797S, *EGFR* C797G, *EGFR* L792H, *EGFR* V802F and *EGFR* V689L. These mutations conferred resistance to almonertinib, whilst maintaining sensitivity to afatinib.

Introduction

Lung cancer is the leading cause of cancer-related deaths globally, with non-small cell lung cancer (NSCLC) making up 80-85% of cases (1). In both male and female cases of malignant tumor-related deaths, ~21% are attributed to lung cancer (1). The risk factors for lung cancer include tobacco use, a family history of the disease, exposure to radiation and the presence of chronic lung conditions (2). Epidermal growth factor receptor (*EGFR*) mutations are the most common driver mutations in patients with NSCLC from the Asian population, occurring in ~47% of cases (3). In clinical practice, first-line therapy with EGFR tyrosine kinase inhibitors (TKIs) is recommended, as it enhances the survival of patients with advanced NSCLC with sensitive *EGFR* mutations (4). There are three generations of EGFR TKIs, each with distinct mechanisms of action. First-generation EGFR TKIs, which include gefitinib, erlotinib and icotinib, function as reversible inhibitors. Second-generation EGFR TKIs, including afatinib and dacomitinib, are ErbB family blockers (5). Third-generation EGFR TKIs, such as osimertinib, almonertinib and furmonertinib, overcome the resistance mechanisms posed by first- and second-generation inhibitors by incorporating an acrylamide group, which alkylates the Cys797 residue of the *EGFR* T790M mutation (6). However, drug resistance is inevitable, even with the use of third-generation TKIs. The mechanisms reported include changes in the EGFR signaling pathway, abnormal activation of bypass and downstream signaling pathways and histological transformation (7,8). Efforts are ongoing to clarify their potential targetability; however, these strategies are still mostly in the research phase. To the best of our knowledge, the present report is the first to describe a case in which afatinib therapy could overcome multiple *EGFR* mutations-mediated resistance to a third-generation TKI (almonertinib).

Case report

Patient. A 57-year-old Chinese female patient was referred to Shenzhen People's Hospital (Shenzhen, China) due to the identification of lung nodules in a routine physical examination in December 2018. Positron emission tomography and CT revealed a 2.7x2.5x2.8-cm density mass in the upper lobe of

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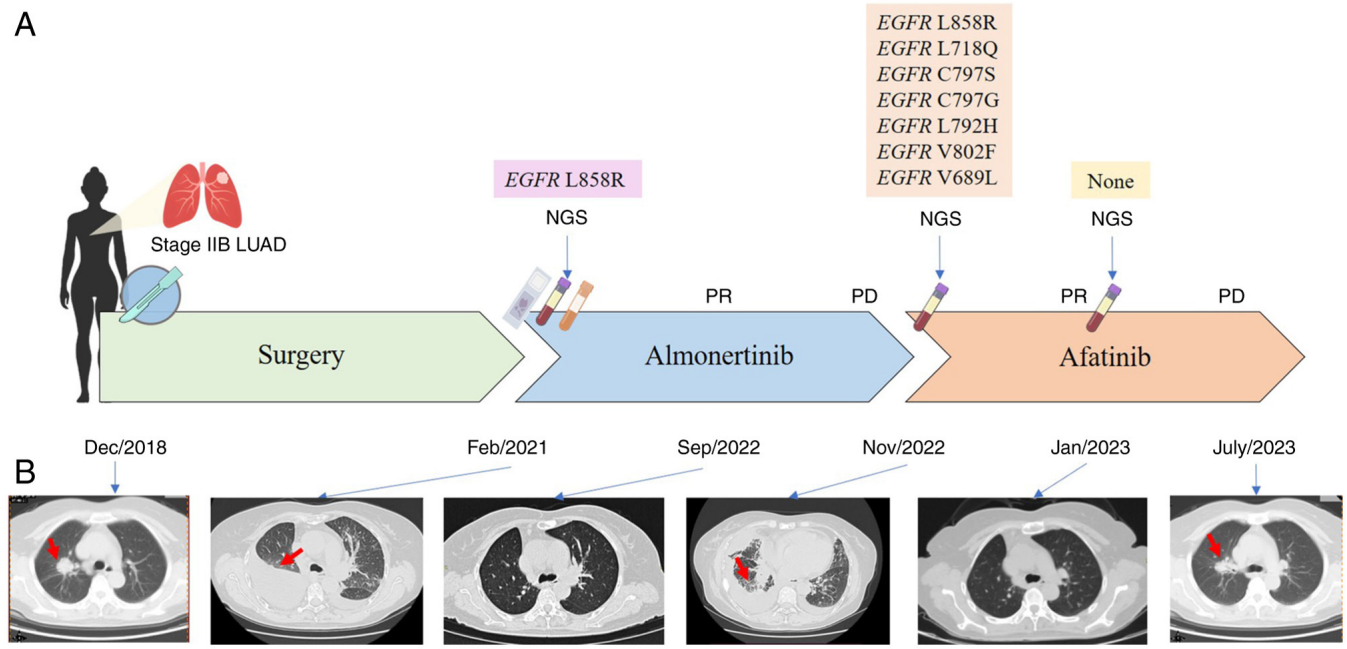

Figure 1. Overview of the treatment history of the patient. (A) Disease timeline illustrating the different treatments received by the patient. (B) Chest CT images, with lung tumors indicated by red arrows. LUAD, lung adenocarcinoma; NGS, next-generation sequencing; PD, progressive disease; PR, partial response; EGFR, epidermal growth factor receptor.

the right lung (Fig. 1A and B). The patient had no underlying medical conditions. Subsequently, thoracoscopic radical surgery was performed for right upper lung cancer, along with a cauterization procedure for right pleural adhesion. Postoperative pathology indicated invasive adenocarcinoma measuring 2.7x2.5x2.8 cm in diameter, which was mainly composed of papillary type (Fig. 2). The patient was diagnosed with stage IIB right upper lung infiltrating adenocarcinoma (T1cN1M0) according to the 8th edition of TNM Staging System (9). As the pathology of the patient was clear and no residual lesions were found, and there were no high-risk characteristics, no adjuvant treatment was administered.

In February 2021, chest CT revealed multiple metastases in both lungs with multiple lymph node metastases in the mediastinum and both lung roots (Fig. 1B). Whole-body emission CT revealed multiple bone metastases in the thoracic vertebrae, bilateral ribs, humerus and scapula. Subsequent targeted next-generation sequencing (NGS) analysis of 425 cancer-related genes (Geneseeq122 Technology Inc.) identified *EGFR* L858R, which had a mutation allelic frequency (MAF) of 17.3% in the plasma, 34.1% in the pleural fluid and 27.8% in the tumor tissue of the upper lobe of the right lung (Table I). Subsequently, the patient was treated with oral almonertinib (110 mg per day) in March 2021, which is a third-generation EGFR TKI. The patient initially achieved a partial response (PR) that was maintained for 20 months (Fig. 1B).

In November 2022, chest CT of the patient revealed that the size of the mass in the upper lobe of the right lung had increased, which indicated progressive disease (PD; Fig. 1B). The patient has several small, spread-out metastatic lesions in both lungs, making a puncture biopsy unsuitable. To identify a more effective therapeutic strategy, targeted NGS was performed using the plasma sample, revealing the presence of *EGFR* L858R (MAF, 27.62%), *EGFR* L718Q (MAF, 8.30%), *EGFR* C797S (MAF, 6.55%), *EGFR* C797G (MAF, 0.56%), *EGFR* L792H (MAF, 0.36%), *EGFR* V802F (MAF, 1.13%) and *EGFR* V689L (MAF, 26.41%; Table I). The patient was then switched to oral afatinib (40 mg per day), a second-generation EGFR TKI, and achieved an initial PR, as indicated by chest CT 2 months later, which revealed marked shrinkage of the lung lesions (Fig. 1B).

In January 2023, follow-up genomic testing revealed that all the genetic alterations of the tumor had disappeared. However, in July 2023, the size of the mass in the upper lobe of the right lung increased, which indicated PD (Fig. 1B). The patient reached a progression-free survival (PFS) of 9 months. During this period, the patient did not receive any other treatment. As the patient refused chemotherapy, immunotherapy was planned for the patient. However, due to economical difficulty, the follow-up treatment was terminated.

Methods

Hematoxylin and eosin staining. Tissue samples were sliced and submerged in 10% neutral buffered formalin. The fixation occurred at 25˚C for 3-6 h. After fixation, the tissue samples were dehydrated, embedded in paraffin, and tissue sections were cut at 4 μm. The paraffin sections were immersed in xylene for 10 min, xylene changed and soaked for another 10 min to dissolve the wax. Samples were rehydrated using a gradient of ethanol concentrations (anhydrous ethanol, 95%, 85%; 70% ethanol), each immersion lasting 5 min. The hydrated tissue sections were cleaned by immersion in PBS solution, each immersion lasting 5 min, repeated three times. Subsequently, tissues were stained in hematoxylin at room temperature for 10 min. Afterwards, excess hematoxylin stain was rinsed with distilled water. The samples were differentiated using 1% hydrochloric acid in ethanol, and the sections were rinsed thoroughly with distilled water. The bluing process was completed using

Table I. Genetic alterations identified through targeted next-generation sequencing in the primary tumor of the upper lobe of the right lung, pleural fluid and serial plasma circulating tumor DNA.

		Baseline			Almonertinib treatment (20 months later)	Afatinib treatment (2 months later)
Gene	Alteration	FFPE, %	Pleural fluid, %	Plasma, %	Plasma, %	Plasma, %
EGFR	L858R	17.3	34.1	27.8	27.62	-
EGFR	L718Q	-	-	-	8.30	-
EGFR	C797S	-	-	-	6.55	-
EGFR	C797G	-	-	-	0.56	-
EGFR	L792H	-	-	-	0.36	-
EGFR	V802F	-	-	-	1.13	-
EGFR	V689L	-	-	-	26.41	-

Mutant allele frequencies are indicated. *EGFR*, epidermal growth factor receptor; FFPE, formalin-fixed, paraffin-embedded; -, not detected.

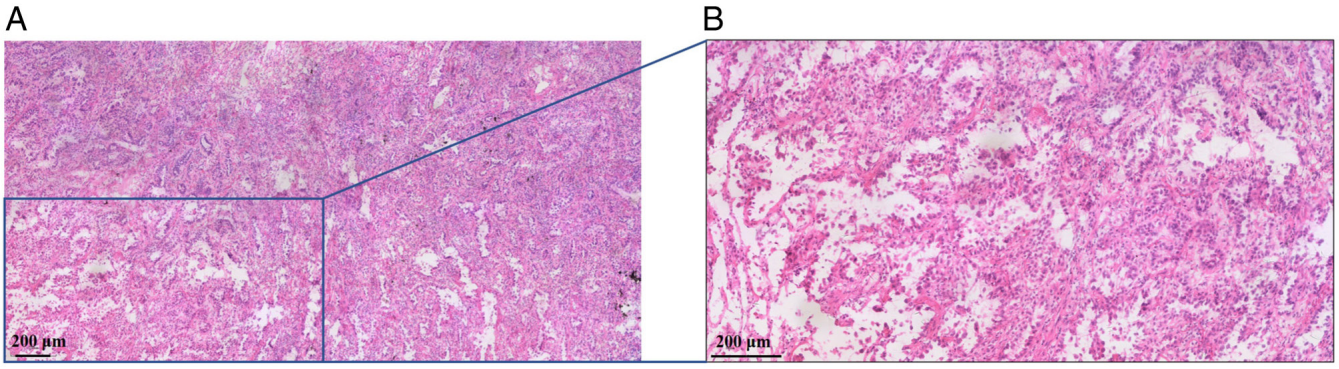

Figure 2. Pathology results. Hematoxylin and eosin staining of the lesion in the right upper lung revealed the presence of lung adenocarcinoma, observed at a magnification of (A) x100 and (B) x200.

0.6% ammonia water, rinsing with clean water, and then rinsing the sections thoroughly with distilled water. The sections were immersed in eosin dye at room temperature for 1 min. The sections were thoroughly rinsed with distilled water, then dehydrated using a gradient of 80% ethanol for 5 sec, 95% ethanol for 2 min and anhydrous ethanol for 2 min. The dehydrated tissue sections were immersed in in xylene twice, each immersion lasting 4 min. Finally, tissue sections were dried and sealed with neutral resin. Images were captured with the Olympus BX43 light microscope (Olympus Corporation).

DNA extraction and targeted enrichment. FFPE genomic DNA was purified using the QIAamp DNA FFPE Tissue Kit (Qiagen). cfDNA was extracted using the NucleoSpin Plasma XS kit (Macherey Nagel) with optimized manufacturer's protocols. Fresh tissue DNA and whole blood DNA were extracted using the DNeasy Blood & Tissue kit (Qiagen GmbH) according to the manufacturer's protocols. The DNA was quantified using the dsDNA HS Assay Kit on a Qubit Fluorometer (Thermo Fisher Scientific, Inc.). Sequencing libraries were prepared using the KAPA Hyper Prep Kit (KAPA Biosystems; Roche Diagnostics), as described previously (10). Indexed DNA libraries were pooled together for probe-based hybridization (11) capture of the targeted gene regions covering 437 cancer-related genes. The finial libraries were quantified by qPCR using the KAPA Library Quantification Kit (KAPA Biosystems; Roche Diagnostics) for sequencing.

Sequencing data processing. Paired-end sequencing of the 300 bp amplicon was performed using the Illumina HiSeq4000 platform (Illumina, Inc.), followed by data analysis as previously described (12). The mean coverage depth was >100x for the whole blood control samples, and >300x for tumor tissues after removing PCR duplicates. For cfDNA samples, the original targeted sequencing depth was >3,000x. The final concentration of the library was determined based on the sample throughput and sample quality. In brief, sequencing data were analyzed by Trimmomatic (13) to remove low-quality (quality <15) or N bases, and were then mapped to the human reference genome, hg19, using the Burrows-Wheeler Aligner (BWA-mem, v0.7.12; https://github.com/lh3/bwa/tree/master/bwakit). PCR duplicates were removed by Picard (available at https://broadinstitute.github.io/picard/). The Genome Analysis Toolkit (GATK 3.4.0; https://software.broadinstitute.org/gatk/) was used to perform

Table II. Literature review of afatinib treatment after progression on third-generation EGFR TKIs in patients with *EGFR*-mutated non-small cell lung cancer.

First author/s, year	Patient no.	Age, years	Sex	Smoker	EGFR mutation	Third-generation TKI treatment	Line of treatment	PFS, months	Resistance mechanism	Afatinib treatment	Line of treatment	PFS, months	(Refs.)
Van Kempen *et al*, 2018	1	36	F	No	Exon 19 deletion	Osimertinib	4	19	EGFR P794L	Afatinib	5	>3.8	(24)
Fang *et al*, 2020	2	55	M	Yes	Exon 19 deletion	Osimertinib	3	3	EGFR G724S	Afatinib	4	>3.8	(25)
Liu *et al*, 2019	3	65	F	NA	L858R, T790M	Osimertinib	2	9	EGFR L718Q	Afatinib	3	4	(26)
Fang *et al*, 2019	4	45	M	Yes	L858R, T790M	Osimertinib	2	8	EGFR L718V	Afatinib	3	>6	(27)
Yang *et al*, 2020	5	69	F	No	L858R, T790M	Osimertinib	3	14	EGFR L718Q	Afatinib	4	4	(28)
Minari *et al*, 2021	6	51	M	No	Exon 19 deletion, T790M	Osimertinib	2	8	EGFR G724S	Afatinib	5	>2	(29)
Zhao *et al*, 2021	7	69	M	No	Exon 19 deletion, T790M	Osimertinib	5	16	EGFR C797S	Afatinib + apatinib	7	10	(30)
Zhang *et al*, 2022	8	72	M	Yes	L858R	Almonertinib + bisphosphonates	1	12	EGFR L718Q	Afatinib + cetuximab	3	7	(31)
Song *et al*, 2022	9	55	F	No	L858R, T790M	Osimertinib	2	10	EGFR L718V	Afatinib + apatinib	4	>18	(32)
Nozaki *et al*, 2022	10	68	M	Yes	L858R	Osimertinib	1	2	High TMB	Afatinib	2	5	(33)
Aredo *et al*, 2022	11	NA	NA	No	L858R	Osimertinib	1	6.6	EGFR L718Q, EGFR L718V	Afatinib	2	2.5	(34)
	12	NA	NA	No	L861Q	Osimertinib	1	8.3	Not tested	Afatinib	2	19.6	
	13	NA	NA	No	Exon 19 deletion	Osimertinib + bevacizumab	5	8	ERBB2 amp	Afatinib	7	1.8	
	14	NA	NA	No	L858R	Osimertinib	1	1.7	None detected	Afatinib + cetuximab	3	1.3	
	15	NA	NA	No	L858R	Osimertinib	2	1.4	Not tested	Afatinib + cetuximab	4	2	
	16	NA	NA	No	L858R	Osimertinib	2	36.4	EGFR R776H	Afatinib + cetuximab	4	2.5	
	17	NA	NA	No	Dupl. exons 18	Osimertinib	4	2.9	Not tested	Afatinib + cetuximab	6	1.3	
	18	NA	NA	No	Exon 19 deletion	Osimertinib + bevacizumab	2	4.7	MAP2K1 K57T	Afatinib + cetuximab	4	1.2	
	19	NA	NA	No	Exon 19 deletion	Osimertinib	2	2	Not tested	Afatinib + cetuximab	4	1.4	
	20	NA	NA	No	Exon 19 deletion	Osimertinib + bevacizumab	2	1.7	Not tested	Afatinib + cetuximab	5	1.9	

Table II. Continued.

First author/s, year	Patient no.	Age, years	Sex	Smoker	EGFR mutation	Third-generation TKI treatment	Line of treatment	PFS, months	Resistance mechanism	Afatinib treatment	Line of treatment	PFS, months	(Refs.)
	21	NA	NA	No	L858R	Osimertinib	5	7.9	EGFR C797S	Afatinib + cetuximab	7	3.8	
	22	NA	NA	No	L858R	Osimertinib	2	7	AKT2 amp	Afatinib + cetuximab	5	4.3	
	23	NA	NA	No	L858R	Osimertinib	2	19.4	Not tested	Afatinib + cetuximab	4	5.6	
	24	NA	NA	No	L858R	Osimertinib	2	2.6	Not tested	Afatinib + bevacizumab	4	2.9	
	25	NA	NA	No	L858R	Osimertinib	2	8.8	Not tested	Afatinib + bevacizumab	5	5.5	
Sanchis-Borja *et al*, 2024	26	61	M	No	L858R, T790M	Osimertinib	2	38.6	EGFR L718Q	Afatinib	3	7.2	(35)
	27	58	F	No	L858R, T790M, G719X	Osimertinib	3	41.5	EGFR L718Q	Afatinib	4	6.1	
	28	65	M	Yes	Exon 19 deletion, T790M	Osimertinib	2	9	EGFR L718Q	Afatinib	4	1.9	

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; PFS, progression-free survival; F, female; M, male; TMB, tumor mutational burden; NA, not available; ERBB2, Erb-B2 receptor tyrosine kinase 2; amp, amplification; Dupl, duplication.

local realignments around indels and base quality reassurance. Gene fusions were identified by FACTERA (14). Somatic SNPs and indels were analyzed by VarScan2 (15) and Mutect2, with the mutant allele frequency cutoff at 2% for tissue samples and a minimum of three unique mutant reads. Common SNPs were excluded using dbSNP (v137) if they were present in >1% population frequency in the 1000 Genomes Project or the Exome Aggregation Consortium (ExAC) 65,000 exomes database. The resulting mutation list was further filtered by an in-house list of recurrent artifacts based on a normal pool of whole blood samples.

Discussion

Almonertinib is a third-generation EGFR TKI with demonstrated activity against *EGFR*-sensitizing and T790M mutations. Its design is a modified version of osimertinib, in which the methyl group on the indole ring is replaced with a cyclopropyl group. This alteration enhances its ability to bind with *EGFR* T790M and improves its transport through the blood-brain barrier (8,16). Despite its efficacy, acquired resistance to almonertinib inevitably develops. A previous study reported that the resistance patterns to almonertinib are similar to those of osimertinib (17). The resistance mechanisms include the following: Loss of the T790M mutation, maintenance of the T790M mutation, *EGFR* mutations (C797S, G724S and L718Q), activation of alternative pathways and histological transformation (6,17,18). In the present case, most acquired resistance mutations to almonertinib were also reported, including L718Q, C797S/G, L792H and V802F. Mutation at *EGFR* L718 has been identified as a factor contributing to resistance against osimertinib, both *in vitro* and *in vivo*. The L718 mutation may mediate drug resistance by causing a substitution at the L718 residue in the ATP-binding site of the EGFR kinase domain. This alteration can lead to steric hindrance that can obstruct osimertinib-EGFR binding (19). The EGFR C797S mutation involves a change from cysteine to serine at codon 797 within the ATP-binding site. This alteration leads to the loss of the covalent bond between osimertinib and the mutant EGFR (20). Mutations in L792 can create steric interference with a methoxy group on the phenyl ring of osimertinib, disrupting its ability to bind to the kinase domain (21). The V802F mutation can displace the first helix adjacent to the hinge region in comparison with the wild-type *EGFR*, leading to minimal effects on osimertinib binding (22). Furthermore, *EGFR* V689L in exon 18 was also observed in the present report, which has been reported to likely be associated with EGFR TKI sensitivity (23). However, its role in mediating third-generation TKI resistance has not been established yet.

Studies have reported cases of patients who received afatinib after progression on third-generation TKIs (osimertinib or almonertinib; Table II). Among 28 patients, most of them had *EGFR* 19del or L858R mutations, several accompanied by T790M mutation, prior to receiving third-generation TKI treatment (24-35). Osimertinib was most often given as second-line therapy. The median PFS was 8 months. After progression, 13 patients exhibited resistance mechanisms dependent on the ErbB family, including *EGFR* L718Q and L718V, *EGFR* R776H, and *EGFR* C797S mutations, as well as amplification of Erb-B2 receptor tyrosine kinase 2 (ERBB2). Certain patients had other mutations, such as *EGFR* G724S, *EGFR* P794L, *ERBB2* amplification, *MAP2K1* K57T and AKT2 amplification. Afatinib was most commonly given as monotherapy in the fourth-line treatment setting for 13 patients. It was used in combination with cetuximab for 11 patients, with bevacizumab for 2 patients and with apatinib for 2 patients. Excluding patients whose PFS was not completely recorded, the remaining patients had a median PFS of 3.8 months (24-35). The patient in the present report had multiple *EGFR* mutations and benefited from afatinib after almonertinib failed. Afatinib is designed to be a multitarget inhibitor that can irreversibly bind to the ATP-binding site of the EGFR tyrosine kinase domain, specifically at Cys797 of EGFR, Cys805 of HER2 and Cys803 of HER4. This binding effectively blocks the downstream transduction signaling pathways (36). A preclinical study reported that 19del, L858R and L718Q mutations were highly sensitive to second-generation TKIs, such as afatinib (31). This has been further validated in other clinical cases, with patients carrying EGFR L858R/L718Q mutations experiencing a PFS of 4-6 months under these treatments (31). The patient in the present report had multiple acquired *EGFR* mutations, including L718Q, C797S/G, L792H, V802F and V689L, and showed a sustained response to afatinib monotherapy. This is in line with previous findings that have indicated that afatinib can be effective in patients with uncommon *EGFR* mutations (37). Therefore, afatinib could be a promising option following third-generation EGFR TKI treatment in these patients.

It is important to acknowledge the limitations of the single-case presentation of the present report. The effectiveness and side effects of almonertinib and afatinib need to be further assessed in larger cohorts. Moreover, the histological test results during the afatinib treatment are missing as only imaging and genetic tests were performed. In the present case, the *EGFR* V689L mutation may have served as a potential resistance mechanism to almonertinib; however, further preclinical studies and clinical evidence are required to support this.

In conclusion, to the best of our knowledge, the present report describes the first case of successful treatment of NSCLC with multiple acquired *EGFR* mutations using afatinib after the patient developed resistance to almonertinib. The patient received afatinib treatment for ~9 months and achieved a sustained PR without any significant side effects. The present case suggests that afatinib may overcome almonertinib resistance and could serve as a promising treatment option for similar patients. However, further investigation is required to determine any additional resistance mechanisms related to EGFR TKIs.

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Availability of data and materials

The sequencing results and raw data generated in the present study may be found in the BioProject database under

accession numbers PRJNA1174043 or at the following URLs: https://www.ncbi.nlm.nih.gov/sra/PRJNA1174043.

Authors' contributions

FY designed this study and collected the data for this case report. JL conceived the present study, analyzed and interpreted of data. MX acquired data. BP made substantial contributions to conception and design. FY and BP confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Written informed consent to publish the clinical details and images were obtained from the patient.

Competing interests

The authors declare that they have no competing interests.

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