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ORIGINAL RESEARCH

Characterization of acquired receptor tyrosine–kinase fusions as mechanisms of resistance to EGFR tyrosine–kinase inhibitors

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Haiyuan Xu[1,2,*]
Jinge Shen[3,*]
Jianxing Xiang[4]
Haiyan Li[4]
Bing Li[4]
Tengfei Zhang[4]
Lu Zhang[4]
Xinru Mao[4]
Hong Jian[5]
Yongqian Shu[1]

[1]Department of Oncology, First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, People's Republic of China; [2]Department of Oncology, Kunshan First People's Hospital affiliated to Jiangsu University, Kunshan 215300, People's Republic of China; [3]Department of Emergency, Shanghai Chest Hospital, Shanghai Jiaotong University, Shanghai 200000, People's Republic of China; [4]Burning Rock Biotech, Guangzhou 510000, People's Republic of China; [5]Department of Oncology, Shanghai Chest Hospital, Shanghai Jiaotong University, Shanghai 200000, People's Republic of China

*These authors contributed equally to this work

Correspondence: Yongqian Shu
Department of Oncology, First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, People's Republic of China
Tel +86 25 8371 8836
Email shuyongqian01@163.com

Hong Jian
Department of Oncology, Shanghai Chest Hospital, Shanghai Jiaotong University, 241 West Huaihai Road, Shanghai 200000, People's Republic of China
Tel +86 21 2220 0000
Email janechest@aliyun.com

Purpose: Responses to *EGFR*-targeted therapy are generally temporary, due to inevitable drug resistance. The prevalence and characteristics of receptortyrosine–kinase (RTK) fusion as acquired resistance to EGFR tyrosine–kinase inhibitors (TKIs) are rarely investigated.

Methods: We retrospectively reviewed genomic profiling data of 3873 *EGFR* (exons 18–21)-mutant lung cancer patients with more than once next-generation sequencing detection. A total of 16 patients who acquired RTK fusions during EGFR-TKI treatment with paired pre- and post-EGFR-TKI samples were identified. Their treatment history was collected.

Results: Newly acquired RTK fusions during EGFR-TKI treatment included *RET* (n=6, 37.5%), *ALK* (n=5, 31.3%), *NTRK1* (n=4, 25.0%), *ROS1* (n=1, 6.3%), and *FGFR3* (n=1, 6.3%). All *RET* and *EML4–ALK* fusions were uncommon variants of *KIF5B-RET* and E2: A20 (V5), respectively. Interestingly, *RET* fusion occurred only after osimertinib treatment, and contributed to drug resistance in 50% (6 of 12) of patients treated with osimertinib, indicating that fusions had different prevalence when functioning as resistance mechanisms to EGFR TKIs. Moreover, we found that in all patients developing drug resistance to EGFR TKIs due to fusion emergence (n=16), those that had a treatment history of third-generation EGFR TKIs accounted for 75% (n=12).

Conclusion: We have extended the current knowledge of resistance mechanisms to EGFR TKIs in non-small-celllung cancer. Detection of RTK fusions should be included in genomic profiling panels to uncover potential resistance mechanisms of EGFR TKIs, which might inform therapeutic strategies, such as combination-therapy approaches, to circumvent tumorigenesis.

Keywords: receptor tyrosine kinase fusions, acquired resistance, EGFR tyrosine kinase inhibitors, lung cancer

Introduction

Improved understanding of the molecular changes that drive tumorigenesis has revolutionized the clinical management of non-small-cell lung cancer (NSCLC).[1,2] Targeted therapy improves outcomes in *EGFR*-mutant NSCLC patients, but clinical responses to these drugs are generally incomplete and temporary, due to inevitable drug resistance.[3,4] Acquired resistance will be the major limitation preventing *EGFR*-targeted therapy from having greater impact. Although several resistance mechanisms to EGFR tyrosine-kinaseinhibitors (TKIs) have been identified, there are still many left to be discovered.[5]

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Acquired resistance, which can be classified as "on-target" and "off-target", likely arises from the acquisition of new alterations under the selective pressure imposed by therapy. The emergence of *EGFR*^{T790M} and *EGFR*^{C797S} during treatment with EGFR-TKIs belongs to on-target resistance, which limits the drug's ability to inhibit EGFR through alterations of *EGFR* itself.[6–8] Alterations activating signaling pathways downstream or in parallel with EGFR lead to off-target resistance, while also sustaining EGFR oncogenic signaling and tumor-cell proliferation, despite the inhibition of original EGFR. Such resistance mechanisms include amplifications in *MET*, *HER2, BRAF*, and fusions in receptor tyrosine kinases -(RTKs).[9–13] Fusions of such RTKs as *RET, ALK*, and ROS1, occurring in 1%–2%, 5%, and 1%–2% of NSCLC patients, respectively, have become potential therapeutic targets. Offin et al reported two *EGFR*-mutant lung cancer patients acquiring *ALK* rearrangements after osimertinib and responding to a combination of osimertinib and an ALK TKI.[12] However, limited cases of acquired RTK fusions as mechanisms in EGFR TKIs have been reported. Meanwhile, the prevalence and characteristics of RTK fusions in acquired-resistance settings are rarely investigated.

We performed a retrospective study of 3,873 *EGFR* (exons 18–21)-mutant NSCLC patients with more than once detection by next-generation sequencing (NGS) and identified 16 patients with newly acquired RTK fusions. We aimed to interrogate the potential characteristics of RTK fusion as a resistance mechanism during EGFR-TKI treatment.

Methods

Patient selection

We retrospectively reviewed genomic profiling data of 3,873 *EGFR* (exons 18–21)-mutated NSCLC patients with more than once NGS detection. Their samples were profiled in a Clinical Laboratory Improvement Amendments–certified clinical molecular diagnostic laboratory (Burning Rock Biotech, Guangzhou, China) from September 2015 to October 2018, with the intent of investigating acquired fusion-related EGFR-TKI resistance. Sequencing panels used in this study were commercial panels provided by Burning Rock Biotech. The study was approved by the institutional review board of the First Affiliated Hospital of Nanjing Medical University. All other centers were covered by this protocol. All patients whose tissues or plasma samples were used in this research had provided written informed consent, in accordance with the Declaration of Helsinki.

DNA isolation

Tissue DNA was extracted using a QIAamp DNA formalin-fixed,paraffin-embedded tissue kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Circulating cell-free DNA was recovered from 4–5 mL plasma using a QIAamp circulating nucleic acid kit (Qiagen). To prepare cfDNA from pleural fluid samples, we first removed cells from pleural fluid by low-speed centrifugation, followed by high-speed centrifugation to remove any debris. The resultant supernatant was then subjected to cfDNA extraction using the circulating nucleic acid kit. DNA concentration was quantified using the fluorometry (Qubit 2.0; Thermo Fisher Scientific, Waltham, MA, US). A minimum of 50 ng DNA from plasma samples and 200 ng DNA from formalin-fixed, paraffin-embedded/pleural fluid samples was required for construction of the NGS library.

NGS-library preparation and capture-based targeted DNA sequencing

DNA shearing was performed using Covaris M220. End repair and A tailing were followed by adaptor ligation. Ligated fragments of 200–400 bp were selected by beads (Agencourt AMPure XP kit; Beckman Coulter, Brea, CA, US), hybridized with probe baits, selected by magnetic beads, and amplified by PCR. Indexed samples were sequenced on a NextSeq500 (Illumina, San Diego, CA, US) with pair-end reads.

NGS data-analysis pipeline

All reads were trimmed with Trimmomatic[14] for adapters and then mapped to the human genome (hg19) with BWA software.[15] Local alignment optimization, mark duplication, and variant calling were performed using the Genome Analysis Toolkit 3.2,[16] Picard, and VarScan.[17] Gene rearrangements were called with FACTERA[18] and copy-number variants analyzed with an inhouse algorithm based on sequencing depth. Variants were filtered using the VarScan filter pipeline, with loci at depth <100 filtered out. At least two and five supporting reads were needed for insertions/deletions in plasma and tissue samples, respectively, while eight supporting reads were needed for single-nucleotidevariants to be called in both plasma and

tissue samples. According to the ExAC, 1000 Genomes, dbSNP, and ESP6500SI-V2 databases, variants with population frequency >0.1% were grouped as single-nucleotide polymorphisms and excluded from further analysis. Remaining variants were annotated with ANNOVAR[19] and SnpEff v3.6.[20]

Results

Patient characteristics

Genomic profiles of 3,873 *EGFR*-mutated (exons 18-21) lung cancer patients who had received at least one NGS detection were reviewed retrospectively. A total of 9,012 samples were included in this study. Overall, 653 samples were sequenced using a 520-gene pan-cancer-related panel, 234 samples using a 295-gene pan cancer-related panel, 6,926 samples profiled using a panel of 168 lung cancer-related genes, 300 samples assessed using a 56-gene panel, and the remaining 899 samples were sequenced using a panel consisting of seven well-known lung cancer–driver genes (*EGFR*, *ERBB2*, *BRAF, ROS1*, *RET*, *ALK*, and *MET*) plus *KRAS*, a well-established prognostic factor. *ALK*, *ROS1*, and *RET* fusions can be detected by all panels, while *NTRK* and *FGFR* fusions can be detected by 168-, 295-, and 520-gene panels.

A total of 93 cases (2.4%) were identified with concomitant *EGFR* alterations and RTK fusions. Among them, 16 patients had positive RTK fusions after EGFR-TKI resistance but negative RTK fusions in pretreatment samples. The 16-patient cohort with paired pre- and post-EGFR TKI–treatment samples was used for following analyses, which investigated kinase fusions as a resistance mechanism to EGFR TKIs (Table 1). The overall workflow for patient selection is demonstrated in Figure 1.

Molecular characteristics of pretreatment samples

In this cohort consisting of 16 *EGFR* and RTK coaltered patients, the median age was 54 years, ranging 45–80 years. Five (31.3%) were males and eleven (68.7%) females. All patients had been diagnosed with lung adenocarcinoma.

Samples profiled at baseline (before EGFR-TKI treatment proceeding to the detection of RTK fusion) consisted of 15 plasma samples and 1 FFPE (case 14). Genomic profiling results revealed that ten (62.5%) cases were positive for *EGFR* exon 19 deletion, four (25.0%) had L858R, and two (12.5%) L861Q. In addition, T790M was identified in ten (62.5%) cases and C797S/G were found in one (6.3%, case 9; Figure 2A).

The 16 patients had different TKI-treatment history after positive *EGFR*-alteration detection. Before the emergence of RTK fusions, three patients (18.8%) received first-generation EGFR-TKI treatment (gefitinib, n=1, 6.3%; erlotinib, n=2, 12.5%), one case (6.3%) received second-generation TKI (afatinib) treatment, and 12 patients (75.0%) received third-generation TKI (osimertinib) treatment (Figure 2B). Of note, we classified case 15 into the osimertinib group, since we thought *ALK* fusion was likely induced by osimertinib.

Molecular characteristics of acquired fusions in posttreatment samples

After EGFR-TKI therapy, all 16 patients acquired RTK fusions. Posttreatment samples contained 15 plasma samples and one pleural effusion specimen (case 10). Among these, the RTK fusions involved consisted of *RET* (n=6, 37.5%), *ALK* (n=5, 31.3%), *NTRK1* (n=4, 25.0%), *ROS1* (n=1, 6.3%), and *FGFR3* (n=1, 6.3%; Figure 3). Of note, one patient had concomitant *ROS1* and *NTRK1* fusions.

Three *RET*-fusion partners were detected in six patients, including *CCDC6* (n=3), *NCOA4* (n=2), and *CDC123* (n=1). The most common *RET*-fusion variant *KIF5B–RET* was not detected in this EGFR TKI–resistant cohort, coinciding with findings from other reports and one of our previous studies.[13,21] Interestingly, we observed that all *RET* fusions occurred after osimertinib treatment, constituting 50% (six of 12) of patients with acquired RTK fusions following osimertinib treatment.

Partners of *ALK* identified in the five ALK^+ patients included *EML4* (n=3), *STRN* (n=1), and *CEBPZ* (n=1). All three *EML4–ALK* fusions emerging after EGFR-TKI resistance were the rare variant E2:A20 (V5). Four fusion partners of *NTRK1* detected in this cohort contained *LRRC71* (n=1), *PLEKHA6* (n=1), *RPL8* (n=1), and *RP11-565P22.6* (n=1). The *ROS1* partner identified here was *DCBLD1* (n=1) and the *FGFR3* partner *TACC3* (n=1). Schematic structures of rearrangements of all RTK fusions are demonstrated in Figure 4.

All the ten cases who had T790M in pretreatment samples received osimertinib treatment. We found five patients with acquired RTK fusions and disappearance of T790M after osimertinib treatment. Five patients still harbored T790M during the emergence of fusions. In addition, two cases (12 and 16) acquired T790M along with RTK fusions

Table 1 Genomic profiles of paired pre- and post-EGFR-TKI treatment samples

Case	Age/sex	NGS results before TKI			NGS results after TKI				
		EGFR	Other mutations	Treatment before fusion detection	*EGFR*	T790M Loss	Fusion	Other mutations	Treatment after fusion detection
Case 1	47/F	L861Q, R776G	NA	Osimertinib	L861Q, R776G	NA	*FGFR3–TACC3*	NA	Continue osimertinib
Case 2	49/F	E746_A750del, T790M	NA	Osimertinib	E746_A750del, T790M	No	*RP11–565P22.6–NTRK1, DCBLD1–ROS1*	*ERBB2* amp	NA
Case 3	67/F	E709K, L858R	NA	Gefitinib	E709K, L858R	NA	*LRRC71–NTRK1*	*EGFR* amp, *MET* amp	NA
Case 4	54/F	E746_A750del	NA	Erlotinib	E746_A750del	NA	*PLEKHA6–NTRK1*	NA	AZD3759
Case 5	47/F	E746_A750del, T790M	NA	Osimertinib	E746_A750del	Yes	*RPL8–NTRK1*	*MET* amp	NA
Case 6	52/F	L858R	NA	Osimertinib	L858R	NA	*CCDC6–RET*	*EGFR* amp	NA
Case 7	45/F	E746_A750del, T790M	NA	Osimertinib	E746_A750del, T790M	NA	*CCDC6–RET*		Continue osimertinib
Case 8	51/M	E746_T751delinsA, T790M	NA	Osimertinib	E746_T751delinsA	Yes	*CCDC6–RET*	*KRAS* amp	Continue osimertinib
Case 9	46/F	C797G, C797S, E746_A750del, T790M	NA	Osimertinib	C797G, C797S, E746_A750del, T790M	No	*CDC123–RET*	*MET* amp	Continue osimertinib, then switch to capmatinib
Case 10	80/F	E746_A750del, T790M	NA	Osimertinib	E746_A750del	Yes	*NCOA4–RET*	NA	NA
Case 11	54/M	L747_T751del, T790M	NA	Osimertinib	L747_T751del, T790M	No	*NCOA4–RET*	NA	NA
Case 12	80/M	L861Q	NA	Afatinib	L861Q, T790M	NA	*EML4–ALK*	*EGFR* amp, $KRAS^{G12D}$	Osimertinib
Case 13	55/F	T790M, L844V, L858R	NA	Osimertinib	L844V, L858R	Yes	*EML4–ALK*	NA	NA
Case 14	57/F	E746_A750del, T790M	NA	Osimertinib	E746_A750del, T790M, C797S	No	*EML4–ALK*	*EGFR* amp	NA
Case 15	64/M	L858R, T790M	NA	Afatinib resistance, then switch to osimertinib	L858R	Yes	*STRN–ALK*	NA	Osimertinib
Case 16	59/M	E746_A750del	NA	Erlotinib	E746_A750del, T790M	NA	*CEBPZ–ALK*	NA	Crizotinib

Abbreviations: NA, not applicable; amp, amplification.

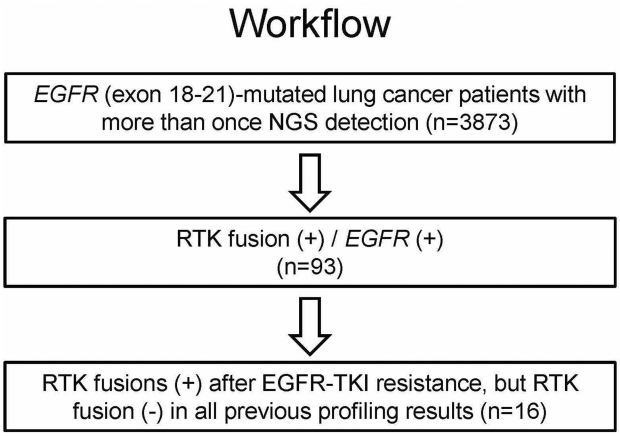

Figure 1 Workflow of patient selection.
Abbreviations: RTK, receptor tyrosine kinase; TKI, tyrosine-kinase inhibitor.

after first- or second-generation EGFR-TKI treatment. Besides T790M, several known resistance mechanisms occurred concomitantly with RTK fusion during EGFR-TKI treatment in eight patients, including *EGFR* amplification (amp; n=4), *MET* amp (n=3), *ERBB2* amp (n=1), *KRAS* amp (n=1), $KRAS^{G12D}$ (n=1), and $EGFR^{C797S}$ (n=1).

Discussion

In this study, we retrospectively investigated acquired RTK fusions as resistance mechanisms to EGFR TKIs in the largest NSCLC cohort with paired pre- and posttreatment samples to the best of our knowledge. It is conventionally considered that oncogenic driver mutations, such as *ALK* or *ROS1* rearrangements, are mutually exclusive with *EGFR* mutations in NSCLC.[22–25] Here, we report that RTK fusions as a result of *ALK*, *ROS1*, *RET, FGFR3*, and *NTRK1* gene rearrangements emerged as resistance mechanisms to EGFR TKIs, providing a basis for the hypothesis that an actionable driver mutation could function as an acquired resistance mechanism to drugs targeting another actionable driver alteration.

We found that all six *RET* fusions emerged after third-generation TKI treatment, and half the patients developing osimertinib resistance in this selected cohort of ours had this caused by *RET* fusions (six of 12, 50.0%). This result corroborates a recent study stating that no *ALK* or *RET* fusions were found in tumor tissue from 174 patients who developed resistance to erlotinib or afatinib, but two *ALK* fusions and one *RET* fusion were found from 14 patients after progression developed during osimertinib treatment.[12] It suggested that the prevalence of fusion events developed during different EGFR-TKI treatments might be distinct.

In addition, for the six patients with acquired *RET* fusions, no fusion variants were *KIF5B–RET*, the most common *RET* fusion. Other groups have also found non-*KIF5B–RET* fusions in *EGFR*-mutant patients with TKI resistance, which coincides with our observations. Reckamp et al studied nearly 33,000 samples and found that *CCDC6–* and *NCOA4–RET* fusions may contribute to

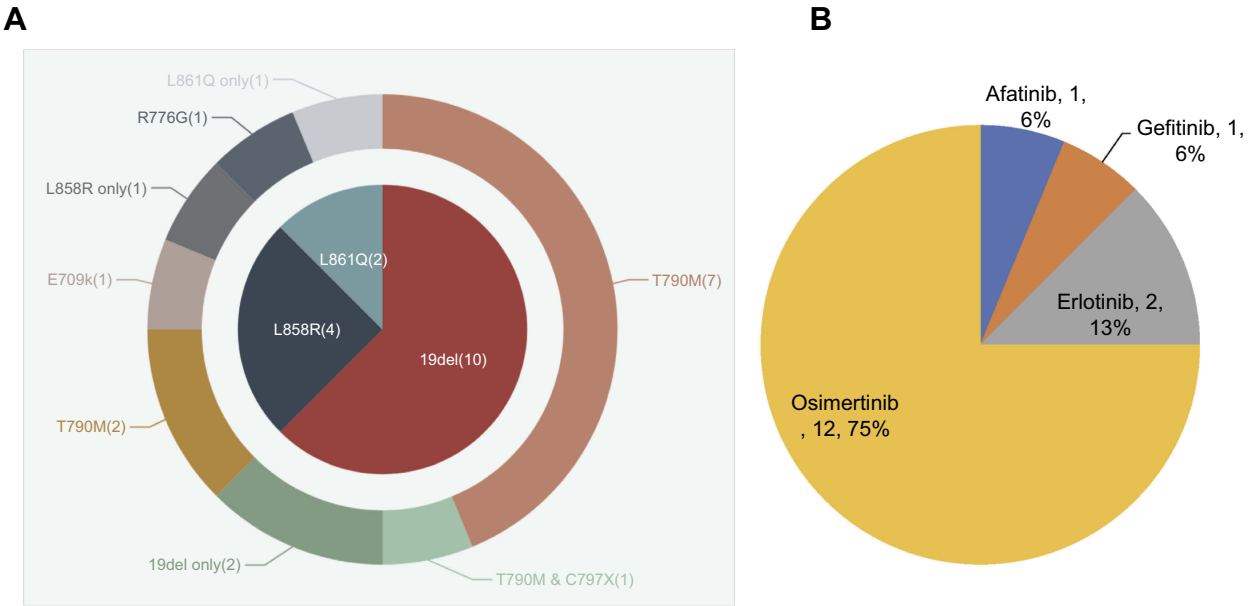

Figure 2 Molecular characteristics in 16 patients who acquired RTK fusions as resistance mechanisms to EGFR TKIs with paired pre- and posttreatment samples.
Notes: (**A**) Distribution of *EGFR* alterations in pretreatment samples; (**B**) treatment history before emergence of fusions.
Abbreviations: RTK, receptor tyrosine kinase; TKIs, tyrosine-kinase inhibitors.

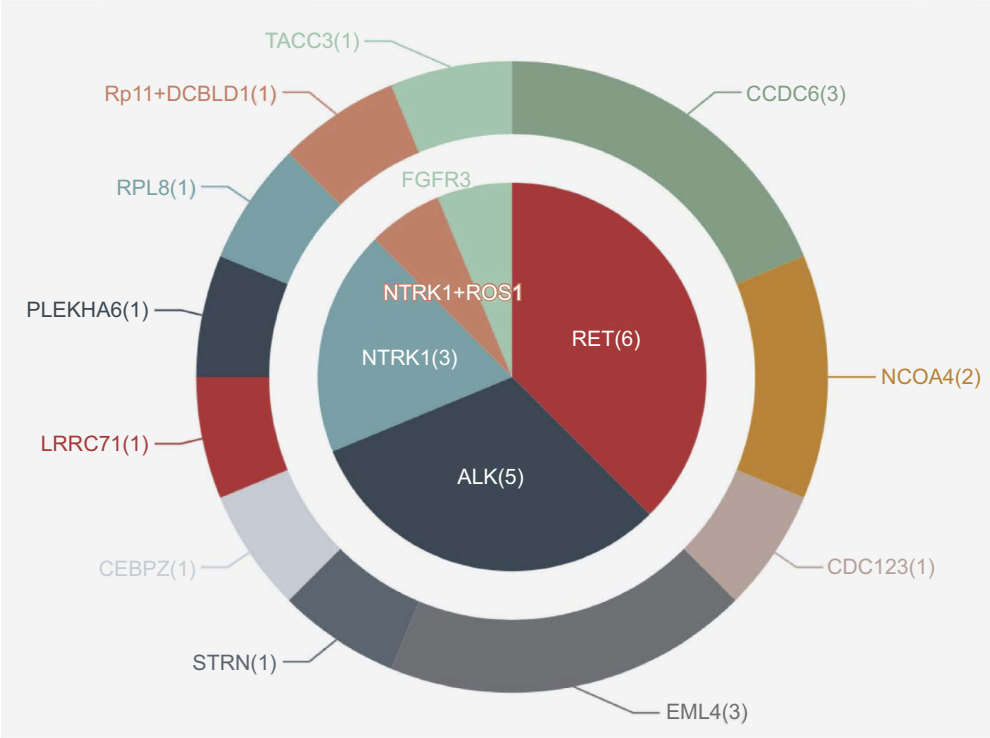

Figure 3 Variant distribution of acquired RTK fusions in EGFR-TKI posttreatment samples.
Abbreviations: RTK, receptor tyrosine kinase; TKI, tyrosine-kinase inhibitor.

anti-EGFR therapy resistance in NSCLC.[21] Schrock et al assessed >3,500 *EGFR*-mutant patients and found that the two NSCLC *RET* fusions emerging after EGFR-TKI treatment were both *CCDC6–RET*.[13] Our results also revealed that all three *EML4–ALK* fusions presented after resistance were the rare variant V5. The absence of common partners/variants in EGFR TKI–resistant patients suggested that common partners/variants themselves were strong enough to drive primary oncogenesis and rare partners/variants more likely functioned as potential acquired resistance mechanisms to EGFR TKIs. Therefore, it is potentially important to use NGS to identify precise fusion partners/variants and further guide treatment strategies, since heterogeneity of clinical responses exists among different fusion partners/variants.[26–29] Owing to the limited number of paired pre- and post-EGFR TKI–treated samples, further studies are needed to validate our new observations.

We observed in our cohort that 75% of patients developed drug resistance after third-generation EGFR-TKI treatment, due to fusion emergence, compared with 18.8 and 6.3% after first- and second-generation EGFR TKIs, respectively. This observation supported the notion that newly acquired RTK fusions were not seen or seen rarely with earlier-generation EGFR TKIs, but could be found at a higher rate with osimertinib resistance.[12] This underscores the necessity to detect fusion status after osimertinib resistance in clinical settings.

In vitro study revealed that *RET* fusions can lead to resistance to osimertinib in *EGFR*-mutant cells and combination treatment with cabozantinib can restore the response to osimertinib.[12] The combination of EGFR TKIs and fusion-based targeted therapy has been reported to be successful in overcoming resistance to EGFR TKIs in clinical cases. Combination treatment with full-dose osimertinib and crizotinib in an *EGFR*-mutant tumor harboring both T790M and *MET*-mediated mechanisms of acquired resistance showed tolerable and effective clinical benefits.[30] Furthermore, an NSCLC patient harboring $EGFR^{L858R}$ and *RET* fusion had an additional 7 months of stable disease after initiation of combinatorial treatment with afatinib and cabozantinib.[13] The potential of such combinations to overcome multiple mechanisms of acquired resistance in *EGFR*-mutant NSCLC should be studied further.

Along with the emergence of RTK fusions during EGFR-TKI treatment, we identified polyclonal resistance alterations in eight cases. This suggests that accurate

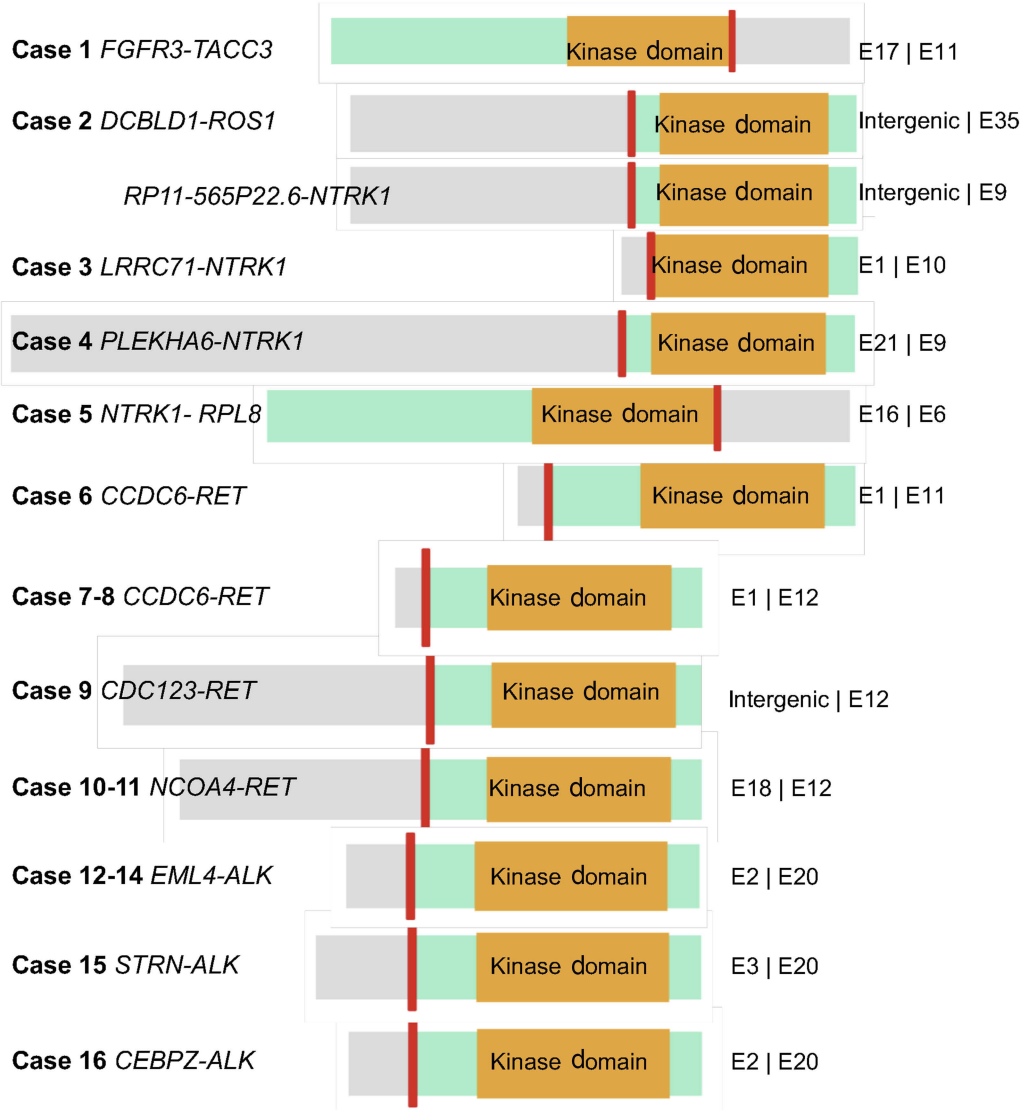

Figure 4 Schematic structure of all RTK fusions.
Notes: The short red line indicates the breakpoint of each fusion. The green box represents the gene thatcontains kinase domain. The gray box represents the fusion-partner gene. The orange box represents the kinase domain. The length of the left and right fragments represents the exon number of two genes in fusion.
Abbreviation: RTK, receptor tyrosine kinase.

assessment of polyclonal drug resistance is limited by the number of resistance mechanisms assessed, the depth of sequencing, and the quantity of tumor DNA assessed in resistant samples. This points to the necessity to apply appropriate NGS panels to detect and monitor all classes of genomic alterations that may guide precise targeted therapies.

There were several limitations of this study. First, although we had a large *EGFR*-mutated NSCLC population at screening, due to the rare occurrence of RTK fusions, the cohort of post
EGFR-TKI–treated RTK fusion–positive samples was still too small fully to reflect the underlying pattern of the fusion resistance mechanism. Studies in larger cohorts are needed to address this phenomenon further. Second, clinical information on patients who received combination therapies with EGFR TKIs and fusion-targeted drugs was unavailable, and thus could not provide clinical evidence for efficacy of such a combination strategy in our cohort.

Taken together, to the best of our knowledge, in the largest NSCLC cohort with paired pre- and post-treatment samples, these results highlighted the unique features of EGFR TKI–resistance mechanisms in EGFR-positive NSCLC and provide the rationale for combinatorial therapeutics tailored to the precise resistance mechanisms identified in patients who relapse on EGFR-TKI treatment.

Disclosure

The authors report no conflicts of interest in this work.

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