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Audit of Molecular Mechanisms of Primary and Secondary Resistance to Various Generations of Tyrosine Kinase Inhibitors in Known Epidermal Growth Factor Receptor-Mutant Non-small Cell Lung Cancer Patients in a Tertiary Centre

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Abstract

Aims: Presently, three generations of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are approved against oncogene addicted EGFR-mutant non-small cell lung cancer (NSCLC). Patients with actionable EGFR mutations invariably develop resistance. This resistance can be intrinsic (primary) or acquired (secondary).

Materials and methods: This was a retrospective study carried out between January 2016 and April 2021 analysing 486 samples of NSCLC for primary and secondary resistance to first- (erlotinib, gefitinb), second- (afatinib) and/or third-generation (osimertinib) TKIs in EGFR-mutant NSCLCs by next generation sequencing (NGS). Tissue NGS was carried out using the Thermofischer Ion Torrent™ Oncomine™ Focus 52 gene assay; liquid biopsy NGS was carried out using the Oncomine Lung Cell-Free Total Nucleic Acid assay. All cases were previously tested for a single EGFR gene with the Therascreen® EGFR RGQ PCR kit.

Results: The results were divided into four groups: (i) group 1: primary resistance to first- and/or second-generation TKIs. This group, with 21 cases, showed EGFR exon 20 insertions, dual, complex mutations and variant of unknown significance, *de novo* MET gene amplification besides other mutations. (ii) Group 2: primary resistance to third-generation TKIs. This group showed two cases, with one showing dual EGFR mutation (L858R and E709A) and EGFR gene amplification. (iii) Group 3: secondary resistance to first- and second-generation TKIs. This group had 27 cases, which were previously reported negative for EGFR T790M by single gene testing. Significant findings were MET gene amplification in four cases, with one also showing MET exon 14 skipping mutation. Three cases showed small cell change and one showed loss of primary mutation. (iv) Group 4: secondary resistance to third-generation TKIs. The latter group was further subgrouped into group 4A: secondary resistance to osimertinib (third-generation TKI) when offered as second-line therapy after first- and second-generation TKIs on detection of T790M mutation. This group had 15 cases. EGFR T790M mutation was lost in 10 (10/15; 67%) cases and was retained in five cases. Patients with T790M loss experienced early resistance (6.9 months versus 12.6 months mean, $P = 0.0024$) compared with cases that retained T790M. Two cases gained MET amplification as the resistance mechanisms. Other mutations that were found when EGFR T790M was lost were in FGFR3, KRAS, PIK3CA, CTNNB1, BRAF genes. One case had EML4-ALK translocation. Two cases showed driver EGFR deletion 19, retained T790M and C797S mutation in Cis form. Group 4B: secondary resistance to osimertinib (when given as first-line therapy) in EGFR-mutant NSCLC. This group had three cases. The duration of osimertinib treatment ranged from 11 to 17 months. Two patients showed additional C797S mutation along with primary EGFR mutation.

Conclusion: This study shows the wide spectrum of primary and secondary EGFR resistance mechanisms to first, second and third generation of TKIs and helps us to identify newer therapeutic targets that could carry forward the initial advantage offered by EGFR TKIs.

Key words: Afatinib; EGFR; erlotinib; gefitinib; next generation sequencing; osimertinib; resistance

Introduction

The dramatic clinical response to tyrosine kinase inhibitors (TKIs) in molecularly driven non-small cell lung cancer (NSCLC) has been a major milestone in precision medicine. It began in 2004, when epidermal growth factor receptor (EGFR) mutations were initially identified in a subset of lung adenocarcinoma [1]. EGFR mutations were found to be sensitive to TKIs and offer improved progression-free survival, overall survival and quality of life advantages over conventional chemotherapy [2].

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Subsequent years have witnessed many newer generations of EGFR-sensitive TKIs, each offering some advantages over the previous generation. Presently, three generations of EGFR TKIs are approved. The first-generation agents are erlotinib, gefitinib and icotinib; second-generation agents are afatinib and dacomitinib; and the recently introduced osimertinib is a third-generation EGFR TKI [3]. After the FLAURA trial, osimertinib was propelled to the first-line choice in EGFR-mutant patients, owing to the advantage offered by better overall survival (hazard ratio 0.63; 95% confidence interval 0.45–0.88, $P = 0.0068$), reduced death rate (37% reduction in the risk of death), decreased grade 3 or higher side-effects (34% versus 45%) and a better central nervous system penetration compared with standard EGFR TKI therapy [4].

EGFR-mutant patients who are treated with TKIs invariably eventually develop resistance. This resistance can be intrinsic (primary) or acquired (secondary). Primary resistance is *de novo* and the TKI treatment does not offer any benefit clinically. Secondary resistance develops after a period of clinical benefit [5]. The common mutations that confer primary resistance to TKIs are the result of non-sensitive EGFR mutation. Some of the known examples of mutations are L747S/D761Y in exon 19, T790M in exon 20 and T854A in exon 21 [6]. Apart from these, other mechanisms could be activation of different pathways by mutations in *HGF* (hepatocyte growth factor) [7,8], *IGF1R* (insulin growth factor 1 receptor) [9], *BIM* (BCL2-like 11) [10], *MET* (MET proto-oncogene, receptor tyrosine kinase) [11] and/or *PI3K/AKT* (phosphatidylinositol-3-kinase and protein kinase B) genes [12]. These mechanisms of primary resistance are seen primarily after the use of first- and second-generation TKIs. There is limited literature available on primary resistance to osimertinib, although occasional case reports on small cell change causing primary resistance have been known [13].

On the other hand, secondary resistance in EGFR mutants post-TKIs is quite common and well documented in the literature. Most of the available data are for post-first (gefitinib and erlotinib) and second (afatinib) generation TKIs. The most common reported mechanism of resistance is T790M mutation in the EGFR gene, which accounts for more than 50% of the cases. We have also published a similar incidence of T790M mutation from our institution [14]. Osimertinib is the drug of choice in EGFR-mutant patients who develop T790M mutation upon progression with first- and second-generation TKIs. This third-generation TKI has shown superior outcomes to chemotherapy in this clinical setting [15]. Rarely, D761Y, T854A and L747S mutations in the EGFR gene have also been reported as a mechanism of secondary resistance [16–18]. Apart from these, other molecular mechanisms of secondary resistance are MET, HER2 MAPK amplification, PIK3CA, BRAF mutations, epithelial-to-mesenchymal transition, small cell transformation and a few other alternate pathway activation mechanisms [19]. Secondary resistance mechanisms to osimertinib differ from its predecessors. It can be secondary resistance when osimertinib is used as a second-line TKI after the development of T790M mutation or when it is used as first-line therapy. There are certain unique known mutations, such as C797S, G796R and certain translocations in RET, FGFR3, ALK genes (which have not been seen as resistance mechanisms in previous generation TKIs) [20,21].

Materials and Methods

Our study comprised 486 samples of NSCLC. As an institutional protocol, all cases of lung cancer undergo basic immunohistochemistry (IHC) for TTF-1, p63/p40 and PDL1. All cases with a histological diagnosis of adenocarcinoma and poorly differentiated (or not otherwise specified) carcinoma were subjected to single gene molecular testing for EGFR by real-time polymerase chain reaction using Therascreen® EGFR RGQ PCR kit from Qiagen, ALK by IHC (VENTANA ALK (D5F3) CDx Assay) and ROS by fluorescent *in situ* hybridisation. This was a retrospective study and included cases selected based on clinician's discretion and were subjected to next generation sequencing (NGS). These cases were selected from primary, secondary or tertiary tissue and/or liquid biopsy with primary or secondary clinical resistance to TKI therapy. New biopsies (preferably from a new metastatic site) after first and second clinical resistance were referred to as secondary and tertiary biopsies, respectively. For tissue NGS, formalin-fixed and paraffin-embedded (FFPE, 5 $\times$, 10 μm) tumour biopsies with more than 20% tumour were chosen (macrodissection was carried out where required). DNA and RNA were isolated using the Promega ReliaPrep™ FFPE gDNA Miniprep System (A2352) and the Promega ReliaPrep™ FFPE Total RNA Miniprep System (Z1002), respectively. For liquid biopsy, 18 ml blood samples were collected in EDTA tubes and centrifuged within 1 h for plasma preparation. Cell-free nucleic acids were extracted using the QIAmp circulating nucleic acid kit (Qiagen) according to the manufacturer's protocol. NGS on tissue biopsy was carried out using the Thermofischer Ion Torrent™ Oncomine™ Focus 52 gene assay and on liquid biopsy was carried out using Oncomine Lung Cell-Free Total Nucleic Acid Assay (12 gene assay including single nucleotide variations, translocation and copy number alterations). The library was prepared according to the user manual. All variants were also analysed using IGV (Integrative Genomic Viewer version 2.3 or higher). The sequences were aligned with the reference genome hg 19. The median coverage depth for tissue samples was $>500\times$ with 5% limit of detection. For liquid biopsy, the assay median coverage depth was $>20{,}000\times$ with 0.1% limit of detection. All the pathogenic mutations were checked in the NCBI and COSMIC databases. Variants of unknown significance were searched on ClinVar, VarSome search engine. *In silico* prediction tools were also used to predict pathogenicity [22,23]. Patient clinical and treatment details were curated from the medical records of the institute.

The cases were divided into four clinical groups, as shown in Figure 1.

Statistical Analysis

Continuous data are reported as median (range), whereas the count data are reported as numbers. Kaplan–Meier curves comparing survival between treatment groups were drawn using Medcalc Statistical Software version 19.6.3 and estimates were reported with 95% confidence intervals. Alpha <0.5 was set as significant beforehand. Swimmer's plot was created using Excel following Peltier Tech charts for Excel version 4.0.

Results

Group 1: Primary Resistance to First- and/or Second-generation Tyrosine Kinase Inhibitors (Table 1)

This group had 21 cases. The median age was 52 years, with an age range of 32–85 years. This included 11 female and 10 male patients. In 11 cases, no mutation was detected on single gene testing. Out of these 21 cases, eight cases were exon 20 insertions (cases 2, 4, 7, 10, 16, 17, 19, 20), a dual mutation – EGFR L833V and H835L (case 3), a rare mutation – EGFR p.(E709_T710delinsD) (case 21) and a variant of unknown significance – EGFR p.(Ile853Ser). There were also two complex mutations (EGFRp.Glu746_-Leu747delinsValPro and EGFR p.Leu747Pro) (cases 1 and 6), which were detected as deletion 19 in the single gene assay but did not respond to either first- or second-generation TKIs. Two cases had additional *de novo* MET gene amplification (cases 8 and 14) apart from the primary mutation. One of the case showed additional T790M mutation apart from deletion 19 and KRAS mutation (case 15). T790M mutation had a variant allele frequency (VAF) of 2% and was not detected earlier by single gene assay. There were additional TP53, PTEN, PIK3CA and KRAS mutations. Three cases did not show any other additional abnormality (cases 5, 11 and 13).

Group 2: Primary Resistance to Osimertinib (Third-Generation Tyrosine Kinase Inhibitor)

This group had just two cases. The median age was 69 years. One case was male and the other was female. The first case (case 1) had dual EGFR mutation (L858R and E709A) together with EGFR amplification on NGS. Initially, only L858R was detected by single gene assay (as E709A was not included in the assay). This patient presented with brain metastasis and was started on osimertinib. She did not respond and was shifted to chemotherapy within 1 month of treatment. Subsequently, she received six cycles of chemotherapy. On progression, 5 months later, NGS showed dual EGFR mutation with EGFR amplification. The patient later showed a partial response to afatinib and was stable for the last 7 months. The second case did not show any additional mutation on NGS testing.

Group 3: Secondary Resistance to First- and Second-Generation Tyrosine Kinase Inhibitors (Figure 2, Supplementary Table S1)

As an institutional policy, most of our cases of secondary resistance to first- and second-generation TKIs are subject to single gene EGFR testing (either liquid/repeat tissue biopsy/or both). Most of the patients who harbour EGFR T790M mutation are offered osimertinib. This group represents patients who were negative for T790M mutation (on single gene testing) and were subjected to NGS to look for other mechanisms of resistance on secondary biopsy.

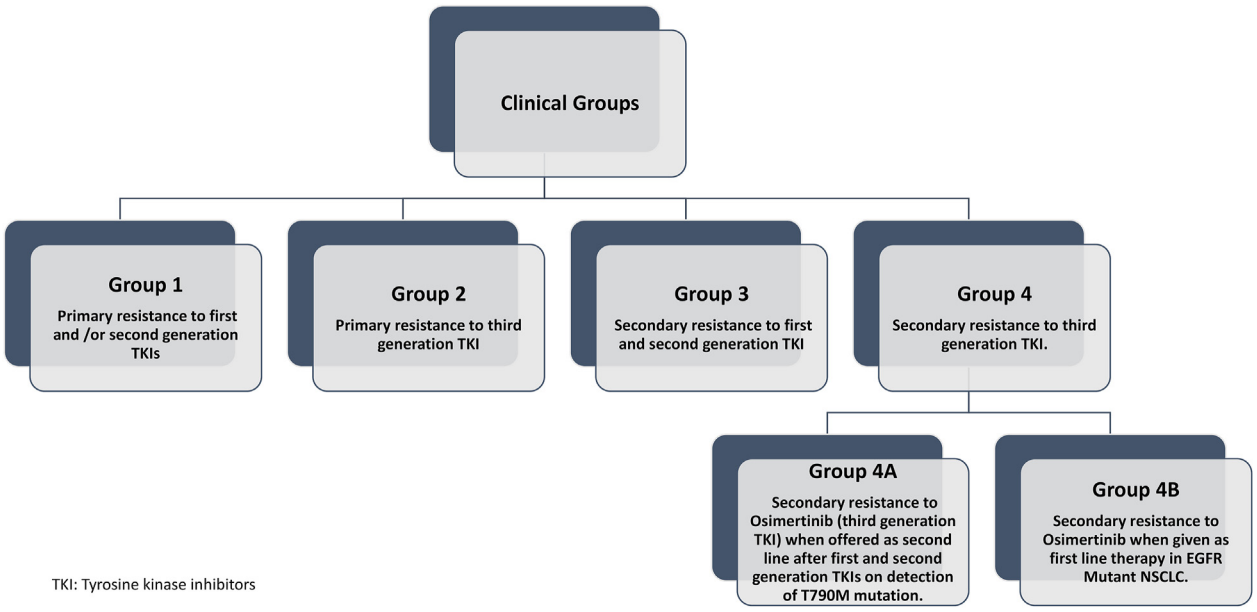

Fig 1. Clinical groups describing primary and secondary resistance to first-, second- and third-generation tyrosine kinase inhibitors (TKIs).

Table 1
Group 1 cases showing primary resistance to first- and/or second-generation tyrosine kinase inhibitors (TKIs)

Serial no.	Age (years)	Gender	EGFR mutation status on single gene testing	Months of TKI treatment	NGS findings	Additional comments
1.	32	F	EGFR deletion 19	3 months	EGFR p.Glu746_Leu747delinsValPro	Complex deletion 19 mutation. No response to gefitinib
2.	47	F	No mutation detected	Not given	EGFR p.(Asp770_771insGly) (exon 20 insertion)	
3.	51	F	No mutation detected	4 months Afatinib	EGFR L833V and H835L mutation	Patient was initially treated with chemotherapy but did not respond. She was offered afatinib therapy and showed a response for 4 months.
4.	53	F	No mutation detected	Not given	EGFRp.(P772_H773insHV) (exon 20 insertion), TP53 p.(R280K), PTEN p.(K267fs)	
5.	74	F	EGFR deletion 19	2 months	EGFR p.(E746_A750del)	
6.	50	M	EGFR deletion 19	3 months	EGFR p.Leu747Pro (c.2239_2240delTTinsCC)	Complex deletion 19 mutation (EGFR c.2239_2240delTTinsCC). No response after 3 months of erlotinib
7.	59	F	No mutation detected	Not given	EGFR p.(Pro772_His773insHisVal) (exon 20 insertion)	
8.	67	M	EGFRL858R	1 month	EGFRL858R and MET amplification	
9.	57	F	EGFR deletion 19	3 months	EGFR p.(E746_A750del) PIK3CA C420_P421del along with	
10.	52	F	No mutation detected	Not given	EGFR, p.(A767_S768insSVD); c.2311_2312insGCG; TGGACA (exon 20 insertion)	
11.	45	M	L858R	3 months	EGFRL858R	
12.	72	M	EGFR exon 19 del	3 months	EGFR p.(E746_A750del)	
13.	56	F	EGFR L858R	3 months	EGFR L858R	
14.	48	M	EGFR L858R	2 months	EGFRL858R and MET amplification	
15.	32	M	EGFR exon 19 del	2 months	EGFR p.(E746_A750del), EGFR T790M(variant allele frequency 2%), KRAS G13C	EGFR T790M mutation was not detected on RT PCR testing (Therascreen® EGFR RGQ PCR kit from Qiagen). Only EGFR p.(E746_A750del) was detected.
16.	51	F	No mutation detected	Not given	EGFR p.(D770delinsES) c.2309_2310insGTC(exon 20 insertion), PIK3CA p.(E542K) c.1624G>A, TP53 p.(S183*) c.546C>G	
17.	77	M	No mutation detected	Not given	EGFR Splice site variant: c.2284 −6C>CTCCAGG AAGCCT leading to exon 20 insertion	
18.	80	M	No mutation detected	Not given	EGFR p.(Ile853Ser)- variant of unknown significance	In silico analysis detected this mutation to be pathogenic. The patient was not offered any TKI therapy for 3 months and was later lost to follow-up.
19.	49	F	No mutation detected	Not given	EGFR p.(P772_H773insHV) c.2321_2322insCCACGT(exon 20 insertion), TP53 p.(R280K) c.839G>A PTEN p.(K267fs)c.801delG	

Table 1 (*continued*)

Serial no.	Age (years)	Gender	EGFR mutation status on single gene testing	Months of TKI treatment	NGS findings	Additional comments
20.	85	M	No mutation detected	Not given	EGFR, p.(A767_S768insSVD); c.2311_2312insGCG; (exon 20 insertion)	
21.	46	M	No mutation detected	3 months	EGFR p.(E709_T710delinsD)	Chemotherapy 6 cycles with no response to afatinib. Not (3 months) detected by RT PCR

EGFR, epidermal growth factor receptor; NGS, next generation sequencing; RT PCR, reverse transcription polymerase chain reaction.

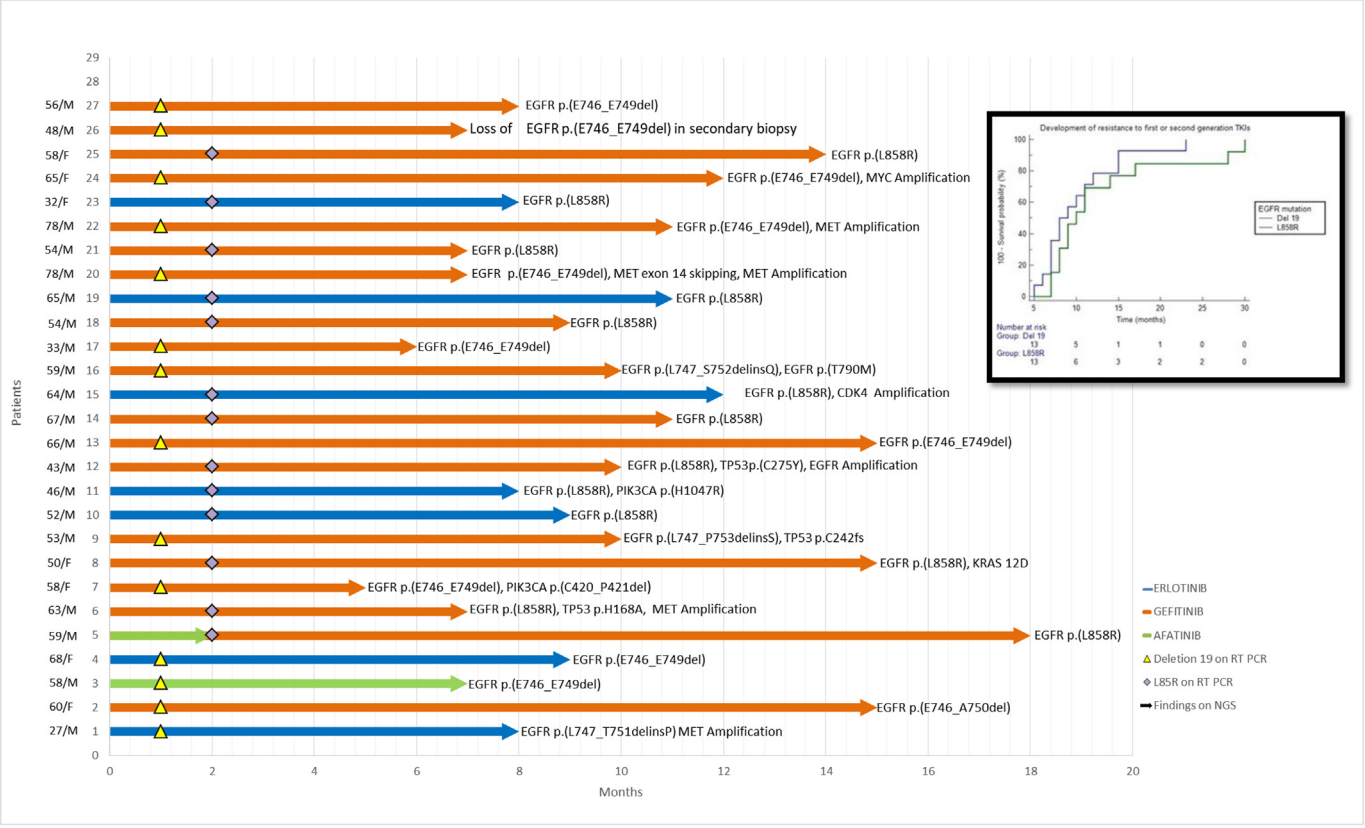

Fig 2. Swimmer's plot of group 3 cases showing secondary resistance mutations to first- and second-generation tyrosine kinase inhibitors (TKIs) in epidermal growth factor receptor (EGFR)-mutant lung cancers. Inset: Kaplan–Meier graph showing no difference in time of development of resistance post first- and second-generation TKI treatment (P = 0.2977) for EGFR L858R and deletion 19 mutations.

We have previously reported our experience with secondary T790M mutation [14]. This group had 27 cases. The median age was 54 years, with an age range of 27–78 years. This included nine female and 20 male patients. The duration of TKI treatment ranged from 5 to 66 months before a second biopsy was carried out. There were 14 patients who had EGFR deletion 19 on primary biopsy and 13 patients who had EGFR L858R. Thirteen cases had only driver mutation on secondary biopsy with no additional mutation. Four cases had MET amplification with one of them (case 20) also showing MET exon 14 skipping mutation. Additional gene amplifications were seen in EGFR (cases 2 and 12), MYC (case 24) and CDK4 (case 15). There was loss of primary mutation in one case (case 26) with poorly differentiated histology. Three cases showed small cell change on secondary biopsy (cases 16, 17 and 27). TP53, PIK3CA and KRAS mutations were also seen in different cases.

Group 4: Resistance to Osimertinib

This group was further divided into two groups.

Group 4A (Table 2): when third-generation TKIs were offered as second line after first- and second-generation TKIs on detection of EGFR T790M mutation. Group 4A comprised 15 patients. The median age was 64 years, with an age range of 32–76 years. This group included four female and 11 male patients. The duration of TKI treatment

Table 2
Group 4A – third-generation tyrosine kinase inhibitors (TKIs) were offered as second-line therapy after first- and second-generation TKIs on detection of EGFR T790M mutation

Serial no.	Age (years)	Gender	EGFR on RT PCR (First biopsy)	Second tissue/liquid biopsy (Single gene assay/NGS)	Duration of osimertinib treatment (months)	NGS on tertiary Biopsy	T790M Lost/ retained
1.	32	M	EGFR p.(E746_A750del)	EGFR p.(E746_A750del) EGFR T790M KRAS G13C(NGS -tissue biopsy)	7	EGFR p.(E746_A750del) FGFR3 p.(G90V)	Lost
2.	46	M	EGFR p.(L858R)	EGFR p.(L858R) EGFR T790M (single gene tissue biopsy)	5	EGFR L858R MET amplification, TP53 p.(F270C)	Lost
3.	45	F	EGFR p.(L858R)	EGFR p.(L858R) T790M (single gene tissue biopsy)	11	EGFR p.(L858R) EGFR T790M	Retained
4.	56	F	EGFR p.(E746_A750del)	EGFR p.(E746_A750del) T790M (single gene tissue biopsy)	8	p.(E746_A750del) EGFR T790M	Retained
5.	54	F	EGFR p.(E746_A750del)	EGFR p.(E746_A750del) T790M (single gene tissue biopsy)	8	EGFR p.(E746_A750del) KRAS p.(G12C) PIK3CA p.(E542K)	Lost
6.	44	M	EGFR p.(E746_A750del)	EGFR p.(E746_A750del) EGFR T790M (single gene tissue biopsy)	5	EGFR p.(E746_A750del) CTNNB1p.(D32N)	Lost
7.	76	M	EGFR p.(L858R)	EGFR p.(L858R) EGFR T790M (single gene liquid biopsy)	6	EGFR p.(L858R) BRAFp.(G469A) KRASp.(G12V)	Lost
8.	54	M	EGFR p.(E746_A750del)	EGFR p.(E746_A750del) EGFR T790M (single gene tissue biopsy)	9	EGFR p.(E746_A750del) MET amplification	Lost
9.	48	M	EGFR p.(E746_A750del)	EGFR p.(E746_A750del) EGFR T790M (single gene tissue biopsy)	15	EGFR p.(E746_A750del, EGFR T790M EGFRp.Cys797Ser(in Cis) Figure 2	Retained
10.	45	M	EGFR p.(E746_A750del)	EGFR p.(E746_A750del) EGFR T790M (single gene liquid biopsy)	8	EGFR p.(E746_A750del)	Lost
11.	76	M	EGFR p.(L858R)	EGFR p.(L858R) EGFR T790M (single gene liquid biopsy)	6	EGFR p.(L858R)	Lost
12.	54	M	EGFR p.(E746_A750del)	EGFR p.(E746_A750del) EGFR T790M (single gene tissue biopsy)	7	EGFR p.(E746_A750del)	Lost
13.	58	M	EGFR p.(E746_A750del)	EGFR T790M (single gene tissue biopsy)	15	EGFR p.(E746_A750del) , T790M &p.Cys797Ser(in Cis)	Retained
14.	48	F	EGFR p.(E746_A750del)	EGFR p.(E746_A750del) EGFR T790M (single gene tissue biopsy)	14	EGFR p.(E746_A750del, EGFR T790M	Retained
15.	70	M	EGFR p.(E746_A750del)	EGFR p.(E746_A750del) EGFR T790M (single gene liquid biopsy)	8	EGFR p.(E746_A750del) EML4-ALK fusion (EML4-ALK.E2A20) (ALK fusion was confirmed by immunohistochemistry. Patient was treated with crizotinib and responded for 4 months)	Lost

EGFR, epidermal growth factor receptor; NGS, next generation sequencing; RT PCR, reverse transcription polymerase chain reaction.

ranged from 5 to 15 months before a second biopsy was carried out. There were 11 patients who had EGFR deletion 19 on primary biopsy and four patients who had EGFR L858R. EGFR T790M mutation was lost in 10 (10/15; 67%) cases, which included seven cases of deletion 19 and three cases of L858R mutation. T790M mutation was retained in five cases, which included four cases of deletion 19 and one case of L858R mutation in EGFR gene. The duration of osimertinib treatment before progression ranged from 8 to 15 months. Patients with T790M loss experienced early resistance (6.9 months versus 12.6 months mean, P = 0.0024; Figure 3) compared with cases that retained T790M. Two of the cases (cases 2 and 8) that lost T790M had gained MET amplification as the resistance mechanisms and one case also had TP53 mutation (case 2). Mutations in FGFR3, KRAS, PIK3CA, CTNNB1, BRAF genes and EML4-ALK translocation were seen in the subgroup where T790M was lost. Three cases which had lost T790M only showed driver EGFR mutation. Three of the five cases that retained T790M mutation had only driver EGFR mutation together with the former (cases 3, 4 and 14). Two cases (cases 9 and 13) showed driver EGFR deletion 19, retained T790M and C797S mutation in Cis form. One case (case 15) showed ALK translocation, which was also confirmed by IHC.

Group 4B: when the patient developed secondary resistance to first-line osimertinib. This was a small group composed of three patients who were all males with a median age of 63.3 years. Two cases (cases 2 and 3) were EGFR deletion 19 and one case was L858R. The duration of osimertinib treatment ranged from 11 to 17 months. One of the patients had irregular treatment. Two patients showed additional C797S mutation (cases 1 and 2). The third patient showed only primary mutation (case 3).

Discussion

EGFR-mutant lung cancer is a single oncogene driven tumour that shows a remarkable response to molecular therapy in most cases. Eventually the tumour outsmarts the targeted therapy and therapeutic resistance develops. This can be primary or at times develop after a period of response. There are various escape mechanisms that could lead to a loss of response. These involve a direct change in the target site of the molecular drug, activation of an alternate upstream or downstream pathway, increased adaptive signalling by the tumour and/or epigenetic alterations [24]. NGS-based technologies with broader panels and a higher depth of coverage have the advantage of detecting low level and uncommon mutations, which give a better insight into the tumour biology.

We carried out a retrospective audit of molecular findings in NSCLC cases that presented at our institute with any kind of resistance in known EGFR gene abnormality. Our data suggested that the most common cause of primary resistance to first- and second-generation TKIs was uncommon EGFR mutations. There were eight cases of EGFR exon 20 insertions including a splice site mutation (group 1). These insertions were not detected by single gene assays. Most of the single gene assays can detect only limited exon

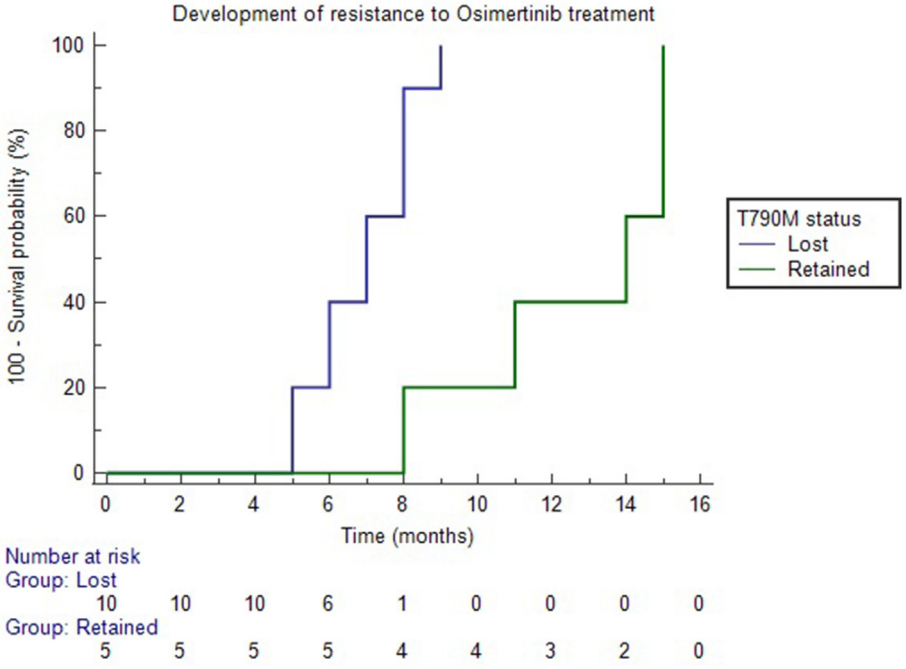

Fig 3. Group 4A – patients who lost epidermal growth factor receptor (EGFR) T790M mutation experienced earlier resistance (6.9 months versus 12.6 months mean) compared with cases that retained T790M (P = 0.0024).

20 insertions. EGFR exon 20 insertions together account for around 4–10% of all EGFR mutations in NSCLC [25,26]. These mutations are characterised by duplications of up to seven amino acid sequences (762–774) in α-C helix and adjacent loop of EGFR gene. These mutations are known to offer de novo resistance to all three generations of approved TKIs. The EGFR gene is activated in these insertions without any decrease in ATP affinity compared with the wild type. This hinders the competitive inhibition offered by common TKIs [27]. Also, a rigid α-C helix configuration formed due to the insertion is known to inhibit drug binding [28]. Only one EGFR exon 20 insertion mutation A763_Y764FQEA has shown a good response to erlotinib in various studies [29]. We did not find this mutation in our samples. There are certain novel agents, such as poziotinib (formerly HM781-36B), mobocertinib (TAK788) and TAS6417 (CLN-081), that can bind to the inactive and rigid ATP-binding site of exon 20 insertion mutations and have shown some promise [30]. Newer dual action agents (EGFR and hepatocyte growth factor receptor or MET inhibitor), such as amivantamab and heat shock protein 90 (Hsp90) inhibitor luminespib (NVP-AUY922), have shown clinical promise [31,32].

Our study also showed that complex EGFR deletion 19 mutations (EGFRp.Glu746_Leu747delinsValPro and EGFR p.Leu747Pro) can also cause primary resistance to TKI therapy. Wu *et al.* [33], in their series of 1261 lung cancer cases, reported two patients who had EGFR p.Leu747Pro mutation. Both patients did not respond to TKI therapy. The true incidence of complex EGFR mutations is largely unknown and has mostly been reported as a part of other rare mutations. Various studies have quoted the incidence to be between 3 and 14% [34]. These complex mutations are diagnosed as deletion 19 mutations on single gene testing. It is only after clinical resistance to TKI therapies and subsequent sequencing of the tumour that the complex nature of these mutations is detected. Most of these complex mutations are unlikely to respond to TKIs. This inadvertently delays appropriate treatment. Two of our cases had de novo MET amplification together with the primary EGFR mutation. MET amplification has been reported in around 3% of patients with EGFR mutation without any prior treatment [35]. MET receptors are encoded by proto-oncogene MET and activate downstream RAS/ERK/MAPK and PI3K-AKT when bound to the HGF ligand. This pathway is essential for cell proliferation, survival, migration, motility and invasive properties. MET gene amplification can dysregulate this pathway, causing resistance to TKIs in EGFR-mutant lung cancer patients [36]. These patients have also shown a response to the combination of EGFR and ALK inhibitors in some studies [37]. One of our cases (case 15) had EGFR deletion 19 together with T790M (variant allele frequency 2%) and KRAS G13C mutation. The baseline T790M mutation was not detected by single gene assay. T790M mutation was below the limit of detection of single gene assay. Such de novo EGFR T790M mutations are a very rare occurrence seen in $<1\%$ of patients and a cause of primary resistance to first- and second-generation TKIs [38]. Two cases showed mutations in PTEN and PIK3CA. These are known to cause aberrant activation in the PI3K/AKT pathway and in turn hinder EGFR TKI to act [39,40]. Apart from these, there are other reported abnormalities, such as MDM2 amplification, BIM polymorphism and germline EGFR mutation T790M and V843I [19,41,42], which can cause primary resistance.

There is limited literature available on the de novo resistance mechanisms of osimertinib. We also had two cases. One of them had dual EGFR L858R and E709A mutations with EGFR amplification. EGFR E709A mutations are rare and over 75% are reported as dual mutations. Preclinical studies have reported a response to afatinib [43]. This is the first time that clinical primary resistance to osimertinib has been reported for this mutation. Our case also later responded to afatinib.

The most common cause of secondary resistance to first- and second-generation TKI is T790M mutation in the EGFR gene [44]. We have also reported a high incidence of T90M in our previous study [14]. MET amplification was the second most common mechanism of resistance. Various studies have reported MET amplification in 5–20% of such cases [19]. Amplification leads to an aberrant excessive hepatocyte growth factor, which is a natural ligand for this gene and reduces the susceptibility to TKIs [45]. One of our cases also showed MET exon 14 skipping mutation together with MET amplification as a resistance mechanism. This is a rare occurrence and has been reported to show some response to a combination of MET and EGFR inhibitor therapy [46]. Other resistance mechanisms, such as EGFR amplification, KRAS, PIK3CA and TP53, have also been previously reported [19]. Three of our cases showed small cell change in the new biopsy (secondary biopsy) taken after the development of resistance. One of these also showed T790M mutation. Most of such cases are known to have a dismal prognosis. There have been occasional reports of a response to third-generation TKIs in such cases [47]. One of our cases showed loss of (driver EGFR deletion 19) mutation with poorly differentiated histology in the secondary biopsy. This is a rare occurrence and has been reported in preclinical studies [48]. This could possibly be due to loss of EGFR gene itself.

Osimertinib is used as a salvage therapy when EGFR T790M mutation occurs as an acquired resistance mechanism to first- and second-generation TKIs. The therapy extends the clinical benefit of the previous TKIs for months. Eventually resistance develops to the third-line also. Some of the resistance mechanisms overlap with first- and second-generation TKIs. Osimertinib shares EGFR, MET and HER2 amplification and PIK3CA, KRAS, BRAF mutations as resistance mechanisms with first- and second-generation TKIs [49]. Our data also showed cases with MET amplification, PIK3CA, KRAS, BRAF mutations. Between 10 and 26% of acquired resistance to osimertinib harbours a new mutation in the EGFR gene. The most common mutation is C797S, observed in 14–21% of cases. We saw this mutation in two (13%) cases. Up to 50% of such cases also experience loss of T790M mutation [49]. We observed T790M loss in 10 (10/15; 67%) cases, whereas this was retained in five (5/15; 33%) cases. The duration of osimertinib treatment varied from 5 to 8 months. T790M mutation was retained in five cases, four of which had deletion 19 and one L858R mutation in

the EGFR gene. The duration of osimertinib treatment before progression ranged from 8 to 15 months. Patients with T790M loss experienced earlier resistance (6.9 months versus 12.6 months mean, P = 0.0024, Figure 3) compared with cases that retained T790M. Oxnard *et al.* [21] have also reported loss of T790M associated with a shorter period of resistance. Two cases (2/15; 33%) that retained T790M also had another mutation C797S in exon 20. Both cases showed C797S mutation in the cis position (Figure 4). This mutation is reported in up to 7% of cases. This mutation is known to cause loss of covalent bond between osimertinib and site of EGFR mutation. Also, mutation in the cis position means that both T790M and C797S are on the same allele. Studies suggest that EGFR C797S rarely responds to first-generation TKIs. Rarely, when this mutation is on a different allele (trans position), combination of a first-generation TKI with osimertinib, which targets T790M, can be used [50]. Other reported mutations are C797G, G796R, G796S, G796D, L792H, L718Q, G724S, S768I and even exon 20 insertions [20,50–52]. Two of our cases (2/15; 13%) showed MET amplification in this group. One was associated with TP53 mutation. This is the most common mechanism of resistance, seen in about 19% of patients [20]. Several preclinical studies have shown that a combination of MET inhibitors, such as crizotinib and osimertinib, can overcome this type of resistance [53].

One case (case 15) showed ALK translocation, which was also confirmed by IHC. This patient was treated with crizotinib and did show a response for 4 months before progression. Fusions have been reported earlier in 3–10% of such cases. Although rare, other previously reported fusions are RET–ERC1, FGFR3–TACC3, CCDC6–RET, NTRK1–TPM3, AGK–BRAF, PLEKHA7–ALK, ESYT2–BRAF and GOPC-ROS1 [21,54,55]. Combining EGFR inhibitor with fusion-specific inhibitor can prolong the therapeutic window offered by targeted therapy [56].

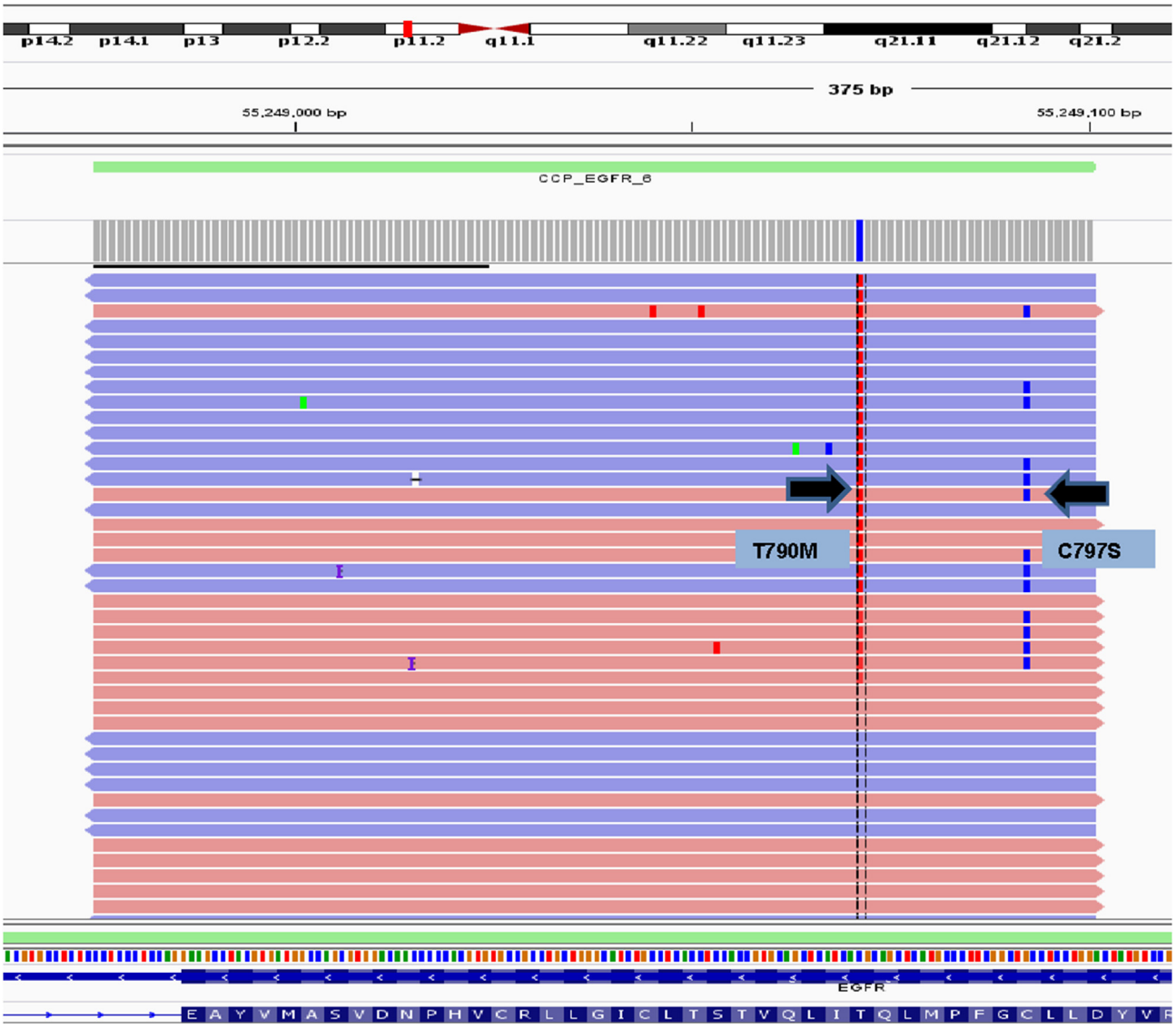

Fig 4. Image from the integrated genomic viewer (IGV) showing the presence of epidermal growth factor receptor (EGFR) T790M and C797S mutation on the same allele.

Secondary resistance to osimertinib showed two cases with C79S mutation together with the driver mutation. This group is known to have lesser frequency of EGFR gene mutations compared with those patients who developed resistance when osimertinib was used in the second line after the T790M mutation developed (6–10% in former versus 10–26% in later). MET amplification is also reported in lesser frequency (8–17% in former versus 5–50% in later). Other mutations, amplifications and rearrangements are reported in almost the same frequency in both groups [49].

Conclusion

This study has shown the wide spectrum of primary and secondary EGFR resistance mechanisms in first-, second- and third-generation TKIs. These resistance mechanisms give deeper insight into the biology of tumour post-TKI treatment and also provide newer therapeutic options.

Ethics Approval

The study was approved by our Institutional Review Board (RGCIRC/IRB/277/2019).

Author Contributions

MS is the guarantor of integrity of the entire study. MS and JJ were responsible for study concepts and design. SM carried out literature research. SD was responsible for clinical studies. JJ carried out experimental studies/data analysis. JJ and MK carried out statistical analysis. MS and JJ were responsible for manuscript preparation. MS edited the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clon.2022.06.003.

References

[1] Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, *et al*. EGF receptor gene mutations are common in lung cancers from “never smokers” and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A* 2004;101:13306–13311.

[2] Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, *et al*. Gefitinib or carboplatinpaclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009;361:947–957.

[3] Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, *et al*. Gefitinib or chemotherapy for non-small cell lung cancer with mutated EGFR. *N Engl J Med* 2010;362:2380–2388.

[4] Wang S, Li J. Second-generation EGFR and ErbB tyrosine kinase inhibitors as first-line treatments for non-small cell lung cancer. *Onco Targets Ther* 2019;12:6535–6548. https://doi.org/10.2147/OTT.S198945.

[5] Soria JC, Ohe Y, Vansteenkiste J, Reungwetwattana T, Chewaskulyong B, Lee KH, *et al*. Osimertinib in untreated EGFR -mutated advanced non-small-cell lung cancer. *N Engl J Med* 2018;378:113–125.

[6] Lovly CM, Shaw AT. Molecular pathways: resistance to kinase inhibitors and implications for therapeutic strategies. *Clin Cancer Res* 2014;20:2249–2256.

[7] Pao W, Chmielecki J. Rational, biologically based treatment of EGFR-mutant non-small-cell lung cancer. *Nat Rev Cancer* 2010;10(11):760–774. https://doi.org/10.1038/nrc2947.

[8] Yano S, Wang W, Li Q, Matsumoto K, Sakurama H, Nakamura T, *et al*. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res* 2008;68:9479–9487. https://doi.org/10.1158/0008-5472.CAN-08-1643.

[9] Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, *et al*. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* 2010;141:69–80.

[10] Costa C, Molina MA, Drozdowskyj A, Giménez-Capitán A, Bertran-Alamillo J, Karachaliou N, *et al*. The impact of EGFR T790M mutations and BIM mRNA expression on outcome in patients with EGFR-mutant NSCLC treated with erlotinib or chemotherapy in the randomized phase III EURTAC trial. *Clin Cancer Res* 2014;20:2001–2010.

[11] Turke AB, Zejnullahu K, Wu YL, Song Y, Dias-Santagata D, Lifshits E, *et al*. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. *Cancer Cell* 2010;17:77–88.

[12] Tan CS, Gilligan D, Pacey S. Treatment approaches for EGFR-inhibitor-resistant patients with non-small-cell lung cancer. *Lancet Oncol* 2015;16:e447–e459.

[13] Zheng LP, Chen LY, Liao XY, Xu ZH, Chen ZT, Sun JG. Case report: Primary resistance to osimertinib in erlotinib-pretreated lung adenocarcinoma with EGFR T790 M mutation. *BMC Cancer* 2018;1070:18. https://doi.org/10.1186/s12885-018-4991-4.

[14] Suryavanshi M, Mehta A, Panigrahi MK, Jaipuria J, Saifi M, Jain K, *et al*. The detection of primary and secondary EGFR mutations using droplet digital PCR in patients with nonsmall cell lung cancer. *Lung India* 2018;35(5):384–389. https://doi.org/10.4103/lungindia.lungindia.

[15] Mok TS, Wu Y-L, Ahn M-J, Garassino MC, Kim HR, Ramalingam SS, *et al*. Osimertinib or platinum-pemetrexed in EGFR T790M-positive lung cancer. *N Engl J Med* 2017;376(7):629–640. https://doi.org/10.1056/NEJMoa1612674.

[16] Balak MN, Gong Y, Riely GJ, Somwar R, Li AR, Zakowski MF, *et al*. Novel D761Y and common secondary T790M mutations in epidermal growth factor receptor-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors. *Clin Cancer Res* 2006;12:6494–6501.

[17] Bean J, Riely GJ, Balak M, Marks JL, Ladanyi M, Miller VA, *et al*. Acquired resistance to epidermal growth factor receptor kinase inhibitors associated with a novel T854A mutation in a

patient with EGFR-mutant lung adenocarcinoma. *Clin Cancer Res* 2008;14:7519–7525.

[18] Costa DB, Schumer ST, Tenen DG, Kobayashi S. Differential responses to erlotinib in epidermal growth factor receptor (EGFR)-mutated lung cancers with acquired resistance to gefitinib carrying the L747S or T790M secondary mutations. *J Clin Oncol* 2008;26:1182–1184.

[19] Stewart EL, Tan SZ, Liu G, Tsao MS. Known and putative mechanisms of resistance to EGFR targeted therapies in NSCLC patients with EGFR mutations – a review. *Transl Lung Cancer Res* 2015;4(1):67–81. https://doi.org/10.3978/j.issn.2218-6751.2014.11.06.

[20] Papadimitrakopoulou VA, Wu Y-L, Han J-Y, Ahn M-J, Ramalingam SS, John T, *et al*. Analysis of resistance mechanisms to osimertinib in patients with EGFR T790M advanced NSCLC from the AURA3 study. *Ann Oncol* 2018;29(Suppl. 8). https://doi.org/10.1093/annonc/mdy424.064. VIII741.

[21] Oxnard GR, Hu Y, Mileham KF, Husain H, Costa DB, Tracy P, *et al*. 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.

[22] Kopanos C, Tsiolkas V, Kouris A, Chapple CE, Albarca Aguilera M, Meyer R, *et al*. VarSome: the human genomic variant search engine. *Bioinformatics* 2018;35:1978–1980. https://doi.org/10.1093/bioinformatics/bty897.

[23] Landrum MJ, Lee JM, Benson M, Brown GR, Chao C, Chitipiralla S, *et al*. ClinVar: improving access to variant interpretations and supporting evidence. *Nucleic Acids Res* 2018;46:D1062–D1067.

[24] Sabnis AJ, Bivona TG. Principles of resistance to targeted cancer therapy: lessons from basic and translational cancer biology. *Trends Mol Med* 2019;25(3):185–197. https://doi.org/10.1016/j.molmed.2018.12.009.

[25] Oxnard GR, Lo PC, Nishino M, Dahlberg SE, Lindeman NI, Butaney M, *et al*. Natural history and molecular characteristics of lung cancers harboring EGFR exon 20 insertions. *J Thorac Oncol* 2013;8(2):179–184. https://doi.org/10.1097/JTO.0b013e3182779d18.

[26] Yasuda H, Kobayashi S, Costa DB. EGFR exon 20 insertion mutations in non-small-cell lung cancer: preclinical data and clinical implications. *Lancet Oncol* 2012;13(1):23–31. https://doi.org/10.1016/S1470-2045(11)70129-2.

[27] Yasuda H, Park E, Yun CH, Sng NJ, Lucena-Araujo AR, Yeo WL, *et al*. Structural, biochemical, and clinical characterization of epidermal growth factor receptor (EGFR) exon 20 insertion mutations in lung cancer. *Sci Transl Med* 2013;5(216):216. https://doi.org/10.1126/scitranslmed.3007205.

[28] Robichaux JP, Elamin YY, Tan Z, Carter BW, Zhang S, Liu S, *et al*. Mechanisms and clinical activity of an EGFR and HER2 exon 20-selective kinase inhibitor in non-small cell lung cancer. *Nat Med* 2018;24(5):638–646. https://doi.org/10.1038/s41591-018-0007-9.

[29] Arcila ME, Nafa K, Chaft JE, Rekhtman N, Lau C, Reva BA, *et al*. EGFR exon 20 insertion mutations in lung adenocarcinomas: prevalence, molecular heterogeneity, and clinicopathologic characteristics. *Mol Cancer Ther* 2013;12(2):220–229. https://doi.org/10.1158/1535-7163.MCT-12-0620.

[30] Pacini L, Jenks AD, Vyse S, Wilding CP, Arthur A, Huang PH. Tackling drug resistance in EGFR exon 20 insertion mutant lung cancer. *Pharmgenom Pers Med* 2021;14:301–317. https://doi.org/10.2147/PGPM.S242045.

[31] Piotrowska Z, Costa DB, Oxnard GR, Huberman M, Gainor JF, Lennes IT, *et al*. Activity of the Hsp90 inhibitor luminespib among non-small-cell lung cancers harboring EGFR exon 20 insertions. *Ann Oncol* 2018;29:2092–2097. https://doi.org/10.1093/annonc/mdy336.

[32] Gonzalvez F, Vincent S, Baker TE, Gould AE, Li S, Wardwell SD, *et al*. Mobocertinib (TAK-788): a targeted inhibitor of EGFR exon 20 insertion mutants in non-small cell lung cancer. *Cancer Discov* 2021;11(7):1672–1687. https://doi.org/10.1158/2159-8290.CD-20-1683.

[33] Wu JY, Yu CJ, Chang YC, Yang CH, Shih JY, Yang PC. Effectiveness of tyrosine kinase inhibitors on "uncommon" epidermal growth factor receptor mutations of unknown clinical significance in non-small cell lung cancer. *Clin Cancer Res* 2011;17(11):3812–3821. https://doi.org/10.1158/1078-0432.CCR-10-3408.

[34] Zhang T, Wan B, Zhao Y, Li C, Liu H, Lv T, *et al*. Treatment of uncommon EGFR mutations in non-small cell lung cancer: new evidence and treatment. *Transl Lung Cancer Res* 2019;8(3):302–316. https://doi.org/10.21037/tlcr.2019.04.12.

[35] Bean J, Brennan C, Shih JY, Riely G, Viale A, Wang L, *et al*. MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. *Proc Natl Acad Sci U S A* 2007;104:20932–20937. https://doi.org/10.1073/pnas.0710370104.

[36] Organ SL, Tsao MS, de Bono JS. An overview of the c-MET signaling pathway. *Ther Adv Med Oncol* 2011;3:S7–S19. https://doi.org/10.1177/1758834011422556.

[37] Suryavanshi M, Shah A, Kumar D, Panigrahi MK, Metha A, Batra U. MET amplification and response to MET inhibitors in stage IV lung adenocarcinoma. *Oncol Res Treat* 2017;40(4):198–202. https://doi.org/10.1159/000457801.

[38] Yu HA, Arcila ME, Hellmann MD, Kris MG, Ladanyi M, Riely GJ. Poor response to erlotinib in patients with tumors containing baseline EGFR T790M mutations found by routine clinical molecular testing. *Ann Oncol* 2014;25(2):423–428. https://doi.org/10.1093/annonc/mdt573.

[39] Sos ML, Koker M, Weir BA, Heynck S, Rabinovsky R, Zander T, *et al*. PTEN loss contributes to erlotinib resistance in EGFR-mutant lung cancer by activation of Akt and EGFR. *Cancer Res* 2009;69(8):3256–3261. https://doi.org/10.1158/0008-5472.CAN-08-4055. Erratum in: Cancer Res 2015;75(9):1922.

[40] Horn S, Bergholz U, Jücker M, McCubrey JA, Trümper L, Stocking C, *et al*. Mutations in the catalytic subunit of class IA PI3K confer leukemogenic potential to hematopoietic cells. *Oncogene* 2008;27(29):4096–4106. https://doi.org/10.1038/onc.2008.40.

[41] Jin Y, Shi X, Zhao J, He Q, Chen M, Yan J, *et al*. Mechanisms of primary resistance to EGFR targeted therapy in advanced lung adenocarcinomas. *Lung Cancer* 2018;124:110–116. https://doi.org/10.1016/j.lungcan.2018.07.039.

[42] Sun D, Zhu Y, Zhu J, Tao J, Wei X, Wo Y, *et al*. Primary resistance to first-generation EGFR-TKIs induced by MDM2 amplification in NSCLC. *Mol Med* 2020;26:66. https://doi.org/10.1186/s10020-020-00193-z.

[43] Heigener DF, Schumann C, Sebastian M, Sadjadian P, Stehle I, Märten A, *et al*, Afatinib Compassionate Use Consortium (ACUC). Afatinib in non-small cell lung cancer harboring uncommon EGFR mutations pretreated with reversible EGFR inhibitors. *Oncologist* 2015;20(10):1167–1174. https://doi.org/10.1634/theoncologist.2015-0073.

[44] Oxnard GR, Arcila ME, Chmielecki J, Ladanyi M, Miller VA, Pao W. New strategies in overcoming acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in lung cancer. *Clin Cancer Res* 2011;17:5530–5537.

[45] Yamada T, Matsumoto K, Wang W, Li Q, Nishioka Y, Sekido Y, *et al*. Hepatocyte growth factor reduces susceptibility to an irreversible epidermal growth factor receptor inhibitor in EGFRT790M mutant lung cancer. *Clin Cancer Res* 2010;16:174–183.

[46] Suzawa K, Offin M, Schoenfeld AJ, Plodkowski AJ, Odintsov I, Lu D, *et al*. Acquired MET exon 14 alteration drives secondary resistance to epidermal growth factor receptor tyrosine kinase inhibitor in EGFR-mutated lung cancer. *JCO Precis Oncol* 2019;3. https://doi.org/10.1200/PO.19.00011. PO.19.00011.

[47] Tang K, Jiang N, Kuang Y, He Q, Li S, Luo J, *et al*. Overcoming T790M mutant small cell lung cancer with the third-generation EGFR-TKI osimertinib. *Thorac Cancer* 2019;10(2): 359–364. https://doi.org/10.1111/1759-7714.12927.

[48] Furugaki K, Iwai T, Moriya Y, Harada N, Fujimoto-Ouchi K. Loss of an EGFR-amplified chromosome 7 as a novel mechanism of acquired resistance to EGFR-TKIs in EGFR-mutated NSCLC cells. *Lung Cancer* 2014;83(1):44–50. https://doi.org/10.1016/j.lungcan.2013.10.003.

[49] Leonetti A, Sharma S, Minari R, Perego P, Giovannetti E, Tiseo M. Resistance mechanisms to osimertinib in EGFR-mutated non-small cell lung cancer. *Br J Cancer* 2019; 121(9):725–737. https://doi.org/10.1038/s41416-019-0573-8.

[50] Yang Z, Yang N, Ou Q, Xiang Y, Jiang T, Wu X, *et al*. Investigating novel resistance mechanisms to third-generation EGFR tyrosine kinase inhibitor osimertinib in non-small cell lung cancer patients. *Clin Cancer Res* 2018;24:3097–3107.

[51] Jiang T, Su C, Ren S, Cappuzzo F, Rocco G, Palmer JD, *et al*, A consensus on the role of osimertinib in non-small cell lung cancer from the AME Lung Cancer Collaborative Group. *J Thorac Dis* 2018;10:3909–3921.

[52] Peled N, Roisman LC, Miron B, Pfeffer R, Lanman RB, Ilouze M, *et al*. Subclonal therapy by two EGFR TKIs guided by sequential plasma cell-free DNA in EGFR-mutated lung cancer. *J Thorac Oncol* 2017;12:e81–e84.

[53] O'Kane GM, Barnes TA, Leighl NB. Resistance to epidermal growth factor receptor tyrosine kinase inhibitors, T790M, and clinical trials. *Curr Oncol* 2018;25:S28–S37.

[54] Le X, Puri S, Negrao MV, Nilsson MB, Robichaux J, Boyle T, *et al*. Landscape of EGFR-dependent and -independent resistance mechanisms to osimertinib and continuation therapy beyond progression in EGFR-mutant NSCLC. *Clin Cancer Res* 2018;24:6195–6203.

[55] Piotrowska Z, Isozaki H, Lennerz JK, Gainor JF, Lennes IT, Zhu VW, *et al*. Landscape of acquired resistance to osimertinib in EGFR-mutant NSCLC and clinical validation of combined EGFR and RET inhibition with osimertinib and BLU-667 for acquired RET fusion. *Cancer Disco* 2018;8: 1529–1539.

[56] Schrock AB, Zhu VW, Hsieh W-S, Madison R, Creelan B, Silberberg J, *et al*. Receptor tyrosine kinase fusions and BRAF kinase fusions are rare but actionable resistance mechanisms to EGFR tyrosine kinase inhibitors. *J Thorac Oncol* 2018;13: 12–1323.