CHALLENGES AND OPPORTUNITIES OF TKIS IN THE TREATMENT OF NSCLC PATIENTS WITH UNCOMMON MUTATIONS

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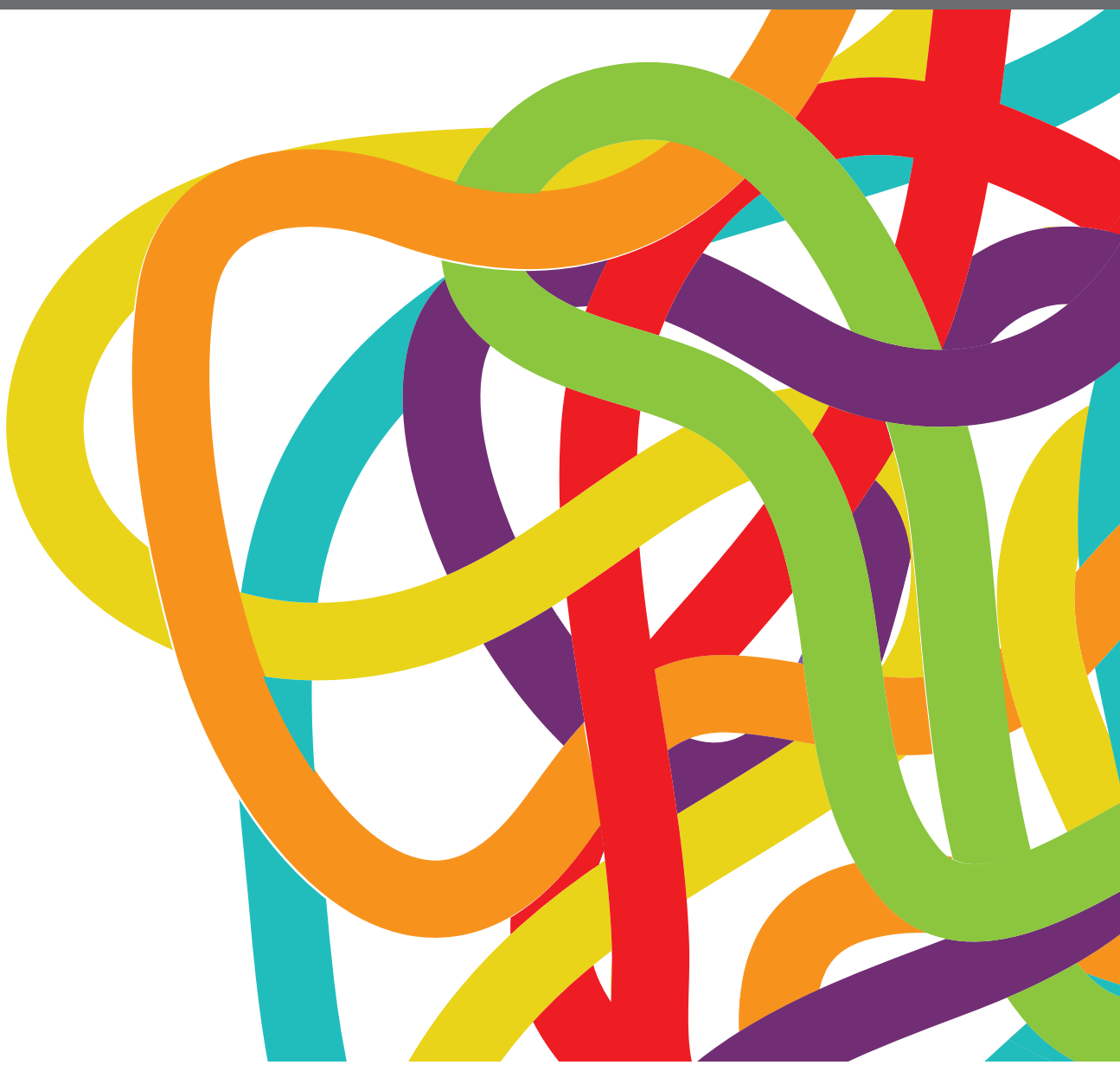

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CHALLENGES AND OPPORTUNITIES OF TKIS IN THE TREATMENT OF NSCLC PATIENTS WITH UNCOMMON MUTATIONS

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Editorial: Challenges and opportunities of TKIs in the treatment of NSCLC patients with uncommon mutations

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non-small cell lung cancer, uncommon mutation, tyrosine kinase inhibitor, targeted therapy, immunotherapy, resistance

Editorial on the Research Topic

Challenges and Opportunities of TKIs in the Treatment of NSCLC patients with Uncommon Mutations

Substantial advances have been made in our understanding of the molecular biology of cancer, leading to profound progression in the fields of diagnosis and treatment of non-small cell lung cancer (NSCLC). More recently, many uncommon mutations are gaining attention in NSCLC, including epidermal growth factor receptor exon 20 insertion (EGFR 20ins), anaplastic lymphoma kinase (ALK), human epidermal growth factor receptor 2 (HER2), rearranged during transfection (RET), v-Raf murine sarcoma viral oncogene homolog B (BRAF), neurotrophic tropomyosin receptor kinase (NTRK), and others (1). Pharmaceutical agents developed to target classical driver mutations detected in NSCLC patients have provided profound and durable responses compared with conventional chemotherapy. This paradigm shift can also be seen in the realm of uncommon mutations. Ongoing studies and clinical trials of tyrosine kinase inhibitors (TKIs) for uncommon targets are gaining insights into possible treatment options to enable long-term survival of patients. Since immunotherapy serves as the backbone treatment in mutation-negative populations, the efficacy of immunotherapy or immunotherapy-based combinations has also been evaluated in patients harboring uncommon targets. Furthermore, other novel therapies and platforms have been developed and tested to provide more possibilities and options for these patients.

In this special issue, we compiled a series of papers including original research, reviews and case reports that focus on recent advances and challenges in the treatment of lung cancer patients with uncommon driver mutations. For rare EGFR mutations, Xu et al. reported that two patient-derived xenografts in zebrafish embryos from two patients

harboring EGFR 20ins received precision treatment. Zebrafish were inoculated with tumor cells and cultured in osimertinib-containing medium to predict a clinical response. Their study demonstrated the applicability of zebrafish models for testing targeted drugs. Yang et al. identified five metastatic NSCLC patients with EGFR p.L747P mutation and found that afatinib achieved numerically longer progression-free survival (PFS). Dynamic simulations and *in vivo* experiments demonstrated that afatinib had the best binding affinity and significantly inhibited p.L747P-mutant tumor growth. Feng et al. reported three locally advanced NSCLC patients with EGFR sensitive mutations switching to aumolertinib, a novel third generation EGFR-TKI, as neoadjuvant therapy after 1-2 cycles of preoperative chemotherapy neoadjuvant therapy. Excellent tumor remission and downstaging were achieved to allow surgical treatment, and no tumor recurrence was observed until the latest follow-up. This may indicate that aumolertinib was clinically applicable and could be a viable option in the neoadjuvant phase of therapy.

Nowadays, ALK-altered NSCLC can be treated with a variety of effective ALK inhibitors and a number of next-generation ALK-TKIs have already been developed. Peng et al. reviewed recent studies and summarized the efficacies and safety profiles of ALK-TKIs and other therapies with data from preclinical and clinical trials. They also proposed several key points regarding treatment sequencing strategies, resistance mechanisms of ALK-TKIs and toxicity problems when giving these drugs.

HER2 aberrations are comprised of three distinct formations: mutation, amplification and overexpression. They were originally discovered in breast and gastric cancer, but their role in lung cancer is gaining increasing attention. Yu et al. reviewed available data and described the biological function of HER2 and its dysregulation in NSCLC, as well as clinical characteristics of patients. Further, they provided a comprehensive overview of traditional and emerging therapies including chemotherapy and monoantibody, non-selective TKIs, new-generation TKIs and antibody-drug conjugates (ADCs). Of note, ADC-based therapy seems to provide the best clinical outcomes among all treatment regimens, which sheds new light on the management of HER2-altered NSCLC.

There are two real-world studies concerning treatment options for RET-fusion positive NSCLC patients. Meng et al. retrospectively analyzed the characteristics and clinical outcomes of patients with RET-fusion-positive NSCLC receiving RET-TKI, multi-kinase inhibitor (MKI), chemotherapy and immunotherapy-based regimens from three centers. The results showed that RET-TKI remained the best choice for a better response rate and PFS. Chemotherapy, especially with angiogenesis inhibitors, was still a good choice, while the other two regimens should not be recommended for this patient group. Zhou et al. reported a case involving a stage IIIA lung adenocarcinoma patient harboring RET rearrangement who was treated with pralsetinib as neoadjuvant target therapy. Pralsetinib exhibited a significant response and transformed the unresectable tumor into a resectable one. Additional clinical trials are warranted to verify the effect of pralsetinib for locally advanced NSCLC.

BRAF activation consists of several distinct forms including V600 and non-V600 mutations, rearrangements, fusions, in-frame deletions and insertions. Yan et al. and Sun et al. discussed the diagnostic challenges of BRAF mutations, therapeutic strategies and post-therapeutic evolutionary pathways of BRAF, and also the mechanisms of resistance to BRAF-TKIs. NTRK fusion has become increasingly studied in lung cancer. Liu et al. provided a panoramic view of the function of NTRK genes, the diagnostic techniques for NTRK fusions, the clinical data on TRK inhibitors and their resistance mechanisms. These reviews provide us with a comprehensive view of the current landscape of BRAF and NTRK alterations in NSCLC, but there are still many unsolved issues to be addressed.

Personalized medicine has revolutionized the therapeutic landscape of lung cancer with molecular alterations in the past two decades. Since most uncommon mutations are associated with poor to moderate efficacy of immunotherapy, their identification and the development of new-generation drugs are pivotal for designing improved treatment strategies. Attempts to treat other rare mutations are being investigated in addition to those discussed above, such as MET, ROS1, FGFR, STK11, etc. The growing use of next-generation sequencing (NGS) platforms has expanded the panel of genes that can be detected and targeted, and noninvasive liquid biopsy techniques will provide more information regarding efficacy monitoring and resistance mechanisms in real-time. With novel therapies springing up, further investigation should continue to evolve and lead to improve patient outcomes.

Finally, we thank all the authors for their inspiring contributions to this Research Topic and hope that these papers will provide our readers with a deep understanding of the current status and future directions in the treatment of NSCLC patients with uncommon mutations.

Author contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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BRIEF RESEARCH REPORT
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Use of Pralsetinib as Neoadjuvant Therapy for Non-Small Cell Lung Cancer Patient With RET Rearrangement

Ning Zhou[1,2†], Tong Li[1,2†], Maoli Liang[3†], Fan Ren[1,2], Hong Ni[1,2], Wei Liu[4], Tao Shi[5], Dongbo Xu[6], Qiusong Chen[7], Haonan Yu[7], Zuoqing Song[1,2], Lingling Zu[1,2], Shuo Li[3] and Song Xu[1,2]**

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RET rearrangements are rare, and occur in 1%-2% of all non-small cell lung cancer (NSCLC) patients. Pralsetinib has a significant anti-tumor effect in patients with advanced NSCLC and a RET rearrangement. Previous studies have confirmed the efficiency of neoadjuvant target therapy for NSCLC. Herein we present a case involving a female patient who was diagnosed with stage IIIA lung adenocarcinoma and harbored a KIF5B-RET rearrangement based on next-generation sequencing. Radiologic downstaging was indicated after pralsetinib treatment. Therefore, a right lower lobectomy and systemic lymphadenectomy were successfully performed. The postoperative pathologic results revealed a response rate of 74% for primary tumor and no residual viable tumor cells were observed in lymph nodes. The tumor, nodes, and metastases (TNM) stage was ypT1cN1M0. The tumor micro-environment (TME) of the primary tumor was also assessed.

Keywords: RET, pralsetinib, locally advanced (stage III) non-small cell lung cancer, neoadjuvant, targeted therapy

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INTRODUCTION

The RET gene was identified as a proto-oncogene in 1985 (1). The RET gene is associated with normal embryonic development (2). RET fusions are rare, occurring in 1%-2% of all patients with non-small cell lung cancer (NSCLC) (3). Patients with RET fusions are prone to brain metastases (4). Because RET fusions occur in lung cancer, RET-targeted therapy has been attempted by clinicians. Unfortunately, the efficacy of some multi-targeted tyrosine kinase inhibitors (TKIs) was not satisfactory, including vandetanib, cabozantinib, and lenvatinib (5). Pralsetinib had a significant effect in patients with advanced NSCLC; specifically, the response rate was 61% (6). Pralsetinib was approved by the Food and Drug Administration (FDA) for treating RET fusion-positive NSCLC in

2020. Several studies have verified the availability of neoadjuvant-targeted therapy for NSCLC patients with ROS1, ALK, and epidermal growth factor receptor (EGFR) alterations (7–9); however, there are no reports regarding pralsetinib as neoadjuvant treatment for NSCLC patients with a RET rearrangement.

CASE REPORT

A 54-year-old female never-smoker was admitted to the hospital for evaluation of a non-productive cough with bloody phlegm for 1 year and persistent chest and back pain for 1 month. Enhanced computed tomography (CT) revealed a mass with a diameter of 42 mm located in the right lower lung with enlarged mediastinal and hilar lymph nodes (stations 2, 4, 7, and 10). The CT findings were confirmed by 18F-fluorodeoxyglucose (FDG) positron emission tomography (PET) as cT2bN2M0, IIIA (AJCC, 8th edition; **Figure 1**). A bronchoscopic biopsy was performed and the pathologic examination revealed that the right lung lesion was a lung adenocarcinoma. We further performed next-generation sequencing (NGS) with a 520-gene panel; a KIF5B-RET fusion was detected. Plasma ctDNA for the RET mutation was also positive with a frequency of 0.15%. After a multiple disciplinary team (MDT) discussion, the patient was diagnosed with a resectable stage IIIA lung adenocarcinoma. Based on the NGS results, we recommended neoadjuvant treatment followed by surgical resection. After obtaining informed consent from the patient, we prescribed pralsetinib at a dosage of 400 mg per day. After 1 month of treatment, a chest CT scan revealed significant shrinkage of the lung tumor (**Figure 1**). A PET-CT scan exhibited significantly decreased F18-FDG uptake in the tumor and no uptake in the hilar and mediastinal lymph nodes (**Figure 1**). In addition, the plasma ctDNA level was also tested, and we showed that the plasma ctDNA was cleared after neoadjuvant treatment (**Figure 2**). During the treatment of pralsetinib, some treatment-related adverse effects were observed, including mild edema and fatigue and moderately increased blood pressure.

Considering that radiologic downstaging was indicated, a right lower lobectomy and systemic lymphadenectomy were successfully performed 1 week after the last dose of pralsetinib (**Figure 1**). Severe adhesions were noted intraoperatively. The postoperative pathologic results showed that although the Ki67 index was significantly decreased, 26% of the tumor cells were still alive in the primary tumor bed however no residual viable tumor cells were observed in lymph nodes. Microscopically, a large number of lymphocytes were infiltrated together with some plasma cells and neutrophils. The lymphatic follicles were formed. Foam cell reactions and cholesterol crystals were observed as well as necrosis and fibrosis. To investigate the change in tumor microenvironment (TME), especially inflammatory and immune cells before and after pralsetinib neoadjuvant treatment, multiple immunohistochemistry (mIHC) staining (Genecast Biotechnology, Wuxi, Jiangsu, China) on the biopsy tissue and surgical sample was performed. The CD68+

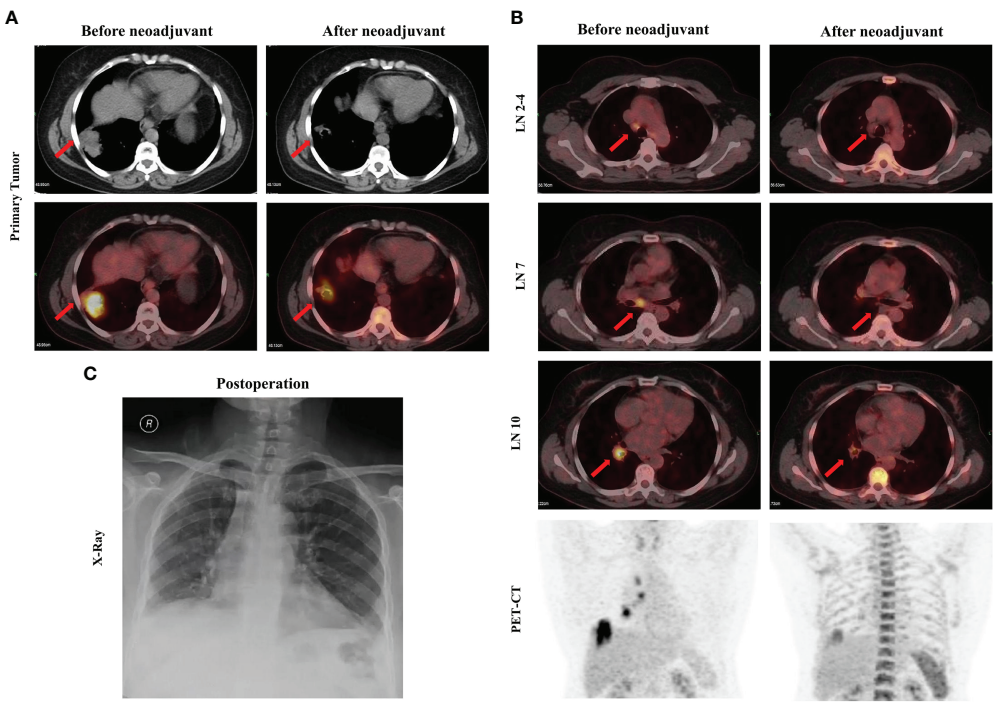

FIGURE 1 | Images before and after neoadjuvant pralsetinib treatment. **(A)** Enhanced CT and PET-CT of the primary tumor. **(B)** PET-CT of the mediastinal lymph nodes and the chest. **(C)** X-ray after surgery.

CD163+ cell population was significantly increased, whereas the proportion of CD8+ T lymphocytes was decreased (**Figure 2**). PD-L1 expression were also decreased after neoadjuvant treatment (**Figure 2**). The patient received four cycles of pemetrexed (500mg/m^2, d1) and cisplatin (75mg/m^2, d1) every 21 days in the adjuvant settings due to the unaffordable economic burden of pralsetinib.

DISCUSSION

In recent years the benefit of neoadjuvant-targeted therapies for EGFR- and ALK-driven NSCLC patients has been identified (10). To our knowledge, this is the first case of neoadjuvant pralsetinib for NSCLC patients with a RET fusion. In our case pralsetinib exhibited a significant response in a patient with NSCLC and an RET fusion and transformed the unresectable tumor into a resectable tumor; however, 26% of the tumor cells were still alive after pralsetinib neoadjuvant treatment, indicating the necessity of complete resection.

In the current study, we also performed mIHC to determine the changes in TME before and after pralsetinib neoadjuvant treatment. The staining data demonstrated that the proportion of M1 macrophages was upregulated, while the number of CD8+ tumor infiltrating lymphocytes (TILs) and the level of PD-L1 expression were decreased significantly after neoadjuvant treatment. Significant changes in other immune cells, such as natural killer (NK) cells, were not detected. The alteration of these factors indicates that the TME is less inflammatory after pralsetinib neoadjuvant treatment. A previous study suggested that high PD-L1 expression and an increased number of CD8+ TILs are related to clinical benefit in immunotherapy (11). In addition, M1 macrophages are thought to have a direct or indirect anti-tumor role (12). However, in our case, the increased proportion of M1 macrophages and decreased number of CD8+ TILs and PD-L1 expression obscured the role of immunotherapy in subsequent treatment. Further therapeutic strategies after resistance of pralsetinib treatment should be seriously considered.

In conclusion, our case, for the first time suggested that pralsetinib neoadjuvant treatment is feasible for locally advanced NSCLC patients with a RET rearrangement. This patient had an apparent radiologic downstaging after neoadjuvant treatment, which was an indication for complete resection. This case report, however, included one patient only. The role for pralsetinib in neoadjuvant treatment for locally advanced NSCLC and postoperative adjuvant-targeted therapy have not been established for early-stage NSCLC. For NSCLC patients with a RET rearrangement, additional clinical trials are

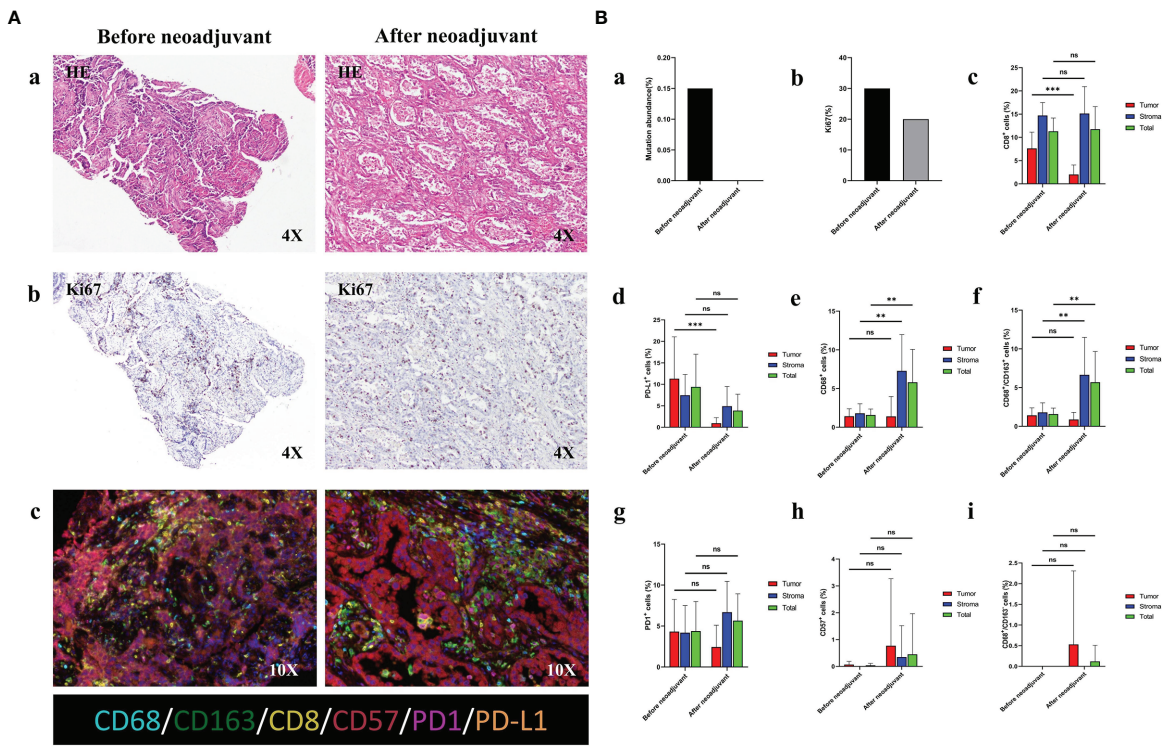

FIGURE 2 | Comprehensive pathological evaluation before and after neoadjuvant pralsetinib treatment. **(A)**. Histochemistry staining before and after neoadjuvant pralsetinib treatment. (a) Hematoxylin and eosin (HE) staining. (b) Ki67 staining. (c) Multiple immunohistochemistry staining on CD68, CD163, CD8, CD57, PD1 and PDL1. **(B)**. Quantitative analysis for plasma ctDNA and staining data. Quantitative analysis for plasma ctDNA (a), Ki67 staining (b), CD8+ (c), PD-L1+ (d), CD68+ (e), CD68+CD163+ (f), PD1+(g), CD57+ (h) and CD68+CD163- (i) cell population. **p < 0.01; ***p < 0.001; ns, not significant.

warranted to evaluate the effect of pralsetinib in locally advanced and early-stage NSCLC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of Tianjin Medical University General Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

NZ: Data curation, Formal analysis, Writing- Original draft preparation. TL: Formal analysis, Writing- Original draft preparation. ML: Data curation, Writing- Original draft preparation. FR: Formal analysis. HN: Visualization. WL: Resources. TS: Resources. DX: Resources. QC: Resources. HY: Resources. ZS: Investigation. LZ: Investigation. SL: Conceptualization, Validation, Visualization. SX: Conceptualization, Methodology, Supervision, Writing- Reviewing and Editing. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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The Lifted Veil of Uncommon EGFR Mutation p.L747P in Non-Small Cell Lung Cancer: Molecular Feature and Targeting Sensitivity to Tyrosine Kinase Inhibitors

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Objectives: The uncommon p.L747P mutation in epidermal growth factor receptor (EGFR) exon 19 reveals to alter the response to tyrosine kinase inhibitors (TKIs) in patients diagnosed with advanced non-small cell lung cancer (NSCLC). However, the underlying mechanism is still not clear. This study aimed to investigate the clinical outcomes, binding affinities, and modes of action of currently available EGFR TKIs towards p.L747P mutation.

Materials and Methods: Clinical data of NSCLC patients harboring p.L747P mutation who had received different generations of EGFR TKIs were collected from medical records. Computational structure of p.L747P was constructed and *in vitro* cellular kinase inhibition assay and mice xenograft experiment were performed to predict and confirm the binding affinities and antitumor activities of diverse EGFR TKIs.

Results: A total of five metastatic NSCLC patients with p.L747P mutation were included in the final analysis. Patients treated with second-generation (2G) TKI afatinib achieved numerically longer progression-free survival (range 2.4-8.5 months) than that with first-generation (1G, range 1.4-5.5 months) or third-generation (3G, range 1.6-7.5 months) TKIs. None of the patients administered 1G or 3G TKIs achieved tumor response, but two-thirds of them treated with afatinib achieved partial response. Dynamics simulation predicted that 2G TKIs presented the best binding affinity to p.L747P mutation. The cellular kinase inhibition assay and mice xenograft experiment confirmed that afatinib could potently inhibit p.L747P-mutant cells and significantly reduce p.L747P-mutant

tumor growth ($P< 0.001$), together with reduced phosphorylation of EGFR and its downstream signalings.

Conclusions: The uncommon p.L747P mutation in EGFR exon 19 resulted in a poor response to first-generation EGFR TKIs. Afatinib revealed a better clinical response and binding affinity compared with osimertinib for this specific alteration.

Keywords: EGFR, tyrosine kinase inhibitor, molecular feature, targeting sensitivity, p.L747P mutation, non-small cell lung cancer

INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality worldwide. New strategies have been developed to target specific alterations in lung cancer in the last decade and hence improved treatment outcomes and survival (1). Classic activating mutations of the epidermal growth factor receptor (EGFR) in non-small cell lung cancer (NSCLC) are found in approximately 47% of patients in Asian-Pacific countries (2). Most of these mutations occur in exons 18 to 21 of EGFR gene, which encode the main EGFR tyrosine kinase binding domain (3, 4). Exon 19 deletion (19del) and exon 21 missense mutation L858R are the two most common activating forms, accounting for nearly 80% to 90% of the total EGFR mutations, which are strong predictors of favorable response to tyrosine kinase inhibitors (TKIs) and viewed as sensitizing EGFR alterations. These mutations are most commonly seen in young Asian females diagnosed with lung adenocarcinoma (LUAD) who never smoked (5–10).

A series of randomized clinical trials have confirmed that NSCLC harboring the classic EGFR mutations responded better to first-generation (1G) TKIs than conventional chemotherapy (11–16). In addition, the second-generation (2G) TKIs afatinib and dacomitinib significantly improved the progression-free survival (PFS) and overall survival (OS) in these patients (17–19). The third-generation (3G) TKI osimertinib showed a clinically meaningful improvement in the PFS over 1G TKIs in the Asian population (20). Therefore, osimertinib is currently recommended as the first-line targeted therapy for advanced NSCLC patients carrying classic EGFR mutations. Based on the awareness of necessity to qualify EGFR mutations, therapeutic approach with EGFR TKIs based on the detection of EGFR sensitizing alterations in the kinase domain has led to a dramatic shift in the treatment paradigm in advanced NSCLC, which has represented the standard of care for EGFR-mutated patients (21, 22).

Nevertheless, a spectrum of uncommon EGFR mutations such as p.G719X, p.S768I, and p.L861Q, affecting about 10% of the NSCLC population (5, 8, 23), have been reported to be more responsive to afatinib (23–25). Uncommon EGFR alterations appeared to carry heterogeneous molecular features with clinically variable responses to TKIs and shorter PFS when compared to EGFR common mutations (26). Furthermore, few details are known about the differences on TKI sensitivity among variable EGFR alteration subtypes, even though some evidence had issued their response and survival benefit to TKIs by clinical appraisal (22). Notably, quite a part of uncommon EGFR mutations are "untested" with the polymerase chain reaction (PCR)-based assay commonly used in clinical practice, together with the adequacy, quality, and heterogeneity of tumor samples in detection techniques, which results in the inaccuracy and bias in the reported incidence of less common EGFR mutations (27). Inevitably, PCR-based commercial assays could only identify "hot spots" or common mutations to predict the responses of TKIs, and are far from sensitivity for testing other uncommon mutations, which has posed significant diagnostic issues (27). Given the urgent need for more comprehensive genetic profiling in advanced NSCLC, the introduction of next generation sequencing (NGS) covering different panels in the clinical setting has significantly improved the detection frequency of uncommon EGFR alterations, and the implement of NGS testing well characterizes the accurate EGFR mutation status (28, 29).

The p.L747P missense mutation, which also occurs in exon 19 of the EGFR gene, is rarely observed in NSCLC. It occurs due to a two-base-pair (bp) mutation (c.2239_2240TT>CC) at codon 747. This causes the substitution of the amino acid proline to leucine, leading to oncogenesis in the same way as other EGFR activating alterations (30). Due to the rarity of p.L747P mutation in NSCLC, its response to different types of EGFR TKIs is unclear and controversial, and most studies suggested that it mediated intrinsic resistance to 1G TKIs while increasing the sensitivity to afatinib (30–37). However, it still remains unclear whether this mutation improves the binding affinity and responds to osimertinib. This highlights the need for further studies to understand the underlying mechanism behind the response to different generations of EGFR TKIs in NSCLC patients with p.L747P mutation.

Therefore, in this study, we aimed to conduct a retrospective cohort study to investigate the therapeutic outcomes of diverse EGFR TKIs in patients with metastatic NSCLC harboring p.L747P mutation. Our findings were compared with published

Abbreviations: ATP, adenosine triphosphate; bp, base-pair; CAMS, Chinese Academy of Medical Sciences; cDNA, complementary DNA; CR, complete response; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; FBS, Fetal Bovine Serum; IC_{50}, half maximal inhibitory concentration; IHC, immunohistochemical; LUAD, lung adenocarcinoma; MM/GBSA, Molecular Mechanics/Generalized Born Surface Area; NGS, next generation sequencing; NSCLC, non-small cell lung cancer; ORR, objective response rate; OS, overall survival; PCR, polymerase chain reaction; PD, progressive disease; PDX, patient-derived xenograft; PFS, progression-free survival; PR, partial response; RECIST, Response Evaluation Criteria in Solid Tumors; RLU, relative light unit; SD, stable disease; TKIs, tyrosine kinase inhibitors; WT, wild type; 1G, first-generation; 2G, second-generation; 3D, three-dimensional; 3G, third-generation; 1L, first-line; 2L, second-line; 3L, third-line; 19del, exon 19 deletion; ΔG_{bind}, binding free energy.

evidence. Furthermore, we also constructed the three-dimensional (3D) computational modeling of p.L747P mutation to simulate its binding activities to EGFR TKIs. The antitumor activities of EGFR TKIs for p.L747P mutation were finally evaluated and confirmed through cellular kinase inhibition assay and mice xenograft experiment.

MATERIAL AND METHODS

Patients and Data Collection

All patients diagnosed with metastatic NSCLC carrying p.L747P mutation treated at the Chinese Academy of Medical Sciences (CAMS)/Cancer Hospital from 2016 to 2020 were included in this cohort study. The p.L747P mutation in this study was identified by NGS testing which was performed in institutional laboratories or qualified third-party genetic testing companies that had acquired the national quality system certification *via* formalin-fixed, paraffin-embedded tissue samples. All of the NGS testing was performed based on the Illumina sequencing system, with same detection of a protein sequence encoded by the EGFR exon 19 with a substitution of the amino acid proline to leucine at codon 747 (p.L747P) and a DNA sequence with a 2-bp cytosine substitution to thymine (c.2239_2240 TT>CC). The medical records of these patients were retrospectively reviewed, and their clinical characteristics and targeted outcomes were recorded. The last follow-up date was July 21, 2021.

Response Assessment

The lesion size and overall disease stage at baseline were obtained through the use of computed tomography images of the chest and abdomen, brain magnetic resonance imaging, and whole-body bone scans. Tumor response to targeted therapy was evaluated after 4 weeks of TKI initiation and subsequently every 8 weeks, and presented as either complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. PFS was defined by the investigators as the time from TKI initiation to the date of documented disease progression or death from any cause (whichever occurred first). The objective response rate (ORR) was the proportion of patients with at least once confirmed CR or PR. OS was defined as the time from the diagnosis of stage IV disease to death from any cause.

Molecular Dynamics Simulation

The 3D-modeling of p.L747P was performed based on the crystal structure of the wild-type (WT) EGFR kinase domain in complex with dacomitinib, using the Schrödinger software (2020-1 Release) (PDB: 4I23). For the prediction of bioactive conformation and binding modes with EGFR TKIs (chemical structures were listed in the **Supplementary Figure**), including afatinib (BIBW2992), dacomitinib (PF299804), osimertinib (AZD9291), poziotinib (HM781-36B), and mobocertinib (TAK-788), we conducted docking simulations using the GLIDE (Schrödinger 2020-1 Release) program from Schrödinger Inc. (Portland, Oregon). The protein preparation wizard of the Maestro (Schrödinger 2020-1 Release) interface in the Schrödinger modeling package was used to prepare the protein. Compounds were constructed using the 3D-sketcher module in Maestro. The computer-based binding free energy (ΔG_{bind}) was calculated with the GlideScore method and the Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) method. The electrostatic energy, van der Waals action, polar solvation energy, and total residual energy contributions were also calculated by the MM/GBSA method.

Genetically Engineered Cell Lines

A431 cells were purchased from Nanjing Cobioer biotechnology Co., Ltd. Dulbecco's Modified Eagle Medium (DMEM), Penicillin-Streptomycin and 0.5% Trypsin-EDTA (10X) were purchased from ThermoFisher (Waltham, MA, USA). Certified Fetal Bovine Serum (FBS) was purchased from Biological Industries (BI). Corning 96 and 384-well cell culture plates were purchased from CORNING, USA. Cell-Titer Glo® was purchased from Promega Corporation (Madison, WI, USA). Complementary DNA (cDNA) of p.L747P-mutant EGFR were transfected into A431 cells using Nucleofector (Lonza), followed by clone selection using puromycin. All cell lines were authenticated by western blot and drug screening. Sequencing analysis was performed to confirm the integration of p.L747P-mutant EGFR. All cell lines used in the study tested negative for mycoplasma as determined by Real-Time PCR (Takara).

Cell Proliferation Inhibition Assay

Cell viability was assessed using the Cell Titer-Glo assay kit from Promega (Madison, WI, USA) by quantitating the adenosine triphosphate (ATP) present in the cell cultures. A431 cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin. Exponentially growing cells were plated in a 384-well plate at a concentration of 1000 cells/ml with 20ul per well, followed by overnight incubation at 37°C, 5% CO_2. Compounds were prepared as 12-point, 3-fold serial dilutions in dimethyl sulfoxide (DMSO), beginning at 2mM. They were further diluted 100 folds with cell culture media and 20 μL were added to each well of cell plate. The final top concentration of compound in the assay was 10uM and that of DMSO was 0.5%. The plates were then incubated for 3 days at 37°C, 5% CO_2. Luminescence was read after 20 minutes of incubation with the SPARK multiple plate reader from TECAN (Switzerland). The half maximal inhibitory concentrations (IC_{50}) of compounds inhibiting cell viability were determined using a sigmoidal dose-response model (variable slopes, four parameters) in Prism 7 (La Jolla, CA) to evaluate the inhibitory ability of compounds on the proliferation of A431 cells.

Mice Xenograft Experiment

The LUAD sample with p.L747P mutation was obtained from one metastatic NSCLC patient from the CAMS/Cancer Hospital and

was transported directly to the laboratory after tumor tissue biopsy. The tumor sample was washed twice with cold phosphate-buffered solution and minced into smaller pieces ($1cm^3$) using scissors before being implanted into a four-week-old female BALB/c nude mice. All animal experiments in this study were conducted under an institutionally approved protocol of the Animal Care and Use Committee of the CAMS/Cancer Hospital. After five generations in nude mice, the mice received an oral gavage of vehicle consisting of 0.5% methylcellulose in water, afatinib (7.5mg/kg/daily), dacomitinib (10mg/kg/daily), osimertinib (25mg/kg/daily), poziotinib (0.3mg/kg/daily), and mobocertinib (7.5mg/kg/daily) for 14 days. The xenograft tumor growth and mice body weight were monitored every three days. All the mice were killed on day 15 to harvest the tumors. The xenograft tumors were fixed in 4% paraformaldehyde for 24 hours, then sliced at a thickness of 5μm for immunohistochemical (IHC) analysis. The slices were subsequently stained using an anti-rabbit p-EGFR antibody (ab40815; Abcam, Cambridge, UK), anti-rabbit p-ERK antibody (4370; Cell Signaling Technology, Danvers, MA, USA), and anti-rabbit p-AKT antibody (4060; Cell Signaling Technology, Danvers, MA, USA) as indicated by the manufacturers' instructions.

Statistical Analysis

Statistical analyses were performed using the SPSS software, version 20.0 (IBM Corp., Armonk, NY, USA) and the GraphPad Prism software, version 8.0 (GraphPad Software Inc., San Diego, CA, USA). The experimental data were presented as the mean ± standard deviation. The Student's t-test was used for comparison between two groups. The two-way analysis of variance was used for comparison between multiple groups. All reported *P*-values were two-tailed, and for all analyses, a *P*-value below 0.05 was considered statistically significant unless otherwise specified.

RESULTS

Patient Characteristics

A total of five patients with metastatic LUAD harboring p.L747P mutation were included in the study. The median age was 52 (range, 41-63) years. Three patients were male, and two were never smokers. All of them received first-, second-, and third-line treatment. As first-line (1L) treatment, three patients received platinum-based chemotherapy, and two patients were treated with 1G TKIs either gefitinib or icotinib. In the second-line (2L) setting, two patients were administered 1G TKIs, one patient received 2G TKI afatinib, and another patient received 3G TKI osimertinib. In addition, as third-line treatment (3L), one patient received afatinib, and the others received osimertinib.

Treatment Response

Among the three patients receiving 1L platinum-based chemotherapy, one achieved PR with a PFS of 5.6 months, while the other two patients only achieved SD as the best response, with a PFS of 3.0 and 4.3 months. For the two patients treated with 1G TKIs in 1L, all had SD as the best response, with PFS of 3.2 and 3.4 months.

All the five patients were treated with 2L targeted therapy, and two receiving afatinib achieved PR, with a PFS of 4.7 and 8.5 months. The patient who was administered with osimertinib as 2L therapy showed best response of SD and a PFS of 7.5 months. The other two patients treated with 1G TKIs had ORR of 0, with PFS of 1.4 and 5.5 months. In the 3L setting, one case received afatinib and achieved a PFS of 2.4 months, with SD as the best response. The other four patients treated with osimertinib achieved a PFS ranging between 1.6 to 6.3 months, with ORR of 0. Up to the last follow-up, all the patients had died. The median OS was 19.7 months (95.0% CI: 18.0-21.4). The treatment responses to EGFR TKIs extracted from published studies were summarized in **Table 1**.

TABLE 1 | Responses to EGFR TKIs in NSCLC patients with p.L747P mutation from published reports.

No.	Age/Sex	Ethnicity	EGFR TKI	Best response	PFS (months)	Reference
1	63/M	Taiwan/Chinese	Gefitinib	PD	0.9	(30)
2	36/M	Taiwan/Chinese	Erlotinib	PD	2.9	(30)
3	69/M	Taiwan/Chinese	Afatinib	PR	12.0	(30)
4	49/M	Taiwan/Chinese	Afatinib	PR	19.8	(30)
5	61/F	Taiwan/Chinese	Afatinib	NE	1.0	(30)
6	NA	Taiwan/Chinese	1G TKI	PD	NA	(34)
7	66/M	Chinese	Gefitinib	PD	0.5	(32)
8	54/F	Chinese	Gefitinib	PD	1.0	(35)
			Osimertinib	PD	1.0	(35)
9	76/F	Italian	Gefitinib	NE	7.0	(36)
10	61/M	Chinese	Erlotinib	PD	1.0	(31)
11	44/F	Chinese	Afatinib	SD	24.0	(37)
12	59/F	Dutch	Gefitinib	SD	6.0	(38)
13	69/F	Japanese	Gefitinib	PD	1.6	(39)
14	80/F	Chinese	Gefitinib	SD	18	(40)
15	69/F	Japanese	Gefitinib	NA	4.0	(41)
			Osimertinib	NA	4.0	(41)

F, female; M, male; NA, not available; NE, not evaluable; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease; TKI, tyrosine kinase inhibitor; 1G, first generation.

Binding Affinity to EGFR TKIs by Dynamics Simulation

To elucidate the structural signature of p.L747P on the EGFR catalytic domain and investigate its affinity to currently available EGFR TKIs, 3D-modeling of p.L747P was constructed (**Figure 1A**) based on the crystal structure of the WT EGFR kinase domain in complex with dacomitinib (**Figure 1B**). The modeling revealed no significant difference in the activating kinase domain, ATP-binding site incorporating the hinge region, C-helix, P-loop, and activation loop between WT and p.L747P (**Figure 1C**). The 3D structure of p.L747P revealed that the amino acid residue leucine at codon 747 was close to the binding pocket, which was located in a key hydrophobic core that stabilized the inactive EGFR state. Compared with the WT of EGFR, no significant structural changes in the binding pocket was observed in p.L747P conformation (**Figure 1D**).

The 1G TKIs (gefitinib, erlotinib, icotinib) showed the poorest binding affinity to p.L747P mutation, with a computer-based ΔG_{bind} of -5.749 ~ -7.387 kcal/mol by GlideScore and -47.56 ~ -56.65 kcal/mol by MM/GBSA. In contrast, the 2G TKIs (afatinib, dacomitinib) conferred the best binding affinity, with a ΔG_{bind} of -7.737~ -7.953 kcal/mol by GlideScore and -61.20 ~ -65.53 kcal/mol by MM/GBSA. The 3G TKI osimertinib showed moderate binding affinity, with a ΔG_{bind} of -6.485 kcal/mol by GlideScore and -59.678 kcal/mol by MM/GBSA. These observations indicate a reduction in the binding affinity for the 1G and 3G TKIs to p.L747P when compared with 2G TKIs. In addition, we simulated the binding affinity of p.L747P with another two novel EGFR TKIs poziotinib and mobocertinib, which are designed to target EGFR exon 20 insertions under ongoing clinical trials. Dynamics simulation revealed that poziotinib and mobocertinib displayed potent and much favorable binding affinity to p.L747P mutation, with a ΔG_{bind} of -67.49~ -81.84 kcal/mol by MM/GBSA (**Table 2**).

By dynamics simulation, the binding affinity of osimertinib for p.L747P was less potent when compared with afatinib. However, the underlying mechanism for this observation has not been explored before. For this purpose, we investigated the energy contribution of residues within 4 Å of the ligand, and 10,000 conformations were extracted in 20 nanoseconds by calculation. We observed that amino acid residues that play a key role in the binding of molecules mainly were Met793 and Cys797 when afatinib (**Figure 2A**) and osimertinib (**Figure 2B**) bound with WT. The ΔG_{bind} for Met793 (-2.157 kcal/mol) and Cys797 (-2.134 kcal/mol) with osimertinib in WT was significantly lower than that for Met793 (0.091 kcal/mol) and Cys797 (0.540 kcal/mol) in p.L747P (**Figure 2C**). Conversely, the ΔG_{bind} for Met793 and Cys797 with afatinib in WT was similar to that in p.L747P (**Figure 2D**), which indicated that osimertinib was less able to bind with p.L747P compared with WT of EGFR.

Subsequent analysis of hydrogen bond occupancy further confirmed that afatinib conferred better binding affinity to

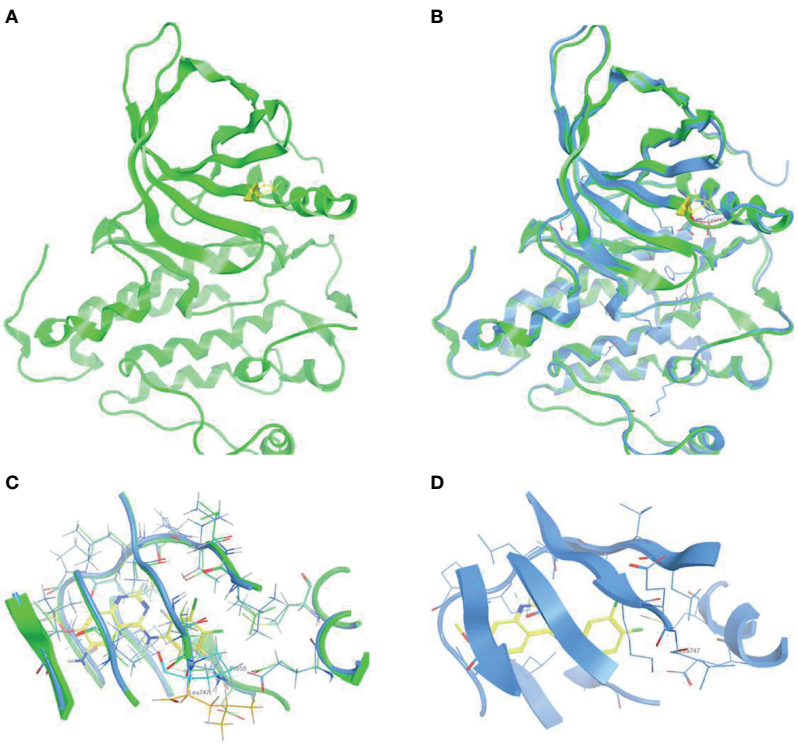

FIGURE 1 | 3D-modeling of p.L747P conformation **(A)** and crystal structure of EGFR wild type kinase domain in complex with dacomitinib **(B)**. ATP-binding pocket in the activating kinase domain of EGFR wild type **(C)** and p.L747P conformation **(D)**.

TABLE 2 | Binding free energies with different EGFR TKIs for p.L747P and WT of EGFR by dynamics calculation.

Molecule	p.L747P		WT	
	GlideScore ΔG_{bind} (kcal/mol)	MM/GBSA ΔG_{bind} (kcal/mol)	GlideScore ΔG_{bind} (kcal/mol)	MM/GBSA ΔG_{bind} (kcal/mol)
Gefitinib	-5.749	-49.47	-6.291	-50.31
Icotinib	-6.320	-47.56	-6.174	-46.21
Erlotinib	-7.387	-56.65	-7.585	-57.67
Afatinib	-7.953	-61.20	-7.261	-58.29
Dacomitinib	-7.737	-65.53	-7.887	-86.24
Osimertinib	-6.485	-59.68	-6.170	-59.73
Poziotinib	-5.159	-67.49	-8.023	-94.46
Mobocertinib	-6.892	-81.84	-7.093	-85.55

MM/GBSA, Molecular Mechanics/Generalized Born Surface Area; WT, wild type; ΔG_{bind}, binding free energy.

p.L747P (**Figure 3B**) than to WT of EGFR (**Figure 3A**), due to its stability in binding with amino acid residues Met793 and Cys797 to form more hydrogen bonds. However, the decreasing binding affinity of osimertinib for p.L747P may be attributed to its unstable binding mode (**Figure 3D**), along with fewer and weaker hydrogen bonds formed between residues Met793 and Cys797 than that with WT (**Figure 3C**). Molecular dynamics calculation demonstrated that the substitution of amino acid proline (0.043 kcal/mol) to leucine (0.032 kcal/mol) at codon 747 in the EGFR kinase domain had little effect on ΔG_{bind} with afatinib, resulting in an inconspicuous impact on affinity both in WT and p.L747P (**Figure 3E**). However, a distinct contribution to ΔG_{bind} was observed with osimertinib when substituting proline (0.009 kcal/mol) for leucine (0.130 kcal/mol), which eventually resulted in the weaker binding affinity to p.L747P (**Figure 3F**).

Sensitivity to EGFR TKIs in p.L747P and EGFR WT Cell Lines

Bioluminescence technique is a rapid test for detecting cellular ATP, which is calculated as the total light emission amount-relative light unit (RLU) *via* chemiluminescence measuring devices (42). The RLU correlates with the amount and survival

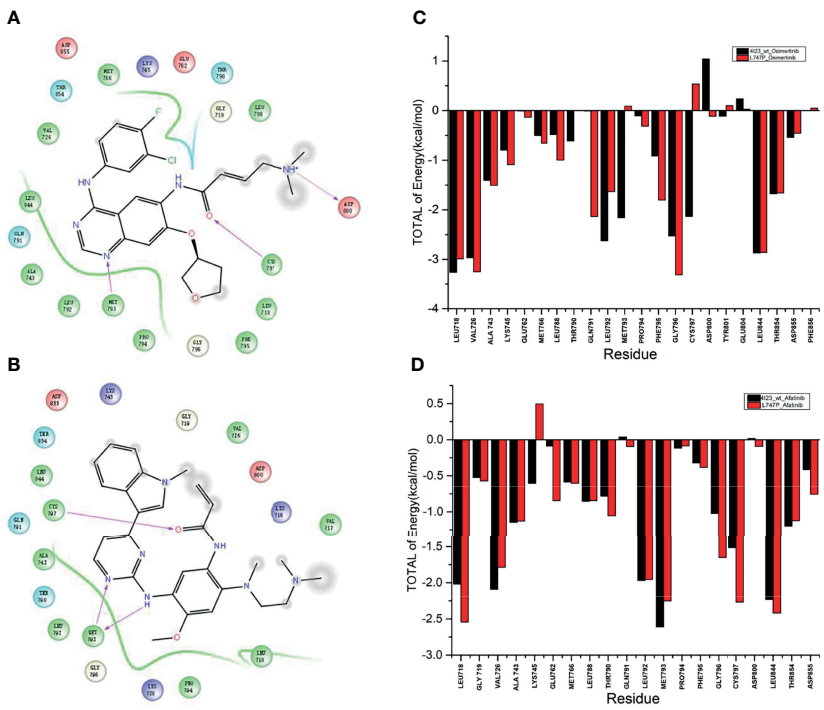

FIGURE 2 | Key amino acid residues binding with molecules in the wild type of EGFR kinase domain with afatinib **(A)** and osimertinib **(B)**. Binding free energy with osimertinib **(C)** and afatinib **(D)** in EGFR wild type and p.L747P conformation.

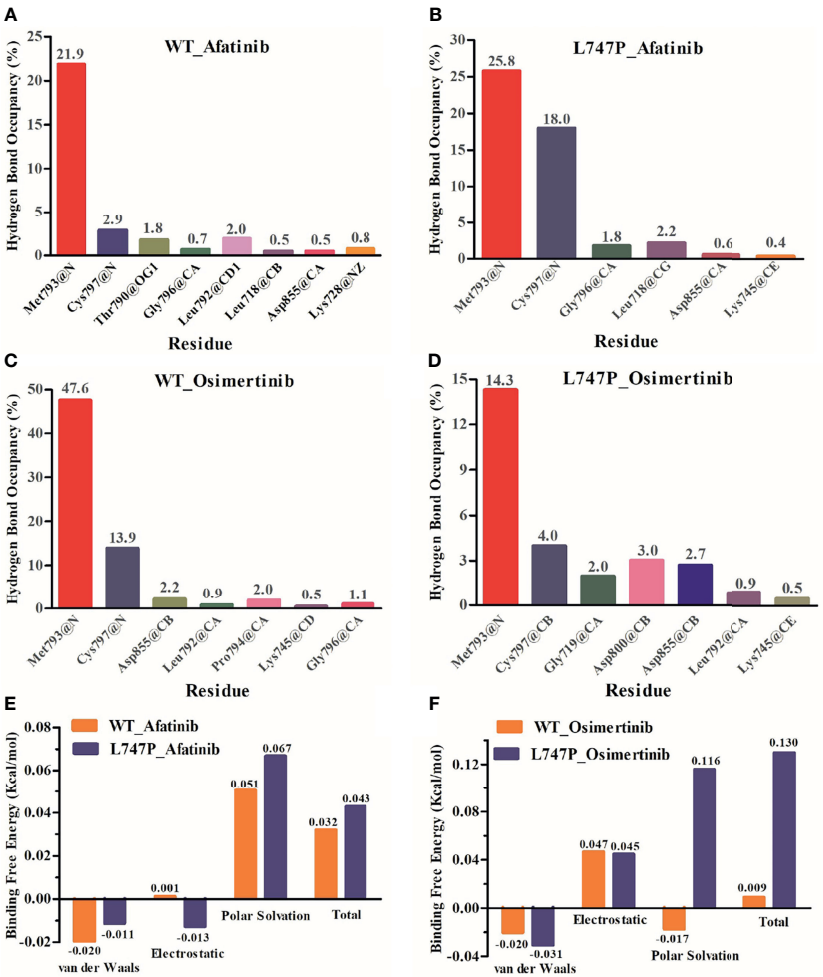

FIGURE 3 | Binding affinity to afatinib in EGFR wild type **(A)** and p.L747P conformation **(B)** by hydrogen bond occupancy analysis. Binding affinity to osimertinib in EGFR wild type **(C)** and p.L747P conformation **(D)** by hydrogen bond occupancy analysis. Dynamics calculations for binding free energies with afatinib **(E)** and osimertinib **(F)** when substituting proline for leucine.

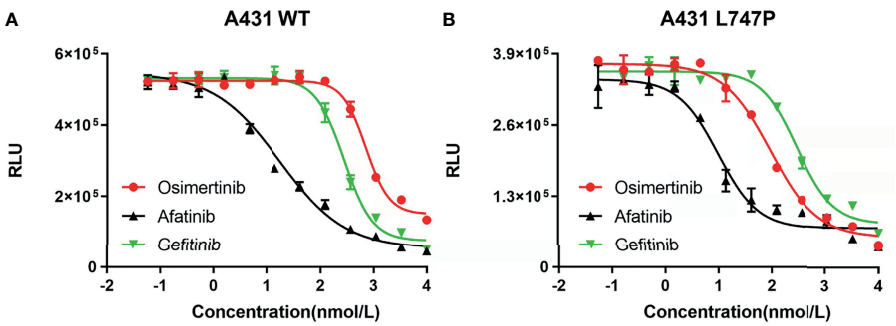

FIGURE 4 | The kinase inhibition activity of 1G to 3G EGFR TKIs against EGFR wild type **(A)** and p.L747P-mutant **(B)** cell lines.

activity of cells, and it showed a significant decrease on afatinib both in WT (**Figure 4A**) and p.L747P-mutant A431 cell lines (**Figure 4B**), indicating that afatinib demonstrated most favorable activity for p.L747P mutation at a small concentration. In addition, the RLU did not decrease until the concentration of gefitinib elevated to 10^2 nmol/L, which issued that it was not a sensitive inhibitor for p.L747P-mutant cells, and with poorest sensitivity to p.L747P when compared with afatinib and osimertinib. The kinase inhibition activity of diverse EGFR TKIs against p.L747P-mutant and WT cell lines was listed in **Table 3**.

Mice Xenograft Experiment

We next assessed the therapeutic efficacy of p.L747P to different EGFR TKIs in a p.L747P-mutant patient-derived xenograft (PDX) model (**Figure 5B**). After five generations in nude mice, mice received oral gavage of vehicle, afatinib, dacomitinib, osimertinib, poziotinib, mobocertinib for 14 days according to the dosing schedule (**Figure 5A**). Consistent with our findings in clinical practice, afatinib significantly attenuated both the growth and size of tumor nodules in the p.L747P-mutant xenograft mouse model when compared with the other groups (P<0.001, **Figures 5C-E**). Notably, dacomitinib and mobocertinib also showed a strong antitumor activity on tumor growth, but they also resulted in a significant weight reduction in the mice when compared with afatinib (P< 0.001, **Figures 5C-F**). As shown in **Figure 5C**, severe skin damage was found in mice treated with dacomitinib. In addition, the IHC results demonstrated that phosphorylated EGFR, ERK, and AKT were significantly decreased in tumors treated by afatinib and dacomitinib when compared with tumors treated by other EGFR TKIs. Yet, osimertinib did not effectively inhibit phosphor-EGFR and its downstream molecules (**Figure 5G**).

DISCUSSION

Due to the rarity of p.L747P mutation in the NSCLC population, it was not possible to accurately determine its incidence. A cohort study conducted in Taiwan, China only identified 12 patients with the uncommon p.L747P or p.L747S mutations among 2031 EGFR-mutant LUAD patients, which resulted in an overall incidence of approximately 0.59% (30). The intrinsic resistance of p.L747P mutation to EGFR TKIs was first reported in 2008 (34). The EGFR kinase 3D structure showed that condon 747 was located at the end of the β3 strand connecting to the C-helix. A cluster of hydrophobic residues contributed to the stabilization of the inactive EGFR kinase form (43).

Consistent with previously published studies, the findings from our cohort study indicated that the p.L747P mutation was associated with poor response to 1G EGFR TKIs, while a better response to 2G TKI afatinib (30–32, 34, 35, 37, 39, 41). None of the patients treated with 1G TKIs showed a tumor response, and their PFS ranged between 1.4 to 5.5 months. According to published studies, 11 patients had received 1G TKIs (gefitinib or erlotinib), and seven cases (63.6%) of them showed *de novo* resistance with PD as the best response and a PFS ranging between 0.5 to 2.9 months (30–32, 34–41). As for the 2G TKIs, most case reports and studies suggested that afatinib revealed the best activity for p.L747P, with a much longer PFS ranging between 12 to 24 months (30, 37). In our cohort study, two patients achieved PR to afatinib in 2L, with a PFS of 4.7 and 8.5 months. These findings indicated a good response to afatinib in carriers of p.L747P mutation, as also identified in the above-mentioned studies. For the 3G TKI osimertinib, case report indicated that one patient with p.L747P mutation failed to respond to it, with a PFS of only 1.0 months (29). Some small-scale studies reported moderate sensitivity to osimertinib in patients with p.L747P mutation (40, 41), yet, the evidence on the use of osimertinib to treat these patients is still insufficient. In our study, one patient received osimertinib as 2L therapy and achieved SD with a PFS of 7.5 months, and four patients were treated with osimertinib as 3L treatment and achieved a PFS ranging between 1.6 to 6.3 months with no response. However, we acknowledged that the sample size in our study was small. Therefore, further studies are warranted to investigate the real efficacy of osimertinib in carriers of p.L747P mutation.

The 3D-modeling of p.L747P constructed in our study revealed no significant difference in the activating kinase domain compared with WT of EGFR. As well, not any significant structural changes in the binding pocket was observed when substituting proline for leucine at codon 747. According to this observation, we speculated that the underlying mechanism for *de novo* drug resistance to 1G EGFR TKIs might be derived from the discrepancies in the free binding energies caused by the p.L747P conformation. As reported recently, 1G TKIs had the highest ΔG_{bind} to L747P compared with other

TABLE 3 | Kinase inhibition activity of diverse EGFR TKIs against p.L747P and EGFR WT cell lines.

Compounds IC_{50} (nmol)	A431 WT	A431 p.L747P
Gefitinib	724.8	147.3
Erlotinib	945.1	167.3
Afatinib	14.5	6.7
Dacomitinib	13.1	5.2
Osimertinib	341.6	80.9
Poziotinib	1.1	1.6
Mobocertinib	17.2	15.8

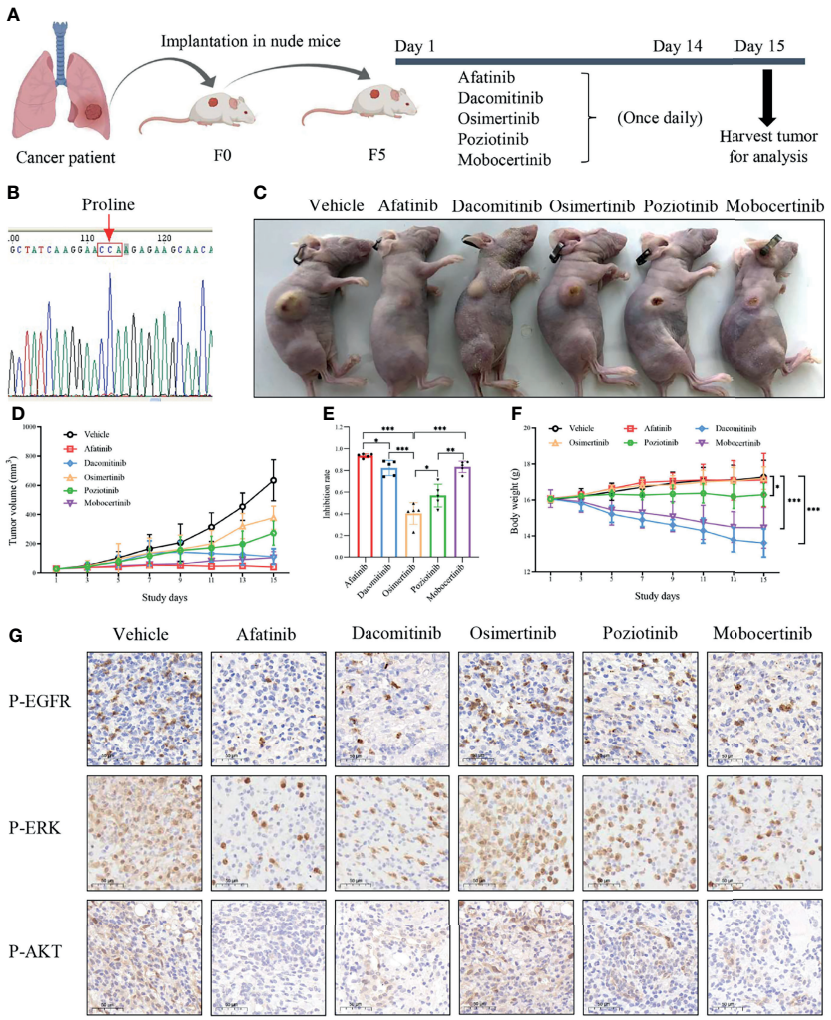

FIGURE 5 | Oral gavage of vehicle, afatinib, dacomitinib, osimertinib, poziotinib, mobocertinib according to the dosing schedule **(A)** in a p.L747P-mutant patient-derived xenograft model **(B)**. EGFR TKIs for the antitumor tumor activity **(C)**, tumor volume **(D)**, tumor inhibition rate **(E)** and mice body weight **(F)** in p.L747P-mutant xenograft mouse model. Phosphorylated EGFR, and its downstream molecules phosphorylated ERK and phosphorylated AKT under inhibition of different EGFR TKIs by IHC analysis **(G)**. *P < 0.05; **P < 0.01; ***P < 0.001.

EGFR TKIs, potentially causing binding instability and markedly decreased van der Waals interaction between EGFR tyrosine kinase and gefitinib and resulting in drug resistance (41). In addition, Ba/F3 cells expressing p.L747P mutation showed higher IC_{50} compared with the 19del and L858R mutant cells. Furthermore, immunoblot analysis has shown that p.L747P mutation was less sensitive to the 1G TKIs. In comparison, the 2G TKIs afatinib and dacomitinib could effectively inhibit phosphor-EGFR and its downstream molecules (41). Notably, dynamics simulation has shown that p.L747P mutation induced a structural change in the C-helix orientation towards the P-loop, facilitating the formation of a salt bridge between K745 and E762 residues to fix the active EGFR conformation (41). Consistent with these reported studies (30, 35, 37, 41), afatinib revealed a lower ΔG_{bind} to p.L747P and was more selective to bind with p.L747P mutation in our study when compared with osimertinib. The energy contribution simulation in our study showed that osimertinib had a significantly higher ΔG_{bind} to bind with p.L747P than that with WT of EGFR. Conversely, the ΔG_{bind} with afatinib was similar to that in WT and p.L747P. Hydrogen bond occupancy analysis further confirmed that afatinib had a better binding affinity to p.L747P due to its increasing hydrogen bonds when compared with osimertinib. All of our molecular dynamics simulation results confirmed that 2G TKIs presented the best binding affinity to p.L747P alteration.

In addition, we performed biochemical and cellular experiment to verify the mechanism of actions of gefitinib, afatinib and osimertinib targeting p.L747P mutation, and finally found that afatinib showed best binding sensitivity and antitumor activity against p.L747P-mutant cells compared with

gefitinib and osimertinib. As well, the compound IC_{50} data with comparison between afatinib, gefitinib and osimertinib confirmed our findings. The mice xenograft experiment further confirmed our clinical investigation and published studies. Afatinib significantly attenuated both the growth and size of tumor nodules in the xenograft mouse model of p.L747P compared to other EGFR TKIs ($P<0.001$). Dacomitinib also showed strong antitumor activity on the p.L747P-mutant tumor growth, but it significantly reduced the weight of mice and caused severe skin damage compared with afatinib ($P<0.001$). We also observed a significant reduction in the phosphorylated EGFR, ERK, and AKT in tumors treated by 2G TKIs compared with those by 1G or 3G TKIs. Interestingly, osimertinib failed to effectively inhibit phosphor-EGFR and its downstream molecules in IHC analysis, which confirmed our investigational results obtained from our cohort study and 3D-based molecular dynamics simulation. Furthermore, according to the mice xenograft experiment, mobocertinib conferred favorable antitumor activity to the p.L747P-mutant tumor. These findings were also consistent with result of binding affinity to p.L747P observed during our dynamics simulation, suggesting that mobocertinib might be a potential inhibitor for p.L747P mutation, although this agent is currently under ongoing clinical trials aiming to target EGFR exon 20 insertions.

This study has some limitations that have to be acknowledged. First, due to the scarcity and limited sample size of patients with p.L747P mutation, it is hard to conduct a prospective study enrolling enough patients. Therefore, our cohort study only included five patients with the p.L747P mutation, potentially leading to a patient selection bias even though our findings were consistent with those reported by previous studies. Furthermore, although we calculated the binding free energies of currently available EGFR TKIs by dynamics simulation to elucidate the underlying mechanism for drug resistance, exploration for molecular features and other possible signaling pathways involved in the drug resistance of p.L747P mutation is still required. Further clinical studies are warranted to confirm our findings.

In conclusion, the uncommon p.L747P mutation leads to a worse response to 1G EGFR TKIs when compared with the classic EGFR exon 19 deletions. Afatinib shows better binding affinity and antitumor activity compared with osimertinib for p.L747P mutation. NGS testing should be recommended to detect this specific mutation and hence guiding the accurate usage of TKIs in clinical practice.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by the Ethics Committee of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College.

AUTHOR CONTRIBUTIONS

Study design and data analysis: GY, CL, and JHu. Experiment administration: JHu, CL, YS, LL, and DL. Data collection: GY, CL, PH, HX, WL, and YY. Paper writing: GY, CL, and JHu. Manuscript modification: NS, JHe, and YW. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.843299/full#supplementary-material

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REVIEW
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NTRK Fusion in Non-Small Cell Lung Cancer: Diagnosis, Therapy, and TRK Inhibitor Resistance

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Neurotrophic tropomyosin receptor kinase (NTRK) gene fusion has been identified as an oncogenic driver of various solid tumors, and it is rare in non-smalll cell lung cancer (NSCLC) with a frequency of approximately less than 1%. Next-generation sequencing (NGS) is of priority for detecting NTRK fusions, especially RNA-based NGS. Currently, the tropomyosin receptor kinase (TRK) inhibitors have shown promising efficacy and well tolerance in patients with NTRK fusion-positive solid tumors, regardless of tumor histology. The first-generation TRK inhibitors (larotrectinib and entrectinib) are recommended as the first-line treatment for locally advanced or metastatic NSCLC patients with positive NTRK fusion. However, TRK inhibitor resistance can eventually occur due to on-target or off-target mechanisms. Further studies are under investigation to overcome resistance and improve survival. Interestingly, NTRK fusion might be the mechanism of resistance to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKI) in NSCLC patients with EGFR mutation. Regarding immunotherapy, the efficacy of immune checkpoint inhibitors in NSCLC patients harboring NTRK fusion has yet to be well described. In this review, we elucidate the function of NTRK genes, summarize the diagnostic techniques for NTRK fusions, and present clinical data for TRK inhibitors; we also discuss potential mechanisms of resistance to TRK inhibitors.

Keywords: non-small cell lung cancer, NTRK fusion, diagnosis, TRK inhibitor, resistance

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INTRODUCTION

Lung cancer is the second most common cancer worldwide but remains the leading cause of cancer-related death according to the latest cancer statistics, accounting for almost one-quarter of all cancer deaths (1). In recent years, targeted therapy with small molecular tyrosine kinase inhibitors targeting the EGFR/ALK/ROS1, and immunotherapy blocking immune checkpoints have been approved to treat patients with nonsmall cell lung cancer (NSCLC), and of note, the overall survival and quality-of-life have been drastically improved (2, 3). In addition, the diagnosis and therapy of gene fusions including ALK and ROS1 were revolutionary for TKI therapy in NSCLC, demonstrating remarkable antitumor effects (4–6). Therefore, the novel gene fusion of neurotrophic tropomyosin receptor kinase (NTRK) family has gained popularity recently for clinical research. NTRK genes involving NTRK1, NTRK2 and NTRK3, encode the proteins of

tropomyosin receptor kinase (TRK) family TRKA, TRKB and TRKC respectively, which are transmembrane receptor tyrosine kinases. NTRK gene fusions including NTRK1, NTRK2, and NTRK3 fusions are identified as oncogenic drivers in various types of tumors (7). The detection of NTRK gene fusion is recommended by the National Comprehensive Cancer Network (NCCN) clinical practice guidelines, and the TRK inhibitors (larotrectinib and entrectinib) are preferred as the first-line treatment for locally advanced or metastatic patients with NTRK-fusion-positive NSCLC (8). In this review, we describe the molecular biology and functions of NTRK gene. We also summarize the diagnostic techniques of NTRK gene fusions and the clinical data of TRK inhibitors, further discuss the therapeutic strategies and potential mechanisms of TRK inhibitor resistance.

NTRK GENE AND NTRK FUSION

NTRK Genes and TRK Receptors

NTRK1 gene is localized on chromosome 1q21–q22 (9), and its encoding protein TRKA binds to the nerve growth factor (NGF) to induce the tyrosine phosphorylation and tyrosine kinase activity of TRKA (10). NTRK2 gene is located on chromosome 9q22.1 (11), and the protein TRKB specifically binds to brain-derived neurotrophic factor (BDNF) (12). Moreover, NTRK3 gene is located on chromosome 15q25 (13), and the TRKC selectively binds to neurotrophin 3 (NT-3) (14). Furthermore, the NT-3 binds to all three TRK receptors, and the interaction between NT-3 and TRKC elicits a more efficient biological response than that with TRKA or TRKB (14, 15). Additionally, each of the TRK proteins is composed of an extracellular domain, a transmembrane region, and an intracellular region containing the tyrosine kinase domain (16). The bind of ligands and TRK receptors causes TRK receptor dimerization, which activates multiple intracellular signaling pathways involving phospholipase C-γ (PLCγ), PI3 kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways (17). These three pathways play important and different roles in cell functioning. MAPK pathway is involved in cell growth and proliferation, while PLCγ pathway regulates neuronal differentiation, survival, and metabolism. PI3K pathway is responsible for metabolism survival and apoptosis prevention (18). There are crosstalks between these signaling pathways to coregulate biological functions of NTRK genes, and the proper activation of TRK receptors is critical to nervous system development and cell survival (**Figure 1**).

NTRK Fusion

Gene fusions are resulted from genomic rearrangements, such as chromosomal inversions, interstitial deletions, duplications, or translocations, promoting the development and progression of cancer (19). As for the NTRK gene fusions, the 3' sequences of NTRK gene is fused to the 5' sequence of a fusion partner gene, which is a typical genetic structure of the oncogenic fusion (20). The resultant novel fusion oncogene is aberrantly expressed, and causes ligand-independent activation of the kinase domain, which is also called constitutive activation. The constitutive activation is generally the result of the 5' fusion partner gene which contains sequences encoding dimerization domains (19). Thus, it leads to persistent activation of downstream signaling pathways which is essential to tumor maintenance. The NTRK gene fusion TPM3-NTRK1 was initially discovered in colorectal cancer in 1986 (21). NTRK gene fusions were then discovered as oncogenic drivers of various adult and pediatric tumors. In a large-scale study, NTRK fusions with 88 unique fusion partners were identified in 134 histological subtypes among 45 types of cancers (7). However, the frequencies of NTRK gene fusions vary by cancer types. For example, ETV6-NTRK3 fusion is highly enriched in patients with cellular congenital mesoblastic nephroma, congenital fibrosarcoma, and secretory breast carcinoma (22–24), indicating a link between NTRK gene fusion and certain types of cancer histology. A case report showed that a patient initially diagnosed with salivary acinic cell carcinoma was finally reclassified as mammary analog secretory carcinoma after next-generation sequencing (NGS) results, suggesting an ETV6-NTRK3 fusion (25). Additionally, NTRK gene fusions are less frequent in NSCLC. Up to now, multiple NTRK fusion partners have been reported gradually in NSCLC. Vaishnavi et al. described two NTRK gene fusions in lung cancer, MPRIP-NTRK1 and CD74-NTRK1, which result in constitutive TRKA kinase activity and are oncogenic (26). Other NTRK1 fusion partners like SQSTM1, TPR, IRF2BP2, BCL9, LMNA and PHF20 were also detected in NSCLC (27, 28). TPM3 was the most common NTRK1 fusion variant, and TPM3-NTRK1 was reported as a resistance mechanism to both first-generation and third-generation EGFR-TKIs in NSCLC patients (28). Additionally, ETV6 and SQSTM1 were common fusion partners identified for NTRK3 in NSCLC (27).

CLINICAL CHARACTERISTICS OF NTRK FUSIONS

Frequency and Clinical Characteristics of NTRK Fusions

NTRK fusions exist in various adult and pediatric malignancies, though it is a rare gene alteration with an overall frequency of less than 1% (7, 29–31). The overall prevalence of NTRK fusion was 0.27%, where 31 cases were fusion positive from tissue samples of 11,502 patients (29). In addition, in a study with 26,000 patients, 76 cases were identified with NTRK fusions, suggesting an overall prevalence of 0.28% (30). Evidence from a large real-world population showed that the overall prevalence of NTRK fusion was 0.30% among 45 cancers types, and it varied by age with a higher prevalence in pediatric patients (1.34%) than adults (0.28%), especially in children <5 years (2.28%) (7). Consistently, a recent research showed that pediatric tumors had a higher frequency of NTRK fusions and a broader panel of fusion partners than adult tumors (32). Yet in another study, the frequencies of NTRK fusions assessed from 13,467 samples were 0.34% in pediatric tumors and 0.31% in adult tumors (31).

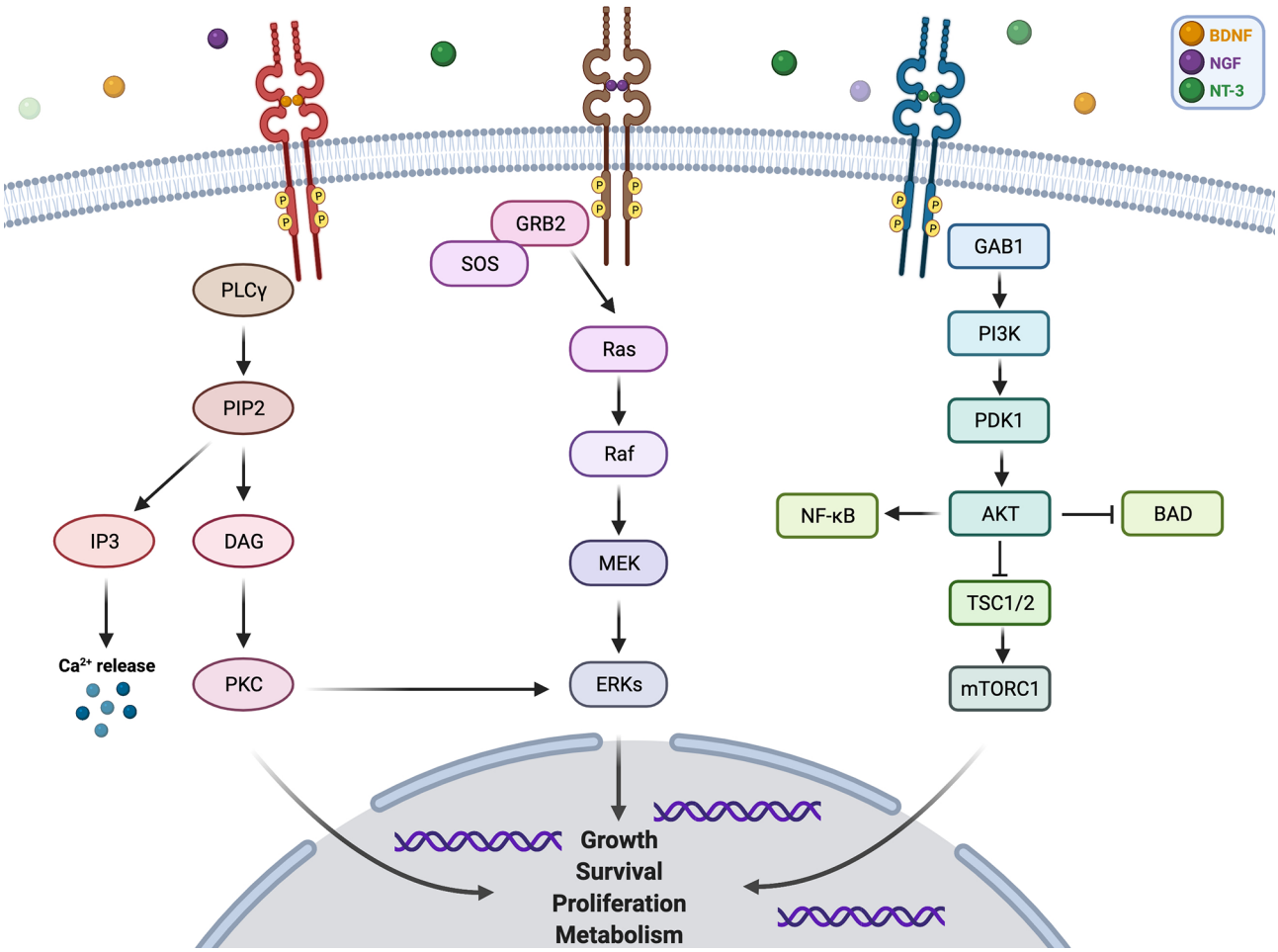

FIGURE 1 | Pathway and function of NTRK gene.

More relevant data are required for confirmed results. Additionally, the frequency of NTRK fusions distinctly varied by cancer type, where rare cancer types such as salivary carcinoma and thyroid cancer had a higher occurrence of NTRK fusions than common cancers like NSCLC (30, 31, 33). In a meta-analysis involving 107 studies, rare cancer types including infantile fibrosarcoma, secretory breast cancer, and congenital mesoblastic nephroma were reported with an incidence of NTRK fusions over 90% (33). However, in other cancer types including NSCLC, nonsecretory breast cancers, pancreatic cancers, renal cell carcinoma, prostate cancer, and melanomas, the frequencies of NTRK fusions were all less than 5%, and most were not up to 1% (30, 33). Furthermore, NTRK fusions were also detected in a large scale of hematologic malignancies with an occurrence of 0.1% in over 7,000 patients, of which a patient with acute myeloid leukemia harboring ETV6-NTRK2 fusion achieved a confirmed response to TRK inhibition therapy (34).

Among the three NTRK genes, NTRK1 and NTRK3 gene fusions can be identified in a wide range of cancer types, NTRK3 fusion is the most common followed by NTRK1 fusion, and ETV6-NTRK3 along with TPM3-NTRK1 are the most common fusion partners (7, 29, 31, 32). NTRK1 fusions are also highly detected in pediatric papillary thyroid carcinomas (32), whereas, ETV6-NTRK3 fusions act as a canonical genetic alteration in secretory carcinoma of salivary glands and breast (24). By contrast, NTRK2 fusions more exclusively exist in central nervous system (CNS) tumors like gliomas, according to a study where NTRK2 fusion was detected in most NTRK fusion-positive patients (9/14) (18, 29). Regarding the co-mutational patterns, NTRK fusions are revealed to barely co-occur with other canonical alterations (7, 35). Previous study revealed that the most frequent co-mutations with NTRK fusions were TP53, PTEN, and PIK3CA mutations, but only one case harbored targetable alterations including EGFR and MET amplification, and 29% (9/31) of patients with NTRK fusion had no other pathogenic alteration (29). Additionally, Rosen et al. described the only one case of 65 cases where NTRK fusion appeared along with activating alterations of classical MAPK pathway oncogenes, yet it later showed a negative expression level of the protein and resistance to larotrectinib (30).

Frequency and Clinical Characteristics of NTRK Fusions in NSCLC

As for NSCLC patients, the prevalence of NTRK fusions reported in multicontinental studies varies from 0.1% to 3.3%. A meta-analysis mentioned above reported that the frequency of NTRK gene fusions in NSCLC was 0.17% (33). Two large-scale studies showed the frequencies of NTRK gene fusions in NSCLC patients were 0.1% (4/4073) and 0.16%, respectively (29, 30). Another study enrolling 4,872 NSCLC patients estimated an NTRK fusion frequency of 0.23% through NGS (27). In addition, a retrospective study investigating driver gene alterations in 7,395 Chinese NSCLC patients found that the NTRK rearrangement frequency was 0.59% among all patients, 0.61% (33/5378) for patients with lung adenocarcinoma, and 0.5% (4/855) for patients with lung squamous cell carcinoma (36). NTRK fusion was also detected in neuroendocrine carcinoma and sarcomatoid carcinoma of the lung (27, 28, 37). In general, NTRK fusions are far less frequent than other canonical gene fusions in NSCLC, namely ALK, ROS1, and RET (36, 38–40). The common NTRK gene rearrangements in NSCLC were NTRK1 and NTRK3 gene rearrangements (27, 36). Specifically, the occurrence of gene fusions in NSCLC was 0.07%–3.3% for NTRK1 (26, 28, 41), 0.02%–0.2% for NTRK2 (27, 42), and 0.08% for NTRK3 (27).

Consistent with the co-mutation pattern mentioned before, NTRK fusions in NSCLC present a mutually exclusive manner with other canonical mutations and fusions. In a study of 11 NSCLC patients with NRTK fusions, 6 were recognized with co-mutation but none were common oncogenic genes such as KRAS, EGFR, ALK, or ROS1 (27). Evidence can also be found in another study of 91 NSCLC patients, of which the tumor with NTRK1 gene fusions had no known oncogenic alterations (26). The common co-occurrence mutations with NTRK1 fusion were TP53, RB1, and NF1 (28). Although NTRK fusions are reported mostly in middle-aged (a median age of 47.6 years) and non-smoking history populations, which resembles to the clinical profiles of many other fusions, they can also be detected in patients of other age groups or with previous smoking histories, suggesting that NTRK fusions are not related to certain clinical features in NSCLC (27). Furthermore, most NSCLC patients with positive NTRK fusions have metastasis at diagnosis (27). Yet the conclusion is drawn from data of only 11 cases with NTRK gene fusions. Due to the rarity of NTRK gene fusion in NSCLC, studies above were mostly small-scale retrospective studies. Therefore, prospective studies with larger sample size are required to investigate the clinical features of NTRK gene in NSCLC.

DIAGNOSIS OF NTRK FUSIONS

Generally, nucleic acid-based sequencing is a priority for detecting NTRK fusions, which can be followed by methods like immunohistochemistry (IHC), fluorescence *in situ* hybridization (FISH), and reverse transcriptase polymerase chain reaction (RT-PCR) as complement or substitution when practice environment is limited. Other diagnosis methods have also risen up, such as circulating tumor DNA/RNA testing and nanostring technology. Each method has its own merits and limitations, and some are limited to certain specific clinical conditions. In the following section, we will introduce and compare these techniques individually.

DNA-Based NGS

NGS shows a great advantage when conducting comprehensive analysis including somatic mutations, insertions, amplifications, deletions, microsatellite instability status, tumor mutation burden, as well as chromosomal rearrangements (43, 44), attributing to its broad capacity of molecular profiling. For example, MSK-IMPACT used in Memorial Sloan Kettering Cancer Center and the FoundationOne CDx test are two broad DNA sequencing panels. Based on hybrid-capture method, the two panels cover the whole coding region of 468 and 324 cancer-related genes, respectively, and are capable of detecting selected fusions including NTRK1, NTRK2, and ETV6-NTRK3 (45, 46). Moreover, high sensitivity and specificity as well as the ability to detect novel fusion partners are advantages of DNA sequencing. Additionally, DNA-based NGS can also function to monitor the development of resistance mutations in patients with NTRK fusions, such as G667C and G595R mutations in NTRK1 gene, and G696A and G623R mutations in NTRK3, which are observed to cause TRK inhibitor resistance (47, 48). However, several technical limitations should be taken into consideration. Practically, the sensitivity is determined by the panel coverage of genomic breakpoints of targeted fusions, and the integrity of its coverage is presented at the breakpoint. Therefore, false negatives could appear because of the limited panel size. In the aforementioned MSK-IMPACT panel, no kinase domain intron of NTRK3 was covered, because the intronic regions of NTRK3 are too long to cover, otherwise the coverage for other genes would be shrunk to reduced overall sensitivity (45). Another reason is that repetitive elements inside some introns are hard to tile and infeasible to assemble (49). Thus, the majority of fusions involving NTRK3 are indirectly detected through identification of the most common fusion partner ETV6, thus the sensitivity is restricted. Furthermore, it is uncertain if novel alterations presented in DNA level can be expressed at the mRNA and protein levels that possess clinical significance (35). Thus, further confirmation by RNA-based sequencing is often necessary. To conclude, broad capacity of molecular profiling, high sensitivity and specificity, and the ability to identify novel fusion partners contribute to the advantages of DNA-based NGS. While limitations of this method include its deficiency to detect NTRK3 fusions, the uncertain RNA-level expression of detected fusions, with the addition of high cost, high sample purity, and long turn-around time.

RNA-Based NGS

Practically, RNA-based NGS is preferred when it comes to the detection of NTRK fusion. As mentioned, even the most advanced DNA-based sequencing is incapable of covering large intronic regions in NTRK3. However, such limitation does not exist in RNA-based NGS, for introns are already spliced out in

RNA. Additionally, sequencing carried out in the RNA level can directly verify in-frame and functionally transcribed genes, which is of potential significance to determine the response to targeted therapy (50). In 232 lung adenocarcinoma samples of which driver alterations were not detected by MSK-IMPACT (DNA sequencing), 36 cases were identified positive for driver alterations through RNA sequencing. Among which, 27 patients were in-frame fusions including two with NTRK3 fusions and one with NTRK2 fusion. Intriguingly, two patients with NTRK fusions receiving larotrectinib treatment achieved confirmed PR or SD (51). Moreover, purity of tumor samples is less required due to the sufficiently high expression of gene fusions. The major disadvantage of RNA-based NGS is the labile nature of RNA extracted from archival samples. In aged materials, the occurrence of RNA fragmentation and degradation is of considerably high probability, which might lead to failure of library preparation and hinder subsequent operations. For instance, a study testing samples of 44 archival cases stated that only 23 cases passed quality control thresholds and were eligible for sequencing (52). Thus, effective quality assessment measures are required to identify potential false-negative results, guaranteeing the test reproducibility (53). Currently, the method termed Anchored multiplex PCR for RNAseq is commercially available and widely applied. In addition to higher sensitivity and specificity, it is effective in detecting single nucleotide variants, copy number variants, insertions, deletions, and gene rearrangements without previous knowledge of the fusion partners (54). It highlights the superiority of RNA-based NGS for NTRK fusion detection to find new fusion partners as well as second resistance in NTRK gene. Thus, RNA-based NGS is preferentially recommended for NTRK fusion detection in tumors where NTRK fusions are uncommon like NSCLC (55). In conclusion, RNA-based NGS can avoid the tough intron issues in the detection of fusions like NTRK3, and is able to directly confirm the transcription of detected fusions, making it an optimal approach for NTRK fusion detection. Yet the unstable RNA quality is a major concern, thus extra labor is required for specimen preservation and quality assessment.

Furthermore, there are some commercially available platforms that are able to simultaneously assess both RNA and DNA. For example, Oncomine Comprehensive Assay by Thermo Fisher and The TruSight Oncology 500 assay by Illumina are hybrid panels including all three NTRK genes (56, 57). Currently, a number of NGS panels based on DNA or RNA are designed for liquid biopsy when no sufficient tumor tissue specimen is available, such as Guardant360 panel (58) and AVENIO Extended ctDNA Analysis Kits (59). However, the sensitivity of such methods still requires future improvement.

Immunohistochemistry

As a method analyzing protein expression, IHC shows several evident advantages. Primarily, IHC is widely used in laboratories, due to its relatively low expense and low implementation threshold with only one single unstained slide and approximately a day of turnaround time. Moreover, IHC presents higher confidence that fusions detected are functionally transcribed and translated, allowing a spatial assessment of the subcellular localization of the fusion protein, which is indicative for oncogenic activity and targeted therapy. In addition, IHC presents high sensitivity and specificity (29, 35, 60, 61). EPR17341 (Abcam, Cambridge, MA, USA), a pan-TRK monoclonal antibody, is mostly used and is able to detect proteins TRKA, TRKB, and TRKC expression (35). However, the utility of IHC is restricted in diagnosis of NTRK fusions. Initially, the exact fusion partners and precise breakpoints cannot be identified, since only TRKs are targeted. Second, false positivity may occur as TRK proteins are not only specific to NTRK fusions. For instance, TRK proteins can also be expressed in normal tissues and tumor tissues with neuronal and smooth muscle differentiation, which do not harbor valid fusions, while the specificity was high for lung cancer (45, 61). Furthermore, sensitivity decrease of IHC for TRKC was revealed. Zoran et al. reported the sensitivity as 55% (29), while Solomon et al. have found a sensitivity of 79% for NTRK3 fusions, in contrast with the sensitivity of 96% and 100% for NTRK1 and NTRK2 fusions, respectively (45). Moreover, there are no monoclonal TRKC antibodies commercially available, thus, identification specific to NTRK3 fusions remains stagnated. Finally, the present estimated sensitivity and specificity data are established on research of small samples with NTRK fusion positive, suggesting that verification from studies with larger cohorts is required. Overall, IHC is a convenient, economic, and effective testing method. The detected fusion proteins could provide significant indications for clinical treatment. However, its incapability to identify fusion partners, ineffectiveness to detect TRKC, and false positive results due to the non-specific expression of TRKs jointly limit the application of IHC. Therefore, IHC mainly perform as a screening tool for NTRK fusion when NGS is not available or serve as an adjunct to nucleic acid testing, but orthogonal confirmation through NGS should be conducted for higher sensitivity if possible.

Fluorescence *In Situ* Hybridization

FISH is extensively used for detecting oncogenic fusions in solid tumors *via* chromosomal rearrangement analysis. In addition to the good sensitivity and specificity, it requires only one or two slides and lower tumor purity and takes only a few-day turnaround time. Notably, FISH is highly effective for identifying ETV6-NTRK3 fusions, which enables its good application in mammary analog secretory carcinoma, infantile fibrosarcoma, and congenital mesoblastic nephroma (52, 62). A break-apart probe (Abbott, Chicago, IL) is used specifically for the detection of ETV6 gene. There are also break-apart probes targeting the three NTRK genes and are commercially available (63, 64). Still, there are demerits in NTRK fusion detection. First, three FISH assays are required to be performed to assess three NTRK genes (65), which consequently costs more expense and time. Second, FISH is unable to ascertain the 5' partner of the fusion, while NTRK fusions involve multiple partners of great clinical significance. Third, higher probability of false-negative results is presented particularly for NTRK1 fusions. According to a study of short inversions and intrachromosomal translocations related to ALK, split lengths separated by the break-apart probe is too short to be distinguished from normal

types (66). Given that most NTRK1 fusions are formed in a intrachromosomal manner, false-negative results could appear by insufficient splitting of FISH (67). Finally, no certainty could be made in FISH that the fusion detected on the DNA level can be functionally transcribed and finally translated. In brief, FISH is a widely-applied fusion-testing approach with credible sensitivity and specificity, and particularly serves as a potent tool for ETV6-NTRK3 fusion detection. Nevertheless, it fails to recognize fusion partners, and its sensitivity for NTRK1 is questionable.

RT-PCR

Reverse transcriptase polymerase chain reaction is a method based on the detection of transcribed RNA, in which either qualitative assay or quantitative real-time PCR could be performed. As fusion partners and corresponding exon breakpoints both required clarification before an RT-PCR assay can be conducted, noncanonical and novel fusions could not be identified. In the past years, it has been used mainly for detecting canonical ETV6-NTRK3 fusions, thus its applicability is limited to cases enriched of such alterations (64, 68, 69). However, its sensibility needs further evaluation. In a study involving 25 cases of salivary gland secretory carcinoma which were proven to be canonical fusion negative *via* RT-PCR, four cases of which were found harboring classical fusion through more sensitive nested RT-PCR, and five atypical ETV6 exon4-NTRK3 exon 14 or ETV6 exon5-NTRK3 exon14 fusions were identified by both PCR and nested RT-PCR (64), which suggests a considerable possibility of false-negative results. To conclude, RT-PCR can perform well in ETV6-NTRK3 fusion detection, but its sensibility still requires improvement. Besides, recognition of non-canonical and novel fusions is beyond its category. Therefore, the utility of RT-PCR is largely limited by the highly variable fusion partners, exons, and breakpoints involved in NTRK fusions.

TRK INHIBITORS AND RESISTANCE

The first-generation NTRK-TKIs (larotrectinib and entrectinib) have demonstrated clinically meaningful antitumor activity (**Table 1**), thus had been approved for the treatment of locally advanced or metastatic patients with NTRK-rearranged solid tumors. According to the NCCN guidelines, both larotrectinib and entrectinib are recommended as standard therapies for the first-line treatment of NTRK fusion-positive patients with advanced or metastatic NSCLC, as well as progressive patients with previous systemic therapies. However, primary or acquired resistance to first-generation NTRK-TKIs is inevitable. The mechanisms of acquired resistance include "on-target" mechanisms, secondary mutations occurring at the TRK kinase domain, and "off-target" mechanisms, such as bypass signaling pathways activation (48, 70, 71). However, the mechanisms of primary resistance remain unclear. Currently, the mechanism of resistance to TRK inhibitors and next-generation TRK inhibitors are under development, and ongoing clinical trials are in search of appropriate therapeutic strategies (**Table 2**).

First-Generation TRK Inhibitors

Larotrectinib, an oral small-molecule and highly selective pan-TRK inhibitor, was initially approved for adults and pediatric patients with locally advanced or metastatic solid tumors harboring NTRK gene fusions without known acquired resistance mutations in the USA in November 2018 (72), as the first tissue-agnostic nod of targeted therapy. The antitumor activity of larotrectinib in patients with locally advanced or metastatic solid tumors harboring NTRK fusions has been explored in three clinical trials, including a phase I adult trial (NCT02122913) (73), a phase I/II pediatric trial (SCOUT, NCT02637687) (74), and a phase II adult and adolescent trial (NAVIGATE, NCT02576431). The phase I dose-escalation study of larotrectinib (NCT02122913) recruited 8 patients with NTRK gene fusions; the overall response rate (ORR) was 100% by independent review, including 2 patients assessed as complete responses (CR) and 6 patients assessed as partial responses (PR) (73). Drilon et al. reported the results of a primary analysis set of 55 patients with TRK fusion-positive solid tumors in 3 trials (NCT02122913, NCT02637687, and NCT02576431). The ORR was 75% (95% CI, 61–85) according to the independent review committee and 80% (95% CI, 67–90) determined by the investigator's assessment (47). Thus, the approval of larotrectinib was based on which. Hong et al. reported the pooled analysis result of the abovementioned three phase I/II clinical trials of larotrectinib (**Table 1**) (75). The ORR was 79% (121/153), the median progression-free survival (PFS) was 28.3 months (95% CI 22.1–NE), and the median overall survival (OS) was 44.4 months (95% CI, 36.5–NE) in the overall population. In the subgroup of NSCLC, the ORR was 75% (9/12). Furthermore, the efficacy of larotrectinib was independent of the NTRK gene. There were 13 (8%) of 159 patients with brain metastases, and a response to larotrectinib was observed in 9 of 12 (75%) of these patients. In patients who received larotrectinib treatment with 0, 1–2, and more than 3 prior lines of therapy, the ORR was 86%, 63%, and 80%, respectively, the median duration of response (DOR) was 27.6 months, not reached, and 32.9 months, respectively, and the median PFS was 29.4, 33.4, and 34.5

TABLE 1 | The efficacy of the first-generation TRK inhibitors.

TRK inhibitor	Overall population					NSCLC		
	N	ORR	PFS	CNS ORR	CNS PFS	*N*	ORR	CNS ORR
Larotrectinib	159	79% (121/153)	28.3 (22.1–NE)	75% (9/12)	NA	12	75% (9/12)	NA
Entrectinib	54	57% (31/54)	11.2 (8.0–14.9)	50% (6/12)	7.7 (4.7–NE)	10	70% (7/10)	NA

NE, not estimable; NA, not available.

TABLE 2 | Ongoing clinical trials for NTRK fusion-positive tumor.

ID	Drug	Phase	Gene fusion	Tumor type	Age	Primary outcome measures	Status
NCT02576431	Larotrectinib	Phase 2	NTRK	Solid tumors	18 Years and older	ORR	Recruiting
NCT04671849	SIM1803-1A	Phase 1	NTRK, ROS1, ALK	Solid tumors	18 Years and older	AEs, dose expansion	Recruiting
NCT03215511	Selitrectinib	Phase 1/2	NTRK	Solid tumors	1 Month and older	Phase 1: recommended dose, MTD Phase 2: ORR	Active, not recruiting
NCT04687423	FCN-011	Phase 1/2	NTRK	Solid tumors	16 Years and older	TRAEs, RP2D, ORR	Recruiting
NCT04996121	XZP-5955	Phase 1/2	NTRK, ROS1	Solid tumors	18 years and older	MTD, AEs, ORR	Not yet recruiting
NCT04094610	Repotrectinib	Phase 1/2	NTRK, ROS1, ALK	Solid tumors, lymphoma	Up to 25 years	Phase 1: DLTs, RP2D Phase 2: ORR	Recruiting
NCT04617054	AB-106	Phase 2	NTRK	Solid tumors	18 Years and older	BOR	Recruiting
NCT01639508	Cabozantinib	Phase 2	RET, ROS1, NTRK	NSCLC	18 Years and older	ORR	Recruiting
NCT04901806	PBI-200	Phase 1/2	NTRK	Solid tumors	18 Years and older	Phase 1: AEs, RP2D Phase 2: ORR	Recruiting
NCT02920996	Merestinib	Phase 2	NTRK	Solid tumors	18 Years and older	ORR	Active, not recruiting
NCT02675491	DS-6051b	Phase 1	NTRK, ROS1	Solid tumors	20 Years and older	AEs	Active, not recruiting
NCT03556228[a]	VMD-928	Phase 1	NTRK1	Solid tumors, lymphoma	18 Years and older	AEs	Recruiting
NCT02637687 (SCOUT)	Larotrectinib	Phase 1/2	NTRK	Solid tumors	Up to 21 years	Phase 1: TEAEs, DLT Phase 2: ORR	Recruiting
NCT02568267 (STARTRK-2)	Entrectinib	Phase 2	NTRK, ROS1, ALK	Solid tumors	18 Years and older	ORR	Recruiting
NCT03093116 (TRIDENT-1)	Repotrectinib	Phase 1/2	NTRK, ROS1, ALK	Solid tumors	12 Years and older	Phase 1: DLTs, RP2D Phase 2: ORR	Recruiting
NCT04655404	Larotrectinib	Early phase 1	NTRK	High-grade glioma	Up to 21 years	DCR, TEAEs, AUC, dose–response relationship	Recruiting
NCT03213704	Larotrectinib	Phase 2	NTRK	Solid tumors, non-Hodgkin lymphoma	12 Months to 21 years	ORR	Recruiting
NCT04302025	Entrectinib	Phase 2	ROS1N, TRK	NSCLC	18 Years and older	MPR	Recruiting
NCT03994796	Entrectinib	Phase 2	NTRK, ROS1	Solid rumors with BM	18 Years and older	ORR	Recruiting
NCT03834961	Larotrectinib	Phase 2	NTRK	Solid tumors, acute leukemia	Up to 30 years	ORR	Recruiting
NCT02650401 (STARTRK-NG)	Entrectinib	Phase 1/2	NTRK, ROS1	Solid tumors	Up to 18 years	MTD, RP2D, ORR	Recruiting
NCT02465060	Larotrectinib	Phase 2	NTRK	Solid tumors, lymphoma, multiple myeloma	18 Years and older	ORR	Recruiting

Inclusion criteria also include NTRK1 gene amplifications or TRKA protein overexpression.

AEs, adverse events; AUC, area under the curve; BM, brain metastases; BOR, best overall response; DCR, disease control rate; DLT, dose-limiting toxicity; MPR, major pathologic response; MTD, maximum tolerated dose; ORR, overall response rate; RP2D, recommended phase 2 dose; TEAEs, treatment emergent adverse events; TRAEs, treatment-related adverse events.

months, respectively, suggesting that the efficacy of larotrectinib is independent of prior treatments (76). In addition, a retrospective analysis showed that larotrectinib can improve PFS for previous treated patients with advanced TRK fusion cancer (77). There are several recruiting clinical trials that tend to further explore the efficacy of the larotrectinib in patients with NTRK fusion, and the tumor types of patients enrolled included acute leukemia, lymphoma, or central nervous system neoplasm (NCT03834961, NCT04655404, NCT03213704, NCT02465060). Interestingly, two cases harboring NTRK1 gene amplification were reported a partial response after treatment with larotrectinib, which indicated that larotrectinib may be effective for patients with NTRK gene amplification as well as NTRK fusions (73, 78). Moreover, there are clinical trials (NCT04879121, NCT02693535) exploring the effect of larotrectinib for patients with locally advanced or metastatic solid tumors harboring NTRK amplification. Adverse events of larotrectinib were predominantly of grade 1 or 2, with the most common adverse events being anemia, an increase in the alanine aminotransferase or aspartate aminotransferase level, and a decrease in the neutrophil count (47, 73, 75). Improvement in health-related quality of life was also observed after treatment with larotrectinib (79).

Entrectinib, an oral selective inhibitor of TRKA/B/C, ROS1, and ALK tyrosine kinases, received its first approval for the treatment of advanced or recurrent adult and pediatric solid

tumors with positive NTRK fusion in Japan in June 2019 (80). Then, entrectinib soon received approval for such indication by the FDA in August 2019 (81). It has also been approved for the treatment of adult patients with advanced ROS1 fusion-positive NSCLC. The safety and efficacy of entrectinib have been explored in four clinical trials: a phase I trial ALKA-372-001, a phase I trial in adults (STARTRK-1, NCT02097810), a phase I/II study in children and adolescents (STARTRK-NG, NCT02650401), and a phase II basket trial in adults (STARTRK-2, NCT02568267). Doebele et al. reported an integrated analysis results of three phases I–II trials (ALKA-372-001, STARTRK-1, STARTRK-2) that evaluated entrectinib in patients with advanced or metastatic solid tumors with fusion-positive NTRK (**Table 1**) (82). In the efficacy-evaluable population, the ORR was 57% (31/54) and the median PFS and OS were 11.2 (8.0–14.9) and 21 (14.9–not estimable) months, respectively. In patients with baseline CNS metastatic, the ORR was 50% (6/12) and the median PFS was 7.7 (4.7–not estimable) months. In the subgroup of NSCLC, the ORR was 70% (7/10). Furthermore, intrapatient comparisons of entrectinib efficacy in the STARTRK-2 trial indicated that the ORR was higher and the median PFS was longer for entrectinib than discontinuation since the last therapy (83). Additionally, a case report showed that a patient with SQSTM1-NTRK1 fusion-positive advanced lung adenocarcinoma was treated with entrectinib, then developed partial response and had a complete remission of all brain metastases (41). In summary, treatment with entrectinib led to clinically significant antitumor activity in patients with positive NTRK fusion. Importantly, entrectinib is also effective for CNS tumors or CNS metastases. This is likely due to sustaining CNS exposure of entrectinib, because it is a weak p-glycoprotein substrate different from crizotinib and larotrectinib which are strong p-glycoprotein substrates with poor brain penetration (84). Currently, a head-to-head study comparing the efficacy of entrectinib and crizotinib in patients with advanced or metastatic ROS1+ NSCLC with and without CNS metastases is recruiting (NCT04603807). As for the safety analysis, most adverse events are grade 1 or 2 and reversible, and the common treatment-related adverse events include dysgeusia, fatigue, dizziness, constipation, etc. The commonly reported grade 3 or 4 adverse events are increased weight and anemia, while cognitive disorder is the most common serious treatment-related event (82). Thus, we conclude that entrectinib is an effective therapy with minor adverse events for advanced patients with NTRK gene fusions, including patients with primary CNS tumors and metastatic CNS diseases. Meaningfully, entrectinib as neoadjuvant therapy in patients with resectable stages II–III NSCLC is currently under investigation (NCT04302025), and the results of which may provide a novel perspective for therapeutic strategies in NSCLC.

First-Generation TRK Inhibitor Resistance

"On-Target" Mechanisms

The secondary mutations occurring at the ATP binding pocket of the TRK kinase domain includes the solvent-front, gatekeeper region, and xDFG motif mutations in the activation loop, also known as 'on-target' mechanisms, which represent the common acquired-resistance mechanisms for the first-generation TRK inhibitors (**Figure 2**). Up to now, several resistance mutations have been reported. In 2015, the solvent-front mutations (G595R) and xDFG motif mutation (G667C) in the TRKA kinase domain were initially reported as acquired resistance mechanisms to entrectinib in a patient with colorectal cancer involving LMNA-TRKA rearrangement (48). Then, a NTRK3 G623R mutation was reported to be related to acquired resistance to entrectinib in a patient with mammary analog secretory carcinoma with ETV6-NTRK3 fusion (25). Later, a novel gatekeeper region (F589L) mutation in TRKA, the xDFG mutations (NTRK1 G667S, NTRK3 G696A), and solvent front mutations (NTRK1 G595R, NTRK3 G623R) were identified as resistance mechanisms to larotrectinib (47). Furthermore, NTRK1 G595R and NTRK1 G667S mutations presented in a NSCLC patient, and a gatekeeper mutation (NTRK3 F617L) presented in a patient with gastrointestinal stromal tumor after disease progression with larotrectinib treatment (73). On-target secondary resistant mutations bring about amino acid substitutions, thus result in sterically preventing the binding of the first-generation TRK inhibitors. Next-generation TRK inhibitors have already been developed to overcome the on-target resistance mutations during treatment with first-generation TRK inhibitors.

"Off-Target" Mechanisms

Off-target mechanisms can develop during TRK inhibitor treatment, which include genomic alterations of downstream pathway mediators and other receptor tyrosine kinases (**Figure 2**). Preclinical study showed that the reactivation of RAF-MEK-ERK signaling was observed in NTRK1-driven pancreatic cancer and lung cancer treated with entrectinib, which was possibly one of the acquired-resistance mechanisms to entrectinib, and combined inhibition of TRKA plus MEK1/2 markedly forestalled the onset of drug resistance in both models (85). Furthermore, BRAF V600E mutation, KRAS G12D mutations, and MET amplifications were also identified as the bypass-mediated resistance mechanisms to TRK inhibitors for patients with NTRK fusions. Dual blockade of TRK and MEK could effectively control tumor growth and delay the emergence of off-target resistance (71). However, the next-generation TRK inhibitor monotherapy was not effective for resistance mediated by bypass pathway mutations (71, 86, 87). In a case of pancreatic adenocarcinoma with CTRC-NTRK1 gene fusion, BRAF-V600E mutation emerged when disease progressed with larotrectinib, which previously achieved a PR at its best, then the tumor continued to progress for 2 months even though the treatment was switched to selitrectinib, a next-generation TRK inhibitor (86). Intriguingly, these data provide clues for combination therapies of blocking both NTRK and MEK in NTRK fusion-positive tumors for future investigations.

Next-Generation TRK Inhibitors

Selitrectinib (LOXO-195), a selective TRK inhibitor, was designed to overcome acquired resistance to first-generation

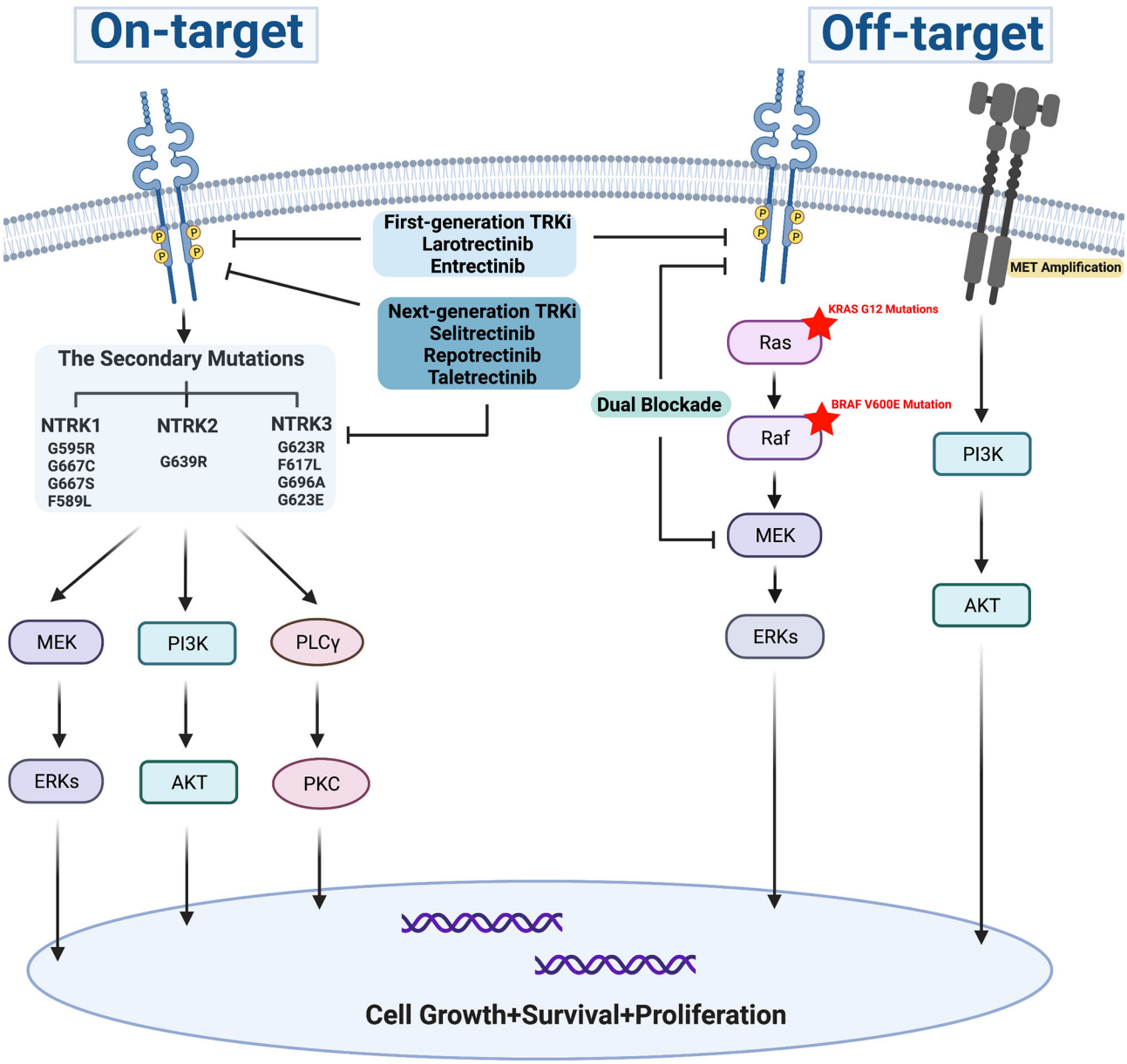

FIGURE 2 | Resistance mechanism for first-generation TRK inhibitors.

TRK inhibitors mediated by secondary mutations in kinase domain. LOXO-195 showed significant inhibitory cellular activity against NTRK fusions and acquired resistance mutations *in vitro*, including TRKA G595R, TRKA G667C, and TRKC G623R (88). Notably, LOXO-195 possessed antitumor activity in two patients that had LMNA-NTRK1 fusion-positive colorectal cancer and ETV6-NTRK3 fusion-positive infantile fibrosarcoma with TRKA G595R- and TRKC G623R-driven acquired resistance to larotrectinib, respectively (88). Furthermore, selitrectinib response was also observed in a patient with NTRK3 G623R mutation and CNS metastasis who has acquired resistance to entrectinib with ETV6-NTRK3 fusion-positive mammary analog secretory carcinoma of the parotid gland (89). In a phase I/II study (NCT03215511, n = 20) and FDA-expanded access single patient protocol (SPP, n = 11), the ORR of LOXO-195 was 34% (10/29) in all evaluable patients, and the ORR was 45% (9/20) in patients with TRK kinase mutation, but the ORR was 0% (0/3) in patients with resistance mediated by identified bypass, and the most common adverse events were dizziness/ataxia, nausea/vomiting, anemia, myalgia, abdominal pain, fatigue, and lymphopenia (87). It suggests that LOXO-195 is significantly effective in patients with resistance to prior TRK inhibitors mediated by mutations in kinase domain but not bypass pathway activation. However, LOXO-195 exhibited

limited response to a pediatric glioma driven by ETV6-NTRK3 fusion with G623A- and G623E-resistant mutations. It was possibly due to the insufficient CNS concentrations of LOXO-195 and trophic microenvironment of the pediatric brain that confers resistance to TRK inhibitors (90). LOXO-195 possessed poor penetration into the brain because of the blood–brain barrier and multidrug efflux transporters, such as ABCB1 and ABCG2 (91, 92). In addition, clinical evidences and preclinical findings revealed that TRKA xDFG motif substitutions, such as TRKA G667A and TRKA G667C, conferred resistance to the next-generation TRK inhibitors including selitrectinib and repotrectinib through impaired drug binding (93). Recently, a case report showed that a patient with DCTN1-NTRK1 fusion-positive undifferentiated pleomorphic sarcoma did not respond to LOXO-195 who harbored acquired NTRK1 G667C mutation after disease progression with larotrectinib (94). Thus, resistance mediated by xDFG mutation remains a major challenge for next-generation TRK inhibitors. Though recent studies report that promising drug compounds designed to overcome multiple resistance possessed potent inhibitory activities to xDFG mutations as well as solvent-front and gatekeeper substitutions *in vitro* and *in vivo* (95, 96), the exploration of new drugs to inhibit xDFG mutation is still facing unmet clinical needs.

Repotrectinib (TPX-0005) is a novel next-generation ALK, ROS1, and pan-TRK inhibitor, which is designed to overcome resistance mutations and potently inhibit wildtype TRK fusions. Repotrectinib is highly potent and selective against wildtype ALK, ROS1, and TRK fusion proteins, as well as their solvent-front substitutions in preclinical studies, including TRKA G595R, TRKB G639R, and TRKC G623R (97, 98). Similarly, a dramatic response to repotrectinib was observed in a patient with NTRK3 fusion-positive mammary analog secretory carcinoma harboring NTRK3 G623E mutation. Notably, repotrectinib achieved partial response in NSCLC patients with ROS1 fusion and intracranial metastasis, who were treatment naive or presented solvent-front mutation-mediated resistance to previous ROS1-TKI, demonstrating an efficient intracranial antitumor activity of repotrectinib (97, 99). Efficient CNS penetration of repotrectinib was observed in patients and mouse models, but inconsistent result was showed in a bioanalytical assay, revealing that repotrectinib possessed very poor penetration into the brain in mouse experiment, probably because of the blood–brain barrier and multidrug efflux transporters, like ABCB1 and ABCG2 (100, 101). The potent intracranial activity of repotrectinib in patients with NTRK-fusion tumors including NSCLC remains unclear, requiring further investigation. A clinical trial of repotrectinib in patients with advanced solid tumors harboring NTRK, ALK, or ROS1 rearrangements (TRIDENT-1, NCT03093116) are currently being conducted, of which the interim data showed evident antitumor activity of repotrectinib in patients harboring NTRK fusion-positive cancers both with and without previous NTRK-TKI treatment (98). Two cases of metastatic NSCLC harboring NTRK3 rearrangement from TRIDENT-1 study achieved durable responses to repotrectinib, with one being NTRK-TKI naive and one with previous entrectinib resistance mediated by G623R mutation (82). What is more, repotrectinib was more potent against wildtype TRK fusions and mutations in TRK kinase domain than selitrectinib in cellular assays and mouse models. Repotrectinib was also the only TRK inhibitor active against TRKA G595R/F589L compound mutation in cis in preclinical Ba/F3 cells (102). This indicates that repotrectinib is more efficient for wildtype TRK fusions and secondary resistance mutations in preclinical studies, though evidence from clinical study is still insufficient. Currently, phase I/II clinical trials (NCT03093116, NCT04094610) are ongoing to explore the efficiency of repotrectinib in patients with advanced solid tumors harboring NTRK, ROS1, and ALK rearrangements.

Taletrectinib (DS-6051b/AB-106) is a selective tyrosine kinase inhibitor of NTRK and ROS1. Preclinical study showed that DS-6051b was significantly effective in inhibiting NTRK and ROS1-rearranged cancers, as well as TKI-resistant tumors with secondary kinase domain mutations, such as G2032R mutation in ROS1 and G595R mutation in NTRK1 (103). However, NTRK1 G667C mutation was resistant to DS-6051b; it was consistent with previous reports claiming G667C mutation in xDFG motif being resistant to next-generation TRK inhibitors (93, 103). Preliminary clinical activity of DS-6051b was observed in TKI-naive and crizotinib-pretreated ROS1+ NSCLC patients and a patient with TPM3-NTRK fusion-positive thyroid cancer who achieved a confirmed partial response of 27 months at the last follow-up (104, 105). The evidence about antitumor effect of taletrectinib in patients with advanced NSCLC harboring NTRK fusion is insufficient; thus, further investigation is required. The most common treatment-related adverse events are elevation of aspartate aminotransferase and alanine aminotransferase, nausea, diarrhea, and vomiting (104, 106).

Next-Generation TRK Inhibitor Resistance

As stated above, resistance mechanisms of tyrosine kinase inhibitors typically include on-target and off-target mechanisms, while resistance mechanisms of next-generation TRK inhibitors are yet to be well described. Two patients, one with TPR-NTRK1-positive NSCLC and the other one with TPM3-NTRK1-positive thyroid cancer, harboring xDFG motif mutations (TRKA G667C, G667S) that emerged as resistance to larotrectinib, did not respond to next-generation TRK inhibitor selitrectinib, which represented one of the primary resistance mechanisms to next-generation TRK inhibitor (93). Furthermore, patients achieved partial response to selitrectinib against TRKA G595R-mediated larotrectinib resistance, while TRKA G667C or TRKA G667A were detected at progression during selitrectinib treatment, indicating that TRKA G667 mutations were responsible for acquired resistance to next-generation TRK inhibitors (93). Consistent results were observed in preclinical models, where NTRK1 G667 mutation was found insensitive to next-generation TRK inhibitors, including selitrectinib, repotrectinib, and DS-6051b (93, 103). Importantly, xDFG motif mutations (NTRK1 G667) were highly sensitive to type II inhibitors, including altiratinib, cabozantinib, and foretinib in preclinical studies (93, 107). Also, foretinib and nintedanib significantly inhibited the growth of cells with TRKA

G667C mutation, and foretinib was also effective against NTRK1-G667C mutation in a brain metastasis model (108). Moreover, Ba/F3 cells expressing TPM3-NTRK1 G667C or TPM3-NTRK1 fusion were sensitive to gilteritinib but it failed to suppress G595R-mutant cells (109). This calls on further studies to overcome G667 mutations. Additionally, in a case with metastatic undifferentiated sarcoma harboring TPM3-NTRK1 fusion, selitrectinib was used to overcome acquired resistance to larotrectinib with a secondary G595R mutation. KRAS G12V mutation and functional activation of KRAS signaling were later identified in the lesion developing resistance to selitrectinib (110). Similarly, a patient with colorectal cancer harboring LMNA-NTRK1 fusion showed emergence of KRAS G12A and G12D mutations when developing acquired resistance to LOXO-195 (71). This indicated that bypass pathway activating *via* KRAS mutations was one of the resistance mechanisms to selitrectinib, and further exploration of other mechanisms is urgently needed for appropriate therapeutic strategies toward resistance to next-generation TRK inhibitors.

NTRK FUSION WITH EGFR-TKI RESISTANCE

Interestingly, NTRK fusions are recognized as a resistance mechanism to EGFR-TKIs in NSCLC patients (28, 111). According to a survey investigating 3,050 EGFR+ NSCLC samples, the emergence of TPM3-NTRK1 was confirmed to follow the initiation of EGFR-TKI erlotinib treatment through the comparison between paired pre- and after-treatment samples (111). Consistent results can also be seen in other studies, where TPM3-NTRK1 fusion was detected in patients with resistance to third-generation EGFR-TKI osimertinib or rociletinib (112, 113). Notably, in a large-scale cohort involving Chinese lung cancer patients, six of twelve patients with NTRK1 fusion-positive NSCLC had co-occurring EGFR mutations and were previously treated with EGFR-TKIs, suggesting that NTRK1 fusions were the potential resistance mechanisms to EGFR-TKIs regardless of its generation (28). A NSCLC patient with EGFR 19del received gefitinib followed by osimertinib because of the emergence of EGFR T790M, then EGFR C797S and LMNA-NTRK1 fusion were detected when resisting to osimertinib. Notably, the patient showed continuous slow disease progression for 9 months with osimertinib combined with crizotinib as an TRK inhibitor (28). Moreover, a patient with IRF2BP2-NTRK1 lung adenocarcinoma achieved a durable stable disease to crizotinib for 16 months (114). It revealed the antitumor effect of crizotinib for NTRK fusion-positive NSCLC, suggesting that combining EGFR-TKIs and TRK inhibitors may be an optional treatment for patients with NTRK fusion-mediated EGFR-TKI resistance. The effect of first- and next-generation TRK inhibitors for EGFR-TKI-resistant tumors with NRTK fusions requires further investigation for better comprehension of resistance mechanism.

NTRK FUSION AND IMMUNOTHERAPY

In recent years, immune checkpoint inhibitors (ICIs) have remarkably changed the treatment landscape of cancers like NSCLC. However, the clinical efficacy and safety of ICIs for patients with NTRK fusion positive remains unknown. There are several studies exploring the relationship between NTRK fusion and biomarkers for ICIs, including PD-L1 expression, microsatellite instability, and tumor mutation burden (TMB), which had been identified as predictive biomarkers for ICIs (115–117). Evidence can be found in 31 cases with NTRK fusions, where PD-L1 expression was detected in 23% of cases with NTRK fusions, but only 2 cases possessed high microsatellite instability (MSI-H) (29). With the exception of colorectal cancer, NTRK fusions was demonstrated to be positively related to MSI-H and mismatch repair deficiency (MMR-D) (7, 118, 119). A study showed that 6 of 7 patients with NTRK fusion-positive colorectal cancers were MSI-H and possessed high median TMB. This is consistent with another finding that of 12 patients with NTRK fusions including two MSI-H colorectal cancers, only a patient with colorectal cancer achieved a complete response to ICIs (30). Additionally, NTRK fusion-positive tumors presented a lower TMB than those with NTRK fusion negative, excluding MSI-H colorectal cancers, which may be due to the uncommon appearance of NTRK fusion co-existing with alternative oncogenic drivers (30). As for lung cancer, previous studies revealed that it had a significantly higher median TMB but a lower frequency of MSI-H compared with other solid tumors (115, 116). However, the association between NTRK fusion and TMB is still unclear in NSCLC. Results from a large real-world study revealed that the median TMB was similar in NTRK fusion-positive and fusion-negative NSCLC. Additionally, a genomic testing of 2,522 lung adenocarcinomas showed that gene fusion was significantly enriched in driver-negative samples with low TMB, the median TMB for fusion-positive and fusion-negative samples were 1.97 and 5.58 mutations/Mb, respectively, yet the analysis was based on all fusion-positive samples and not specific to NTRK fusion (51). As for immunotherapy, a patient with lung adenocarcinoma harboring NTRK fusion receiving anti-PD1/PDL1 treatment achieved stable disease (30). However, inconsistent result emerged in a case report, where a patient with advanced lung adenocarcinoma harboring a novel NCOR2-NTRK1 fusion showed disease progression after receiving two cycles of anti-PD-1 inhibitor monotherapy, although the presence of high TMB (58.58 mutations/Mb) and positive PD-L1 expression (20%–30% of the tumor cells) was also observed in this case. Predominantly, the patient showed a partial response after switching to TRK inhibitor larotrectinib (120). It indicates that TRK inhibitor is more effective than anti-PD-1 inhibitor monotherapy for patients with NTRK fusion-positive NSCLC in spite of higher TMB and positive PD-L1 expression simultaneously. However, there is no sufficient evidence to draw conclusions based on this single case report. Regarding the efficacy comparison of TRK inhibitors and ICIs, further investigations are required. Whether TRK inhibitors

combining with PD-1/PD-L1 inhibitors have superior performance than monotherapy is also worthy of exploration.

CONCLUSION

NTRK gene fusions are identified as oncogenic drivers of various adult and pediatric solid tumors, and the prevalence of NTRK fusions varies by tumor types. In NSCLC, NTRK fusions are rare, with an overall prevalence of below 5% and mostly less than 1%. No clear evidence has been linking NTRK fusion to certain clinical features, but it has been revealed that NTRK fusion is mutually exclusive with other canonical mutations. The first-generation TRK inhibitors (larotrectinib and entrectinib) showed remarkable efficacy and good safety for locally advanced or metastatic patients with NTRK fusions, thus they had been approved for the treatment of NTRK fusion-positive solid tumors by the FDA. However, resistance is developed inevitably, and the typical mechanisms of resistance to first-generation TRK inhibitors include secondary mutations in TRK kinase domain and bypass signaling activation. Subsequently, next-generation TRK inhibitors (selitrectinib, repotrectinib, and taletrectinib) are designed to overcome acquired resistance mediated by secondary mutations in TRK kinase domain, which are predominant against wildtype TRK and secondary mutations. Previous studies have revealed that xDFG motif substitutions in TRK induce resistance to next-generation TRK inhibitors, but are high sensitivity to type II inhibitors, which highlights areas for future study. Interestingly, NTRK fusion was reported as a potential resistance mechanism to EGFR-TKIs, suggesting that combining EGFR-TKIs with TRK inhibitors may be an optional treatment for patient with NTRK fusion-mediated EGFR-TKI resistance. Thus, it indicates the importance of detecting NTRK fusions, secondary mutations, and bypass signaling in patients with NSCLC, which provides clues for appropriate therapeutic strategies. Also, the RNA-based NGS is preferentially recommended for NTRK detection in tumors including NSCLC. In terms of immunotherapy, no response was observed in two cases with NSCLC, the efficacy of ICIs in patients with NTRK fusion has not been well described, and whether combination of TRK inhibitors with ICIs possesses better efficacy and safety is not yet clear, thus, further investigation is urgently required to address these issues more fully.

AUTHOR CONTRIBUTIONS

FL carried out the primary literature search and drafted and revised the manuscript. YW and HZ contributed to drafting and revising of the manuscript. JJ and PZ helped modify the manuscript. JJ and QC carried out the literature analysis and revised the manuscript. All authors read and approved the final manuscript.

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REVIEW
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HER2-Altered Non-Small Cell Lung Cancer: Biology, Clinicopathologic Features, and Emerging Therapies

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Multiple oncogenic molecular alterations have been discovered that serve as potential drug targets in non-small cell lung cancer (NSCLC). While the pathogenic and pharmacological features of common targets in NSCLC have been widely investigated, those of uncommon targets are still needed to be clarified. Human epidermal growth factor receptor 2 (HER2, ERBB2)-altered tumors represent a highly heterogeneous group of diseases, which consists of three distinct situations including mutation, amplification and overexpression. Compared with breast and gastric cancer, previous studies have shown modest and variable results of anti-HER2 treatments in lung cancers with HER2 aberrations, thus effective therapies in these patients represent an unmet medical need. By far, encouraging efforts towards novel treatment strategies have been made to improve the clinical outcomes of these patients. In this review, we describe the biological and clinicopathological characteristics of HER2 alterations and systematically sum up recent studies on emerging therapies for this subset of patients.

Keywords: HER2, ERBB2, non-Small Cell Lung Cancer (NSCLC), targeted therapy, immunotherapy

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INTRODUCTION: THE HISTORY OF HER2 AT A GLANCE

In the 1980s, researchers in Robert Weinberg's laboratory isolated a cDNA clone (which was termed as Neu) from carcinogen-induced tumors and found it displayed a protein structure that highly resembled epidermal growth factor receptor (EGFR) as earlier identified. From that point, successful efforts by three independent laboratories to identify EGFR-related cDNA sequences yielded the human orthologue of Neu (now called HER2 or ERBB2) (1). However, unlike the mutation in the sequence of rodent Neu reported by Weinberg, human HER2 is usually amplified in tumors. Based on preclinical studies of breast cancer cell lines, HER2 amplification was found in a subset of patients with breast cancers and emerged as an important predictor of resistance to hormonal and chemotherapy regimens, time to relapse and overall survival (OS) (2). This significant finding was continuously confirmed by later studies and extended to gastric cancer (3).

HER2 amplification and protein overexpression lead to the dimerization of the receptor and activation of several signaling pathways that drive tumorigenesis. Consequently, targeting HER2 has been investigated as a promising therapeutic strategy. In 1998, trastuzumab, a monoclonal antibody (mAb) against HER2, was the first HER2-targeted agent approved by the FDA for treating metastatic breast cancer, which represented the beginning of a turnaround for the poor clinical

outcomes of HER2-positive disease. Later on, considerable progress has been made as several categories of HER2-targeting agents, including additional mAbs, signal transduction inhibitors, novel tyrosine kinase inhibitors (TKIs) which showed excellent efficacy in both early-stage and metastatic HER2-positive breast cancer. The second malignancy suitable for trastuzumab-based therapy as a standard of care was gastric cancer. Additionally, HER2 amplification/overexpression has also been observed in other solid tumors including the biliary tract, ovarian, endometrial, bladder, colon and NSCLC (4). Disappointingly, targeting HER2 with traditional anti-HER2 agents which were proved to be effective in breast and gastric cancer has failed in other tumor types, indicating the histological and biological diversity of HER2 alternations in distinct malignancies (5).

In the subsequent sections, we will review available data and describe the biology of the HER2 pathway in normal and tumorigenesis processes, trying to provide a comprehensive overview of its dysregulation, clinical implications, as well as recent studies of emerging therapies for NSCLC patients with HER2 alterations.

THE BIOLOGY OF HER2 AND ITS DYSREGULATION IN NSCLC

The ERBB family is comprised of four members that belong to the transmembrane tyrosine kinase receptors (TKR), including EGFR (also known as HER1), HER2, HER3 and HER4. HER2 encodes a transmembrane TKR which consists of three domains: an extracellular domain (ECD), a transmembrane domain (TMD) and an intracellular tyrosine kinase domain (TKD). Ligand binding results in heterodimerization or homodimerization between the ERBB receptors, and it sequentially stimulates the transactivation of the intracellular tyrosine kinase domain and activates downstream signaling pathways concerning cellular proliferation, differentiation, migration and apoptosis. However, HER2 lacks specific endogenous ligands and retains in the active conformation, making it continuously available for dimerization and to be the preferred heterodimerization partner. In contrast, HER3 has several ligands but lacks intrinsic tyrosine kinase activity. Interestingly, HER2-HER3 pairing displays the highest potency regarding the interaction strength and downstream signaling cascade, suggesting a complementary action between them (6–8).

HER2 is a common oncogene identified in various cancer types and dysregulation of HER2 signaling can be caused by mutation, amplification and overexpression. All three types of HER2 dysregulation could appear in NSCLC with almost no overlap between mutation and amplification (9). Thus, associations of the three types of HER2 alterations are much more complex. This phenomenon partially explains the observed poor outcomes of classic HER2-targeted therapies in the setting of NSCLC than in breast cancer, where its oncogenesis predominantly relies on HER2 overexpression attributed to gene amplification.

HER2 mutation is identified in 2%-4% of NSCLC and encompasses heterogeneous alterations distributed in the ECD, TKD and TMD. Exon 20 insertions that occurred within the kinase domain are the dominant forms of all the HER2 mutations and these insertions might account for about 1.5% of NSCLC. The most common variant is a 12-base-pair (encoding YVMA) in-frame duplicated insertion at codon 775 of exon 20, which affects the αC-β4 loop of the kinase domain and is identified as an early event in lung adenocarcinoma (LUAD) tumorigenesis (10). The $HER2^{YVMA}$ subtype accounts for 34%-83% of HER2-mutated NSCLC, followed by G778_P780dup and G776delinsVC (11–13). In addition, there are more types of point mutations affecting the TKD but with a lower prevalence. Mutations in the TKD lead to conformational changes of ATP-binding pocket, which enhances kinase activity and downstream signaling. Other rarer mutations could also affect the ECD (mostly S310 in exon 8) and TMD (mostly V659 and G660 in exon 17) (14, 15). Generally, different mutation variants have heterogeneous behaviors, which could affect inhibitor binding affinity and sensitivity (13). In rare cases, HER2 mutations could be found in germline and cause hereditary and sporadic LUADs (16). Concomitant EGFR somatic mutations are often detected in EGFR/ERBB2 germline mutations, suggesting that patients carrying EGFR/ERBB2 germline mutations could also acquire somatic mutations in EGFR that eventually drive tumorigenesis (17).

HER2 amplification accounts for 2%-4% in NSCLC, which is far less common compared with breast cancer (18). Studies have shown that HER2 amplification and HER2 mutations were distinct molecular targets that may have different therapeutic and prognostic values. But they could co-exist in very few cases (9, 19). Though an official consensus is not available, generally HER2 amplification is defined as HER2/CEP17 ≥ 2.0 by fluorescent *in situ* hybridization (FISH) testing. Notably, it should be distinguished from HER2 copy number gain (CNG), which happens even more frequently as a consequence of chromosome 17 polysomy and is not supposed to drive tumorigenesis (9, 20, 21). Chromosome 17 polysomy leads to the increased copy number of HER-2 per cell, so as to the activity of upstream promoter, resulting in the larger expression of HER2 gene. HER2 CNG is usually defined by HER2/CEP17 < 2.0 and HER2 gene copy number ≥ 6 per cell. By far, the predictive or prognostic role of HER2 CNG in NSCLC remains unclear. Apart from *de novo* tumorigenesis, HER2 amplification is one of the most frequent acquired resistance mechanisms following the EGFR T790M mutation in EGFR-mutant NSCLC treated with first- or second-generation EGFR-TKIs, which accounts for about 10% of cases (22). It can also confer resistance to osimertinib therapy, with a lower incidence rate of 2%-5% (23). A combination of osimertinib and anti-HER2 agent trastuzumab emtansine was reported to overcome osimertinib resistance in T790M-positive EGFR-mutated NSCLC cell lines which gained HER2 amplification (24).

The incidence rate of HER2 overexpression is reported with a wide range of 2.5%-34% in NSCLC, probably due to the inconsistency among different methods for positivity

assessment and low concordance assessment by pathologists (9, 25–27). Currently, there is no consensus on how to define HER2 overexpression using IHC in NSCLC. Immunohistochemistry (IHC) scoring system and H-score are both applied for the assessment, with the former used more widely. Contrary to breast cancer, the co-occurrence of HER2 overexpression and amplification has not been well confirmed in lung cancer. However, an overlap between IHC 3+ staining and HER2 amplification was reported in NSCLC, albeit IHC low/negative has been also shown to be FISH positive. In the IHC 2+ cases, perhaps mechanisms other than gene amplification (HER2 polysomy, mutation and unknown reasons) cause the immuno-positive results (8, 26). Therefore, HER2 overexpression in NSCLC represents a heterogeneous group with distinct molecular features, making it a less accurate indicator of inhibitor sensitivities and patient outcomes. Further analyses need to be done to discriminate among these possibilities. The associations of HER2 mutation, amplification and overexpression are depicted in **Figure 1A**.

CLINICAL CHARACTERISTICS OF HER2-ALTERED NSCLC

Mutations in HER2 are more frequent in females, never smokers and lung adenocarcinoma, similar to those observed in patients with EGFR mutations. However, HER2-mutated patients have a worse prognosis than their EGFR and ALK counterparts, partially due to the lack of highly-selective targeted agents (28–30). HER2 mutations also exhibit a tendency of brain metastases on treatment (31). Similarly, another study found that the $HER2^{YVMA}$ subtype was associated with a higher estimated 12-month brain metastasis incidence compared with the non-YVMA group (40.2% vs. 3.6%, P=0.002) (32). *De novo* HER2 mutations in NSCLC are supposed to be mutually exclusive with other driver genes. But the oncogenic role of HER2 varied among different domains, with most of them occurring in the kinase domain. A study demonstrated that the frequency of EGFR or KRAS co-mutation was significantly higher in the non-TKD mutation compared to the TKD mutation, but OS was comparable between the two groups (28). Another retrospective database study revealed that patients harboring TMD mutations were diagnosed at more advanced stages (P<0.001) and had poorer OS (median OS 10.0m vs. 61.6m, P<0.001) than non-TMD mutations (33). TP53 aberrations were the most prevalent co-mutations in HER2-mutated patients, followed by aberrations in the PI3K/AKT/mTOR pathway. Both two co-mutation variants were correlated with shorter progression-free survival of afatinib treatment (11). This trend was also observed in another study which reported an impaired OS in patients with co-mutations in the cell-cycle pathway especially TP53 (34). These observations indicated different clinical implications of HER2 mutation variants and their co-mutations.

De novo HER2 amplification and HER2 overexpression were detected more often in smokers and male patients, implying their inconsistent origins of tumorigenesis with HER2 mutations (29, 35). HER2 amplification seemed to have a controversial role on the prognosis of NSCLC, while HER2 overexpression was a marker of poor prognosis, especially for adenocarcinoma, early-stage NSCLC and small cell lung cancer (SCLC) as suggested by a meta-analysis (36). Compared with their mutation variants, co-mutations of other driver genes were more frequently seen in HER2 amplification and overexpression tumors but still remained at a relatively low incidence rate. Concomitant HER2 alterations were proved to have an impact on the efficacy of targeted therapies. A retrospective study demonstrated that patients with concurrent EGFR mutation and HER2 amplification had a longer median time on treatment with EGFR-TKIs than those with EGFR

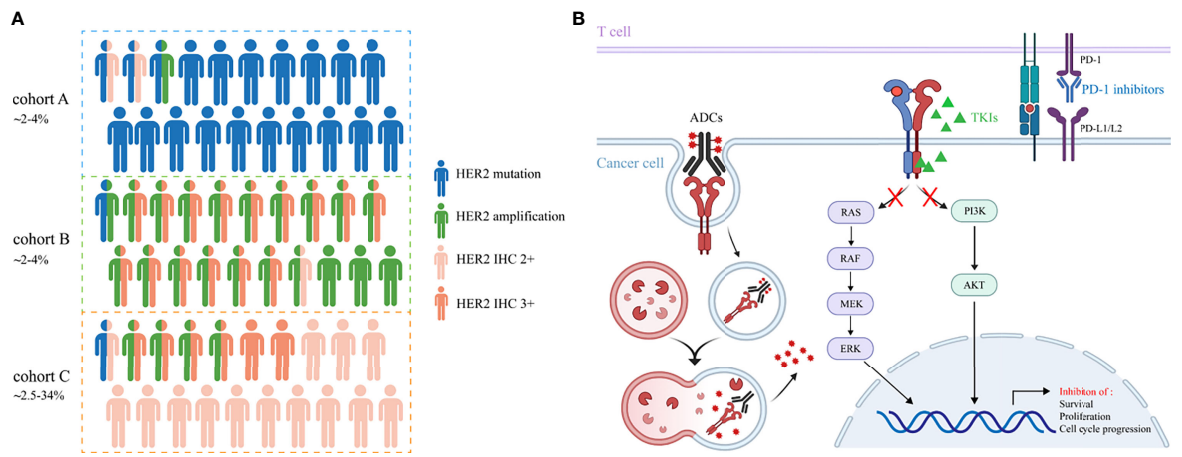

FIGURE 1 | **(A)** The incidence rates and associations of HER2 alterations in NSCLC. Cohort A, B and C represent HER2 mutation, amplification and overexpression, respectively. Cohort C includes patients with IHC 2+ and 3+. **(B)** The underlying mechanisms of TKIs, ADCs and immune checkpoint inhibitors to cope with HER2-altered NSCLC.

mutation without HER2 amplification (846 days vs. 286 days, P =0.004) (37). However, in the post-hoc subgroup study of HER-CS, HER2 expression in EGFR-mutant patients may negatively impact the time-to-treatment failure (TTF) of EGFR-TKIs in the subgroup with a performance status (PS) of 2 (38).

TARGETING HER2 IN NSCLC: PERPLEXITY AND PROGRESS

Chemotherapy and mAbs

Chemotherapy represents the most conventional treatment strategy of NSCLC patients with HER2 alterations before the arrival of targeted therapy. Several studies revealed no association between HER2 status and objective response to chemotherapy (27, 39). While another retrospective study reported the inferior outcomes of HER2-mutant NSCLC compared with ALK/ROS1-rearranged group, with a median PFS of 5.1 months in pemetrexed-based first-line chemotherapy. Additionally, subgroup analysis suggested that PFS had a trend to be inferior in the $HER2^{YVMA}$ group compared with other variants, albeit not statistically significant (4.2 vs 7.2 months, P=0.085) (40). The retrospective study of the EUHER2 cohort showed the ORR and median PFS for patients receiving first-line conventional therapies (including chemotherapy and EGFR-TKIs) were 43.5% and 6 months. For those receiving second-line therapies, ORR and median PFS were 10% and 4.3 months (41). A Chinese study revealed that the median PFS and OS were comparable between first-line chemotherapy and HER2-targeted agents in HER2-mutant patients (5.9 vs 4.6 months, P=0.63; 9.8 vs 10.8 months, P=0.40, respectively) (42). While Xu et al. reported that compared with HER2-TKIs, chemotherapy achieved better outcomes both in the first-line and second-line setting. This trend was also observed in the $HER2^{YVMA}$ subgroup (43).

Trastuzumab is a humanized IgG monoclonal antibody that could selectively inhibit the proliferation and survival of HER2-addictive tumors by targeting the extracellular domain of HER2 (44). A phase II study launched by the Eastern Cooperative Oncology Group (ECOG) evaluated the efficacy of combining carboplatin, paclitaxel and trastuzumab in advanced NSCLC patients with HER-2/neu positivity (1+ to 3+). The reported ORR, median PFS and median OS were 24.5%, 3.3 months and 10.1months, respectively. Notably, patients with 3+ HER-2/neu expression experienced a survival exceeding that of historical data, suggesting potential application for trastuzumab in this rare subgroup (45). Similarly, in a randomized phase II trial, the addition of trastuzumab to gemcitabine–cisplatin chemotherapy was beneficial for HER2 3+ or FISH-positive patients, but this subgroup is too small to provide definitive information (46). A phase IIa multiple basket study (Mypathway) also demonstrated a moderate efficacy of dual blockade with trastuzumab and pertuzumab in HER2-altered NSCLC patients (47). HOT1303-B trial was a multicenter, single-arm phase II study of trastuzumab for pretreated HER2-altered NSCLC patients, which were defined as HER2 mutations, IHC 3+ or IHC 2+/dual color *in situ* hybridization [DISH]+. Disappointingly, trastuzumab monotherapy did not produce any response (ORR was 0%) in this cohort, although DCR was 70.0% and the median PFS reached 5.2 months (48). A multicenter, phase II study (IFCT 1703-R2D2 trial) enrolled HER2-mutated advanced NSCLC patients progressing after platinum-based treatment and evaluated the efficacy and safety of triple therapy with trastuzumab, pertuzumab, and docetaxel (49). The ORR, median PFS and median OS were 29%, 6.8 months and 17.6 months, with tolerable toxicity, suggesting this triple therapy regimen becoming an option for pretreated patients.

In general, the efficacy of traditional chemotherapy for the HER2-altered population is far from satisfactory. The successes of trastuzumab observed in breast and gastric cancer might not be replicated in NSCLC. The reasons for differences in efficacy are complicated, but could possibly be explained by a different spectrum of HER2 alterations and other aspects of disease biology among cancer types. A combination of trastuzumab, pertuzumab, and docetaxel seemed to compete favorably with single mAb or chemotherapy, but this regimen remained to be defined. Other targeting strategies and agents for NSCLC patients with HER2 alterations are urgently needed.

Non-Selective TKIs

Second-generation irreversible TKIs developed for the treatment of EGFR mutations represented early attempts to target HER2 in NSCLC. De Grève and colleagues reported the clinical benefits of afatinib in HER2 ex20ins LUAD patients (50). Among the five identified patients, three patients evaluable for the response all showed an objective response. Later on, the efficacy of afatinib was evaluated in a global named patient use program that enrolled HER2-mutant patients who had exhausted other treatments. Median TTF was 2.9 months, ORR and DCR were 19% and 69%, respectively. Notably, for the group of $HER2^{YVMA}$ subtype, median TTF was 9.6 months and 40% continued treatment for more than one year (51). Conversely, other studies presented different perspectives, showing that the clonality status of HER2 ex20ins and the $HER2^{YVMA}$ subtype were potential indicators for poor response to afatinib, while G778_P780dup and G776delinsVC subtypes derived favorable outcomes from afatinib, suggesting a further investigation into the clinical implications of mutation variants to help optimize outcomes with HER2-targeted therapies (11, 34). A prospective phase II NICHE trial exploring the efficacy of afatinib in pretreated advanced HER2-mutant NSCLC patients reported that ORR and DCR were 7.7% and 53.8%. Thus, the accrual into the trial was terminated with 13 patients enrolled altogether. Median PFS and OS were 15.9 and 56.0 weeks, respectively (52). Additionally, another phase II trial aimed to investigate the efficacy of afatinib among the Asian population with HER2-mutant NSCLC and consisted of two parts. In total, 18 patients were recruited and received afatinib in Part A. None of them achieved PR, 11 patients achieved SD and 6 patients had progressive disease as their best response. Median PFS and OS were 2.76 and 10.02 months, respectively. No patients met the Part B inclusion criteria, resulting in the termination of this study (53). The study of the EUHER2 cohort assessed the efficacy of chemotherapy and/or HER2-targeted agents in 101 advanced NSCLC patients with HER2 ex20ins. Sixty-five patients received HER2-targeted therapies.

Eleven patients were treated with afatinib, with an ORR and median PFS of 18.2% and 3.9 months, respectively (41). Modest clinical activity of afatinib was also observed in a retrospective multicenter study, in which 27 patients included showed an ORR and median duration of response (DOR) of 13% and 6 months (54). A Chinese retrospective study included patients harboring HER2 mutation and amplification treated with afatinib and reported an ORR of 24%. Median PFS and OS were 3.3 and 13.9 months, respectively (55). Collectively, studies above showed inconsistent efficacy of afatinib for HER2-mutant patients. A meta-analysis study integrated and reanalyzed the existing data regarding afatinib treating HER2-mutant lung cancers. Pooled ORR and DCR were 21% and 66%, respectively, with the HER^{YVMA} subtype deriving greater clinical benefit (56). Thus, afatinib was not recommended as the regular application for treating NSCLC patients with HER2 mutation.

Dacomitinib is a pan-HER2 TKI that could bind to EGFR, HER2 and HER4 tyrosine kinases. A prespecified cohort from a phase II study enrolled NSCLC patients with HER2 mutations (n=26) or amplification (n=4). Three of 26 HER2-mutant patients had partial responses, but no partial responses were observed in four patients with HER2-amplified tumors. Median PFS and OS for HER2-mutant patients were 3 and 9 months, respectively. Interestingly, two patients harboring p. P780_Y781insGSP changes showed the longest responses, and the remaining patient with a partial response was identified with a p. M774delinsWLV mutation. No responses occurred in the most common $HER2^{YVMA}$ subtype (57). Subsequently, a preclinical study demonstrated that the IC50 values of the HER2 ex20ins Ba/F3 cells harboring the dacomitinib-sensitive mutations (InsGSP, InsWLV, and InsCPG) were significantly lower than the other HER2 mutants or Wildtype HER2 (P=0.031), consistent with the previous clinical data (58).

Neratinib is another type of TKI which was applied and evaluated in HER2-mutant NSCLC. A preclinical *in vitro* study assessed the activity of drugs in HER2 mutation variants and found neratinib and afatinib more effective than other inhibitors for the $HER2^{YVMA}$ subtype (59). A randomized 2-stage phase II study compared neratinib monotherapy and the combination of neratinib with temsirolimus in stage IIIB/IV HER2-mutant NSCLC patients. Twenty-seven patients were enrolled in stage 1, and 3 of 14 patients (21%) in the combination group had a response, resulting in a median PFS of 4 months (60). In the subsequent expansion cohort, the dual inhibition group obtained an ORR of 19%, a median PFS of 4.1 months and a median OS of 15.8 months. The incidence of grade 3 diarrhea was 12% and could be managed with loperamide prophylaxis (61). In the SUMMIT phase II basket trial, neratinib was evaluated across multiple cancer types. In patients with lung cancer (n=26), only one patient harboring L775S kinase missense mutation was observed with objective response (ORR=3.8%), suggesting its limited efficacy in HER2-mutated lung cancer. The median PFS in recurrent NSCLC was 5.5 months with 6 patients continuing therapy for more than one year (62).

The activities of non-selective TKIs in HER2-mutant NSCLC patients yield moderate or even disappointing results, though sporadic responses have been reported as HER2 mutation location could affect the drug binding affinity. This may be explained because HER2 ex20ins seem to tighten the drug-binding pocket, restricting the binding of large-sized inhibitors. Therefore, structurally novel pan-HER2 TKIs have been developed to achieve better outcomes in NSCLC with HER2 alterations.

New-Generation TKIs

As a covalent and irreversible EGFR/HER2 inhibitor, poziotinib has a smaller size and flexible structure. A preclinical study compared the activity of different TKIs in Ba/F3 cells with HER2 exon 20 mutations, poziotinib showed the most potent activity. Also, the secondary C805S mutation was identified as a potential mechanism of acquired resistance to poziotinib (63). Another study indicated that HER2 20 exon insertions tightened the size of the drug-binding pockets and restricted the binding of large, rigid inhibitors using 3D modeling. Poziotinib can avoid these spatial changes owing to its small size and flexibility, thus becoming a potent inhibitor of the most common HER2 variant. Its efficacy was further confirmed *in vitro* and *in vivo* studies (64). Similar trends were also reported in the pan-cancer landscape and functional analysis of HER2 mutation by Robichaux and colleagues (12). In addition, in their preclinical models, poziotinib upregulated HER2 expression at the cell surface and potentiated the T-DM1 activity. Early results from a phase I study showed an encouraging activity of poziotinib and further justified its application in patients with EGFR/HER2 alterations (65). Based on this study and preclinical evidence, a phase II clinical trial of poziotinib in NSCLC patients with EGFR and HER2 exon 20 mutations was initiated. All HER2-mutant participants enrolled harbored the Y772dupYVMA or G778dupGSP insertions. Response was confirmed in 5 of 12 HER2-mutated patients (confirmed ORR=42%). The DCR and median PFS were 83% and 5.6 months. No patients discontinued treatment due to poziotinib-related toxicity (12). A poziotinib expanded access program enrolling NSCLC patients with EGFR or HER2 ex20ins showed a median PFS of 5.6 months and a median OS of 9.5 months. The ORR was higher in HER2 subgroup (50% vs. 23%). Grade 3 AEs were reported in 66% of the patients, and the toxicity rate was high leading to frequent dose interruption and reduction (66). A single-arm, open-label, phase II study assessed the efficacy and safety profiles of poziotinib in HER2-mutant advanced NSCLC. The confirmed ORR was 27% with responses observed across mutation subtypes. Median PFS and OS were 5.5 and 15.0 months, respectively. One possible treated-related death due to pneumonitis was reported (67). ZENITH20 study evaluated poziotinib in previously treated NSCLC patients with HER2 exon 20 insertions. In cohort 2, patients received poziotinib once daily, the ORR and DCR were 27.8% and 70.0%, respectively. Most patients (74%) had tumor reduction, with a median PFS was 5.5 months. Clinical benefits were seen regardless of types and lines of previous treatment, presence of brain metastasis and HER2 mutation variants. Severe treatment-related AEs (grade≥3) included rash (48.9%) diarrhea (25.6%), and stomatitis (24.4%) (68). Updating results from cohort 4 of ZENITH20 presented a promising efficacy in treatment-naive patients, with an ORR of 44% and a median PFS of 5.6 months, the safety profile was similar to cohort 2 of this study (69).

Pyrotinib is an oral, irreversible pan-HER TKI. The enhanced antitumor activity of another irreversible pan-HER TKI pyrotinib was observed in organoids as well as patients-derived xenograft (PDX) models relative to afatinib and trastuzumab-emtansine (T-DM1). In a phase II cohort of 15 HER2-mutant NSCLC patients, pyrotinib resulted in an ORR and a median PFS of 53.3% and 6.4 months. AEs were all grade 1-2 and no dose reduction or treatment discontinuation occurred (70). In another single-arm prospective study, pyrotinib exhibited promising efficacy and acceptable safety in 27 advanced HER2-amplified NSCLC patients, reaching a confirmed ORR of 22.2%, a median PFS of 6.3 months and a median OS of 12.5 months. Of note, Patients who received pyrotinib as a first-line treatment achieved a median PFS of 12.4 months. Treatment-related AEs occurred in all patients, but no grade 4 or higher AEs were documented (71). A larger phase II study evaluated the efficacy and safety profiles of pyrotinib in stage IIIB-IV LUAD patients harboring HER2 mutations after the failure of platinum-based chemotherapy. Independent reading committee-assessed ORR was 30.0%, with a favorable ORR observed across all HER2 subtypes and between patients with and without brain metastases (25.0% vs. 31.3%). The median PFS and OS were 6.9 and 14.4 months, respectively. Grade 3-4 treatment-related AEs occurred in 28.3% of patients and diarrhea (20.0%) appeared to be the most common type (72). In addition, the encouraging efficacy of pyrotinib was also reported in a study in which patients with EGFR mutation and HER2 amplification could obtain clinical benefits from combining EGFR-TKIs and pyrotinib, suggesting the potential of pyrotinib in targeting the HER2 pathway in patients after progressing on EGFR-TKIs (73).

The hypoxia-activated prodrug (HAP) tarloxotinib is a potential treatment for NSCLC with HER2 alterations. Prior work demonstrates that the tarloxotinib can be converted into tarloxotinib-E as its active form in a hypoxic tumor microenvironment. Preclinical studies showed that tarloxotinib-E could interfere with cell signaling and proliferation by inhibiting phosphorylation and activation of ERBB heterodimers in PDX models. *In vivo*, tarloxotinib inhibited tumor growth and progression. The pharmacokinetic analysis also confirmed the accumulations of tarloxotinib-E in tumor sites than plasma or skin (74). Another *in vitro* study demonstrated that the IC50 of tarloxotinib for wildtype HER2 was 180 times higher than that of tarloxotinib-E, suggesting a wide therapeutic index of tarloxotinib (75). The phase I RAIN-701 trial (NCT03805841) enrolled advanced patients with HER2 activating mutations (cohort B). First results showed that 2 of 9 patients experienced confirmed PR (22%) and 4 patients had SD. Grade 3 TEAEs included prolonged QTc (34.8%), increased ALT (4.3%), diarrhea (4.3%) and rash (4.3%) (76).

Mobocertinib (TAK-788) is a research-based oral EGFR/HER2 inhibitor designed against ex20ins. The IC50 of mobocertinib was higher than poziotinib and comparable with pyrotinib, neratinib and afatinib in HER2 ex20ins cell lines. Mobocertinib exhibited the lowest HER2 ex20ins IC50/wildtype EGFR IC50 ratio, implying its excellent selectivity profile. Additionally, lung cancers with HER2 G776delinsVC subtypes reported a superior response to mobocertinib than the YVMA subtypes. The combination of ado-trastuzumab emtansine (T-DM1) and mobocertinib had a synergistic function in $HER2^{YVMA}$ tumors (77). A phase I/II trial (NCT02716116) showed promising antitumor activities of mobocertinib in advanced NSCLC patients harboring EGFR ex20ins, with a similar safety profile compared to other EGFR-TKIs (78, 79). Results from the expansion cohort 2 of this study which enrolled NSCLC patients with HER2 exon 20 alterations are still awaited.

Compared with non-selective TKIs originally developed for EGFR mutations, these novel TKIs have shown greater activities and broader anti-tumor effects across exons in HER2-mutant NSCLC. For patients who had previously received platinum-based chemotherapy where there are limited therapeutic drugs, new generation TKIs could be an option to consider.

Antibody-Drug Conjugates

ADCs are characterized by the covalent coupling of drugs to mAbs as an alternative to naked antibody-targeted therapy. The development of ADCs represents groundbreaking progress in the treatment of malignancies with actionable targets. After their successes in breast and gastric cancer, these agents are gradually attracting much attention in NSCLC with HER2 alterations.

Trastuzumab emtansine (T-DM1) is a second-generation anti-HER2 ADC composed of trastuzumab and emtansine (DM1), which is an inhibitor of microtubule aggregation. This complex enters into HER2-positive cells *via* receptor-mediated endocytosis. Proteolytic degradation of the antibody moiety in lysosomes leads to the release of conjugated agents (80). In a phase II study of T-DM1 in relapsed HER2-positive NSCLC (IHC 3+, IHC 2+/FISH+, or exon 20 mutations), among fifteen assessable patients, only one patient achieved a PR (ORR=6.7%). The median PFS and OS were 2.0 and 10.9 months, respectively. Grade 3-4 AEs included thrombocytopenia (40%) and hepatotoxicity (20%), with no treatment-related deaths (81). However, another phase II basket trial enrolled 18 advanced LUAD patients harboring HER2 mutations. The treatment with T-DM1 might achieve an ORR as high as 44% and a median PFS of 5 months. Responses to T-DM1 could be seen across all the subtypes. The toxicities included grade 1-2 elevated hepatic transaminases, thrombocytopenia and infusion reactions (82). Peters et al. evaluated the efficacy and safety of T-DM1 in 49 advanced HER2-overexpressing NSCLC patients (29 IHC 2+ and 20 IHC 3+) who were previously treated. Although there were no treatment responses presented in the IHC 2+ cohort, four PR were observed in the IHC 3+ cohort (ORR=20%). Median PFS and OS were similar between the two cohorts (median PFS: 2.6 and 2.7 months; median OS: 12.2 and 15.3 months). Forty-five patients (92%) reported an AE of any grade and ten patients (20%) reported grade 3 AEs. One patient with a history of brain metastases reported grade 4 seizures while receiving seizure therapy. There were no deaths due to AEs (83).

Trastuzumab deruxtecan (T-Dxd, also known as DS-8201a) is a novel HER2-ADC composed of trastuzumab and a novel topoisomerase I inhibitor (MAAA-1181) linked by an enzymatically cleavable peptide. The drug moiety of T-Dxd could bind to topoisomerase I-DNA complexes and induce DNA double-strand breaks. T-Dxd has a drug-to-antibody ratio (DAR) of 8, which is almost two-fold higher than T-DM1 (DAR of 3-4). Preclinical results suggested that T-Dxd had antitumor activities towards a broad

range of HER2-positive models and acceptable safety profiles. Featured by a highly membrane-permeable payload, it can exert the by-stander effect and is favorable in treating tumors that are insensitive to T-DM1. Thus, T-Dxd is expected to be a promising therapy to cope with HER2-positive or HER2-low-expressing tumors that do not respond to T-DM1 (84, 85). The first evidence of T-DM1 in NSCLC from a dose-expansion phase I study suggested that T-Dxd had great potential for HER2-expressing/mutant solid tumors. In the HER2-mutant/HER2-expressing NSCLC subgroup, 10 of 18 patients (55.6%) had a confirmed objective response, with a median PFS of 11.3 months. Among the subset of HER2-mutant NSCLC patients, the confirmed ORR even reached 72.7% (8/11) and the median PFS was 11.3 months. Notably, NSCLC patients with documented HER2 mutations had more pronounced tumor shrinkage than those without mutations, regardless of IHC status. All patients experienced at least 1 AE, and 2 of 18 patients (11.1%) had serious AEs. Three patients were adjudicated as interstitial lung disease (ILD) related to the study drug, and one patient experienced an AE of respiratory failure which was associated with a fatal outcome (86). The DESTINY-Lung01 phase II trial investigated the efficacy of T-Dxd in NSCLC patients and consisted of two cohorts. Cohort 1 contained patients with HER2 overexpression (IHC 2+ or IHC 3+), and cohort 2 contained patients with HER2 mutation. Results from cohort 1 showed an ORR and a median PFS of 24.5% and 5.4 months. Response rates were comparable according to HER2 IHC expression levels (ORR 25.6% vs. 20.0% in IHC2+ and IHC3+ patients, respectively). All patients had at least one treatment-emergent AEs, and grade 3 AEs were reported in 73.5% of patients. There were 8 cases of drug-related ILD as adjudicated by an independent committee. Treatment-emergent AEs were associated with dose interruption in 26 patients (53.1%), dose reduction in 17 patients (34.7%), and treatment discontinuation in 11 patients (22.4%) (87). Recently published results from cohort 2 were promising and more spectacular in comparison with cohort 1. Among 91 patients enrolled, centrally confirmed ORR was 55%, the median PFS was 8.2 months and the median OS was 17.8 months (88). Preclinical findings revealed that HER2-activating mutations facilitated receptor-mediated endocytosis of the HER2-ADC complex (89). This may provide the mechanistic foundation for its higher efficacy in HER2-mutant NSCLC patients in contrast to the lower response rates among HER2-overexpressing patients. Regarding T-Dxd toxicity, common events included gastrointestinal and hematologic events, decreased appetite, and alopecia. Grade 3 or higher drug-related AEs occurred in 42 patients (46%). Twenty-three patients (25%) discontinued treatment because of investigator-reported, drug-related AEs, including pneumonitis in 12 patients and ILD in 5 patients (88). Given the exciting results from DESTINY-Lung01, a randomized, open-label, phase 3 trial (DESTINY-Lung04; NCT05048797) is underway to further evaluate the efficacy and safety of T-Dxd compared to standard of care (pembrolizumab combined with chemotherapy) in non-squamous NSCLC patients harboring a HER2 exon 19 or 20 mutations.

By far, ADC-based therapies seem to provide the highest response rates and best clinical outcomes among the anti-HER2 agents, both in HER2-mutant and HER2-overexpressed NSCLC patients. However, drug-related ILD is observed during the treatment and further studies should be carried out to determine which patients are at great risks and how to manage this potentially fatal AE.

Immunotherapy

Immune checkpoint inhibitors (ICIs) demonstrated significant improvements in overall response and survival for driver-negative NSCLC patients, while their application in oncogene-addicted tumors remains to be elucidated. The presence of HER2 mutations in NSCLC is correlated with a "cold" tumor microenvironment, characterized by a relatively lower PD-L1 positive expression rate and tumor mutation burden (TMB), similar to those of EGFR-dependent tumors (90, 91). The efficacy of ICIs monotherapy was evaluated in several studies. A retrospective study identified 26 patients who were treated with ICIs. ORR was 12% (3/26), the median PFS and OS were 1.9 and 10.4 months, respectively. Of the three responders, none had a HER2 YVMA mutation, two had PD-L1 ≥50%, and two had TMB ≥median (92). In the IMMUNOTARGET registry, 29 NSCLC patients with HER2 mutation received ICIs and the ORR was 7%. The median PFS was 2.5 months and was significantly associated with positive smoking status (3.4 months in smokers vs. 2 months in non-smokers, P=0.04) (93). The French Lung Cancer Group (GFPC) reported 6 out of 23 relapsed HER2-mutant NSCLC patients had objective responses to ICIs, with a median DOR of 15.2 months. Survival data were close to previous reports, with a median PFS of 2.2 months and a median OS of 20.4 months (94). Other studies also showed moderate or even dismal responses and survival outcomes of ICIs monotherapy against HER2 mutations (90, 91, 95).

Considering the synergistic antitumor effects of combining ICIs and chemotherapy, ICIs-based therapies were explored and been evaluated. A multicenter retrospective study enrolled 26 HER2-mutant NSCLC patients, most of which received immunochemotherapy combination regimens. The ORR, DCR and median PFS were 38.5%, 84.6% and 7.4 months, respectively (96). Additionally, results from another study demonstrated that the ORR, median PFS, and one-year OS rate of ICIs combined with chemotherapy for treatment-naive HER2-mutant NSCLC were 52%, 6 months and 88%, respectively (97). Tian et al. reported similar clinical outcomes of chemo-immunotherapy in 13 stage IV HER2 ex20ins LUAD patients, with an ORR of 31% and a median PFS of 8.0 months. They also found a higher TMB, a trend toward lower clonality of tumors and a trend toward lower TCR diversity of peripheral blood in responders compared with non-responders (P=0.0067, 0.071 and 0.085, respectively). Patients with baseline TMB-high combined with mutations in DNA damage repair-related pathways or SWI/SNF complex was associated with favorable outcomes of chemo-immunotherapy combinations (98).

Evidence on the efficacy of ICIs in HER2-mutant NSCLC patients remains limited and is heavily derived from retrospective results. These studies do not encourage the use of ICIs monotherapy as a therapeutic strategy in HER2-mutant NSCLC patients. Immunotherapy-based approaches could be a potential treatment option for this patient group and further clinical trials are required to confirm these results. Clinical trials of anti-HER2 agents in NSCLC patients mentioned in this review are summarized in

TABLE 1 | Clinical trials of anti-HER2 agents in NSCLC patients.

References	Agents	Clinical trials	N	Population	HER2 alterations	ORR n (%)	Median PFS months, (95% CI)	Median OS months, (95% CI)
Langer et al. (45)	Trastuzumab + CT	Phase II study	53	Recurrent, Stage IIIB/IV NSCLC	HER2 positivity (1+ to 3+)	13 (24.5)	3.3 (NA)	10.1 (6.7-14.6)
Gatzemeier et al. (46)	Trastuzumab + CT	Phase II study	50	Untreated stage IIIB/IV NSCLC	HER2 IHC 2+/3+ or serum HER2 ECD positive	18 (36)	6.1 (0.1-19.6)	12.2 (0.1-19.6)
Hainsworth et al. (47)	Pertuzumab + Trastuzumab	Phase IIa basket study (MyPathway)	16	Refractory, metastatic NSCLC	HER2 amplification or overexpression	2 (13)	NA	NA
			14		HER2 mutation	3 (21)	NA	NA
Kinoshita et al. (48)	Trastuzumab	Phase II study (HOT1303-B)	10	NSCLC patients pretreated with ≥2 regimens	HER2 IHC 3+, IHC2+/DISH+ or mutation	0 (0)	5.2 (1.4-6.3)	NA
Mazieres et al. (49)	Pertuzumab + Trastuzumab + Docetaxel	Phase II study (IFCT-1703 R2D2)	45	Advanced NSCLC, progressed after ≥1 platinum-based treatment	HER2 mutation	13 (29)	6.8 (4.0-8.5)	17.6 (11.6-18.9)
Peters et al. (51)	Afatinib	Global Named Patient Use Program	28	Heavily pretreated, stage IV NSCLC	HER2 mutation	3/16 (19)[a]	NA	NA
Dziadziuszko et al. (52)	Afatinib	Phase II study (NICHE)	13	Pretreated, advanced NSCLC	HER2 mutation	1 (7.7)	3.7 (1.4-8.3)	13.1 (3.8-NE)
Fan et al. (53)	Afatinib	Phase II study	18	Pretreated, advanced NSCLC	HER2 mutation	0 (0)	2.76 (1.87-4.60)	10.02 (8.47-10.08)
Kris et al. (57)	Dacomitinib	Phase II study	26	Advanced NSCLC, 83% pretreated with CT	HER2 mutation	3 (11.5)	3 (2-4)	9 (7-21)
			4		HER2 amplification	0 (0)	1,1,5,5	5,7,15,22
Besse et al. (60)	Neratinib (N) ±Temsiromilus (TEM)	Phase II study	13	Stage IIIB/IV NSCLC (N)	HER2 mutation	0 (0)	2.9 (1.4-NE)	NA
			14	Stage IIIB/IV NSCLC (N + TEM)		3 (21)	4.0 (2.9-9.8)	
Gandhi et al. (61)		Phase II study (Expansion cohort)	17	Stage IIIB/IV NSCLC (N)	HER2 mutation	0 (0)	3.0 (1.4-6.9)	10.0 (4.9-19.0)
			43	Stage IIIB/IV NSCLC (N + TEM)		8 (19)	4.1 (2.9-5.6)	15.8 (10.8-19.5)
Hyman et al. (62)	Neratinib	Phase II basket study (SUMMIT)	26	Pretreated, advanced NSCLC	HER2 mutation	1 (3.8)	5.5 (NA)	NA
Robichaux et al. (12)	Poziotinib	Phase II study	12	Metastatic, recurrent NSCLC	HER2 mutation	5 (42)	5.6 (NA)	NA
Prelaj et al. (66)	Poziotinib	Phase II study	8[b]	Advanced NSCLC	HER2 mutation	4 (50)	5.6 (3.6-6.7) [c]	9.5 (5.3-NE) [c]
Elamin et al. (67)	Poziotinib	Phase II study	30	Stage IV or recurrent NSCLC, 90% of patients were pretreated	HER2 mutation	8 (27)	5.5 (4.0-7.0)	15 (9.0-NE)
Le et al. (68)	Poziotinib	Phase II study (ZENITH 20)	90	Pretreated, advanced NSCLC	HER2 mutation	25 (27.8)	5.5 (3.9-5.8)	NA
Cornelissen et al. (69)			48	Treatment naïve, advanced NSCLC	HER2 mutation	21 (44)	5.6 (NA)	NA
Wang et al. (70)	Pyrotinib	Phase II study	15	Pretreated, advanced NSCLC	HER2 mutation	8 (53.3)	6.4 (1.60-11.20)	12.9 (2.05-23.75)
Song et al. (71)	Pyrotinib	Prospective, single-arm trial	27	Stage IIIB/IV NSCLC	HER2 amplification	6 (22.2)	6.3 (3.0-9.6)	12.5 (8.2-16.8)
Zhou et al. (73)	Pyrotinib	Phase II study	60	Pretreated, advanced NSCLC	HER2 mutation	18 (30)	6.9 (5.5-8.3)	14.4 (12.3-21.3)
Hotta et al. (81)	T-DM1	Phase II study	15	Pretreated, advanced NSCLC	HER2 IHC 3+, IHC2+/FISH+ or mutation	1 (6.7)	2.0 (1.4-4.0)	10.9 (4.4-12.0)
Li et al. (82)	T-DM1	Phase II basket study	18	Advanced NSCLC, 83% pretreated with CT	HER2 mutation	8 (44)	5 (3-9)	NA
Peters et al. (83)	T-DM1	Phase II study	29	Locally advanced or metastatic NSCLC,	HER2 IHC 2+	0 (0)	2.6 (1.4-2.8)	12.2 (3.8-23.3)
			20	pretreated with ≥1 CT	HER2 IHC 3+	4 (20)	2.7 (1.4-8.3)	15.3 (4.1-NE)
Tsurutani et al. (86)	T-DXd	Phase I study	18	Pretreated, advanced or recurrent NSCLC	HER2 overexpression or mutation	10 (55.6)	11.3 (7.2-14.3)	NR (17.3-NE)
Nakagawa et al. (87)	T-DXd	Phase II study	49	Pretreated, metastatic NSCLC	HER2 overexpression	12 (24.5)	5.4 (2.8-7.0)	NA
Li et al. (88)		(DESTINY-Lung01)	91	Pretreated, unresectable or metastatic NSCLC	HER2 mutation	50 (55)	8.2 (6.0-11.9)	17.8 (13.8-22.1)

N, number; ORR, objective response rate; PFS, progression-free survival; OS, overall survival; CT, chemotherapy; IHC, immunohistochemistry; ECD, extracellular domain; DISH, dual color in situ hybridization; FISH, fluorescence in situ hybridization; NA, not available; NE, not estimable; NR, not reached.

[a]Tumor response data were available for 16 patients. [b]This phase II study enrolled 30 patients, 22 had EGFR 20 exon mutations and 8 had HER2 mutations. [c]PFS and OS data were evaluated based on the whole cohort (n=30).

Table 1. Promising strategies to cope with HER2-altered NSCLC and their underlying mechanisms are shown in **Figure 1B**.

DISCUSSION

HER2 alterations, including mutation, amplification and overexpression, have emerged as novel potential targets for anti-HER2 agents in NSCLC. Following new drug designations rapidly changing the treatment landscape of this particular subset of NSCLC patients, several matters still need to be discussed and addressed.

First of all, the inconsistency in the clinical efficacy of anti-HER2 drugs among HER2 variants implies their distinct molecular entities and features. Currently, most studies evaluating anti-HER2 agents in NSCLC patients are relatively small-sampled and do not distinguish the three types of HER2 alterations. Thus, it leaves us the question of how we define the term 'HER2-positive' and which types of 'HER2-positive' lung cancers are suitable for receiving anti-HER2 targeted therapy. As mentioned above, apart from breast and gastric cancers, the patterns of HER2 staining in other cancer types have not been thoroughly investigated. This probably explains the variations of IHC overexpression rates reported in different studies. Consequently, IHC expression is not a definitive biomarker for anti-HER2 activity in NSCLC. In order to define HER2 alterations and determine their response patterns, it is crucial to advocate for standardized methods in the future to establish the definitive formulation of HER2 activating mutation, amplification and overexpression. Patient cohorts enrolled for evaluating HER2-targeted drugs should also be distinguished by the particular HER2 alteration present.

Secondly, given the substantial variant efficacy and safety profiles of anti-HER2 drugs, it is imperative to investigate the pharmacodynamic and pharmacokinetic properties of these agents. Studies also reported that HER2-mutated NSCLCs are associated with central nervous system (CNS) metastases during treatment in approximately half of the patients. Therefore, frequent monitoring for the early identification of CNS involvement along with the attentive assessment of the intracranial activity of both existing and forthcoming anti-HER2 drugs are of great importance.

Thirdly, most of the current studies focused on the efficacy of single anti-HER2 agents. But preclinical studies have demonstrated the additional or synergistic effects of combining ADCs with irreversible TKIs or immunotherapy. Li et al. reported that co-treatment with irreversible pan-HER inhibitors promoted receptor ubiquitination and consequent internalization of ADC complex, resulting in improved efficacy. Switching from T-DM1 to T-Dxd, which exhibited a different cytotoxic payload, could achieve durable responses after developing resistance to T-DM1 (89). Moreover, T-Dxd increased tumor-infiltrating CD8+ T cells and enhanced the expression of PD-L1 and MHC-I on tumor cells. Combining T-Dxd and PD-1 inhibitors is more effective than single-agent monotherapy in mouse models. These findings provide evidence for the rationale of combination therapy in patients with HER2-altered NSCLC (99). Therefore, a paradigm shift from monotherapies towards combination therapies will probably change the current treatment landscape for HER2-addicted NSCLC. ADC-based therapies seem to provide the highest response rates and the best survival outcomes in HER2-altered NSCLC patients by far. Several clinical trials (NCT03334617, NCT04686305) have been launched to address this issue. Also, with more agents in the pipeline are waited to be approved, the sequencing of these novel therapies is attracting more attention.

Another issue is the not-infrequent situation of concomitant mutations in HER2-altered NSCLC patients (for example, TP53), which can impair the efficacy of anti-HER2 agents and affect prognosis. Furthermore, acquired HER2 mutation and amplification are considered to be the main mechanisms of bypass pathways driving EGFR-TKIs resistance, suggesting the expansion of TKIs-induced selection of HER2-driven cell clones. Under these circumstances, concomitant treatment of EGFR-TKIs and HER2-targeted agents could become a promising therapeutic strategy. Further investigations are needed to provide the rationale for targeting HER2 in these clinical settings.

Not long ago, HER2 alterations were considered poor targets in NSCLC and were deemed to have inferior clinical outcomes than those with other genetic alterations. Nowadays, owing to advances in preclinical biology-based drug development, the wide array of the investigating HER2-targeted agents is a glimmer of hope shooting through the past prejudice. With emerging therapies requiring further clinical validation, exploration of targeting HER2 in NSCLC is underway.

AUTHOR CONTRIBUTIONS

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CASE REPORT
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Case Report: Aumolertinib as Neoadjuvant Therapy for Patients With Unresectable Stage III Non-Small Cell Lung Cancer With Activated EGFR Mutation: Case Series

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Background: There is no standard treatment for stage III lung cancer due to its low surgical resection rate, and improving PFS and survival of patients with III NSCLC has become an urgent challenge in clinical treatment. For EGFR mutation-positive patients, targeted therapy has the remarkable feature of high efficiency and low toxicity compared with first-line standard chemotherapy, and targeted neoadjuvant therapy needs to be further explored.

Method: We report 3 diagnosed cases of locally advanced unresectable NSCLC with EGFR-sensitive mutations who first received 1–2 cycles of preoperative chemotherapy neoadjuvant therapy and were treated with 110 mg daily of 3rd-generation EGFR-TKI aumolertinib instead because of poor efficacy or safety intolerance.

Result: After 2 cycles of aumolertinib treatment, all 3 patients achieved symptomatic remission and significant tumor size reduction and achieved downstaging to allow surgical treatment. No additional operative difficulties were added during the surgery. They continued to receive adjuvant therapy with the original dose of aumolertinib after surgical treatment, and no evidence of tumor recurrence was found until the most recent imaging examination. In addition, the course of neoadjuvant and adjuvant therapy was free of serious adverse effects.

Conclusion: Perioperative treatment of these three cases of locally advanced unresectable NSCLC with EGFR-sensitive mutations with the third-generation EGFR-TKI aumolertinib showed significant efficacy and excellent safety and may be a new option for targeted therapy in the perioperative period.

Keywords: aumolertinib, NSCLC, neoadjuvant, EGFR, adjuvant

INTRODUCTION

Patients with stage III non-small cell lung cancer (NSCLC) have considerable disease heterogeneity (1, 2). Treatment options include neoadjuvant therapy followed by surgical resection, adjuvant chemotherapy after surgery, or radical radiotherapy. A large meta-analysis confirmed the survival benefit of preoperative chemotherapy (3). However, the lung damage caused by preoperative chemotherapy may also make subsequent surgical resection more difficult (4, 5). No consensus exists with regard to optimal treatment approaches, particularly for unresectable advanced NSCLC characterized by single-site or multisite lymph node metastases identified by preoperative staging; the role of surgical resection after preoperative therapy remains controversial (6–8).

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) significantly prolong progression-free survival (PFS) in patients with advanced EGFR mutation-positive (EGFRm) NSCLC compared to chemotherapy as first-line therapy (9–11). Aumolertinib (HS-10296), a third-generation EGFR-TKI, has attracted much attention due to its reliable antitumor activity and favorable safety profile (12–14).

Although EGFR-TKI was established as an effective first-line therapy for advanced EGFRm NSCLC (9–11), the efficacy and tolerability of EGFR-TKI preoperative induction therapy remain uncertain. We report three cases of stage III NSCLC with confirmed EGFR mutations, who reached a descending stage after preoperative neoadjuvant treatment with aumolertinib and had successful surgical resection of the malignant tumor.

PRESENTATION

Case 1

A 64-year-old Chinese male, a 40-year smoker and hypertension, presented to a local hospital in January 2021 with an irritating cough and sputum with blood in the sputum, and a computed tomography chest (CT) scan showed a 7.8 × 4.9-cm left upper lung space. Lung puncture biopsy showed squamous carcinoma, and immunohistochemistry showed high PD-L1 expression, TPS ≥ 90%, EGFR mutation (exon 19 deletion) by NGS, and clinical stage IIIC (T3N3M0). PET/CT showed left lung cancer with obstructive pneumonia, enlarged lymph nodes in the left clavicular region, posterior to the anterior vena cava, aortic window, left paratracheal area, and both hila, and increased FDG metabolism. Magnetic resonance imaging (MRI) of the brain showed no evidence of central nervous system (CNS) involvement, which made her malignancy compatible. **Figure 1** shows the imaging findings of case 1.

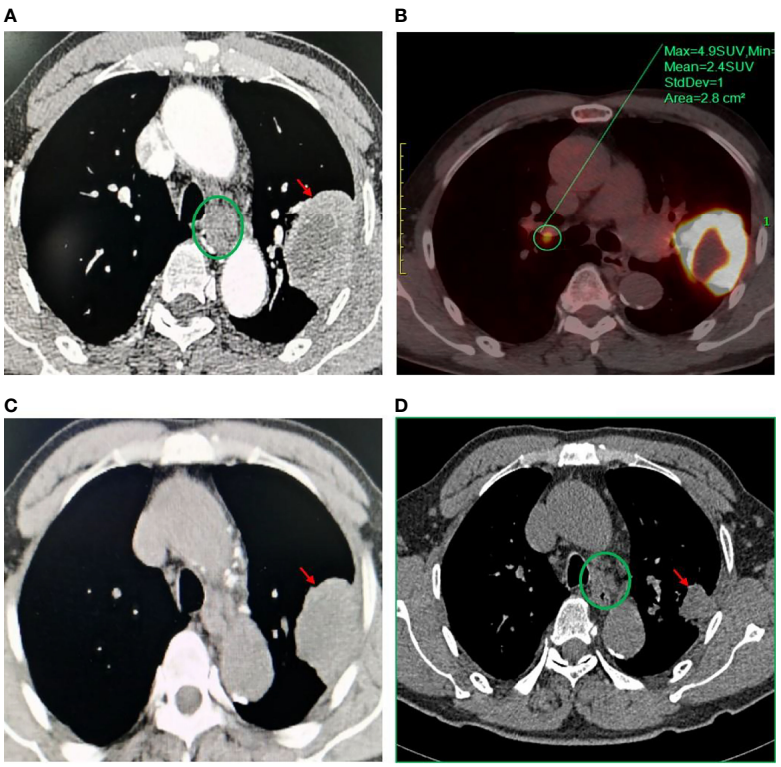

FIGURE 1 | Disease status before and after neoadjuvant chemotherapy and aumolertinib treatment. Computed tomography chest (CT) scan showed **(A)** pulmonary nodule in the left upper lobe of the lung, size 7.8 × 4.9 cm before chemotherapy. PET/CT showed **(B)** lymph node enlargement in the right hilar and pulmonary nodule in the left upper lobe before chemotherapy. **(C)** Stable disease (SD) based on imaging findings after 2 cycles of chemotherapy. **(D)** Pulmonary nodule in the left upper lobe of the lung, size 2.2 × 2.0× 1.8 cm after almost 2 months of aumolertinib treatment, with tumor remission reaching partial remission (PR).

Systemic chemotherapy was started in January 2021 with an initial dose of albumin-bound paclitaxel 400 mg and carboplatin 0.4 mg. Rash and pruritus developed during the second chemotherapy infusion of carboplatin, so treatment with carboplatin was discontinued. After 2 cycles of chemotherapy, objective tumor remission was evaluated as disease stabilization (SD) by RECIST 1.1 (efficacy evaluation criteria for solid tumors). After nearly 8 weeks of oral administration of the third-generation EGFR-TKI aumolertinib (110 mg/day) started in February 2021, imaging showed significant tumor regression, lesion volume reduction to 2.2 × 2.0 × 1.8 cm, partial remission (PR), and clinical stage reduction to stage IB (T2N0M0). After evaluation by the surgeon, the patient was eligible for surgery and underwent VATS left upper lung lobectomy + lymph node dissection with the patient's consent in April 2021. Postoperative pathology showed interstitial fibrous lung tissue with numerous lymphocytic infiltrates, with large necrotic tissue and <10% residual tumor cells (major pathological response, MPR), which was considered as posttreatment changes of squamous carcinoma. There was no metastasis of cancer tissue in any of the lymph nodes examined. After discussion with the patient, he was willing to receive adjuvant therapy with aumolertinib, and the disease was judged to be stable by imaging results in February 2022.

Case 2

A 68-year-old Chinese male with grade 3 hypertension was admitted to the hospital in December 2020 with a 1-week history of pulmonary occupancy found on physical examination. Chest CT revealed a left lingual lobe occupancy with obstructive pneumonia and enlarged mediastinal and left axillary lymph nodes; the size of the tumor was 4.0 × 3.8 × 2.3 cm, and cranial MRI, abdominal ultrasound, and bone scan was unremarkable. Lung puncture biopsy was diagnosed as adenocarcinoma, and immunohistochemistry showed a negative PD-L1 expression with TMB: 6.38 mut/bp; NGS revealed that the patient had EGFR exon 21 L858R point mutation, combined with HEBB2 amplification, EGFR amplification, and TP53 mutation; and the clinical stage was IIIA (T2N2M0). The patient's enlarged lymph node in the left hilum was completely fused with the left pulmonary artery trunk and could not be completely removed surgically, which was an unresectable stage III lung cancer. After 2 cycles of pemetrexed combined with carboplatin chemotherapy in December 2020, the tumor did not shrink significantly according to chest CT. Considering that the patient had EGFR 21 exon L858R mutation, she was treated with oral aumolertinib 110 mg/day in January 2021, and after nearly 2 months of treatment, chest CT indicated significant regression of the left hilar lymph nodes, and the mass volume was reduced to 1.2 × 1.0 × 1.5 cm, achieving PR; clinical stage was reduced to stage IB (T2N0M0); and the tumor was clearly demarcated from the left pulmonary artery trunk, which could be considered for surgical radical treatment. **Figure 2** shows the imaging findings of case 2.

Therefore, VATS left upper lung lobectomy and lymphatic dissection were performed on February 22, 2021. Postoperative pathology showed moderately differentiated adenocarcinoma with elastic plaque formation, tumor involvement in 0/10 lymph nodes, and residual tumor cells <10% (MPR). The postoperative pathological stage was stage IA (T1N0M0). After surgery, the patient had been receiving adjuvant therapy with aumolertinib for 11 months, and no evidence of malignancy recurrence was seen on spiral CT of the chest.

Case 3

A 60-year-old Chinese female with grade 2 hypertension was admitted with cough for 1 month. Chest CT showed a left lower

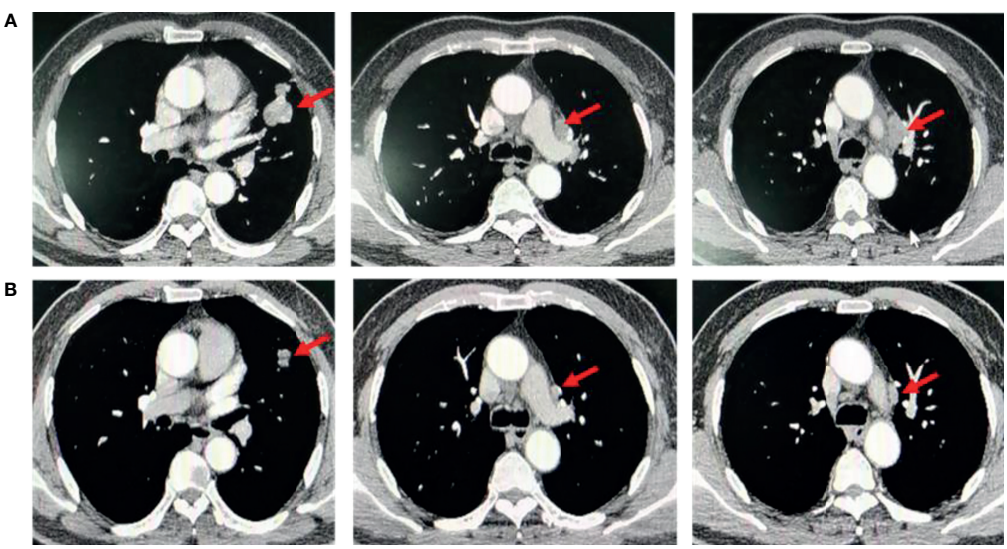

FIGURE 2 | Disease status before and after neoadjuvant almonertinib therapy. Computed tomography chest (CT) scan showed **(A)** left lingual lobe occupancy with a size of 4.0 × 3.8 × 2.3 cm, and subaortic lymph nodes before aumolertinib treatment. **(B)** Left lingual lobe occupancy with a size of 1.2 × 1 × 1.5 cm, and subaortic lymph nodes after aumolertinib treatment.

lung occupancy with pulmonary atelectasis; PET/CT showed a left lower lung soft tissue mass (10.9 × 8.9 × 6.5 cm), left lower lobe bronchial truncation, aortic window, and multiple lymph node enlargement in the left hilar, and left lung cancer with obstructive pneumonia was considered. Lung puncture biopsy and immunohistochemistry diagnosed squamous carcinoma; immunohistochemistry results showed negative PD-L1 expression, and NGS test results were EGFR L858R mutation combined with TP53 mutation, clinical stage IIIB (T4N2M0). Thoracic surgery specialists consulted and judged that there was no indication for surgery for the time being and recommended medical treatment. **Figure 3** shows the imaging findings of case 3.

The patient was treated with a chemotherapy regimen of albumin paclitaxel combined with carboplatin for two cycles in November 2020, and a chest CT review showed that the lesion was still stable, but the tumor did not shrink significantly. Therefore, the patient received oral aumolertinib (110 mg/day) for 2 cycles from January 2021, and the tumor shrank significantly from before to 3.0 × 4.0 × 4.0 cm, reaching PR and clinical stage down to stage IB (T2N0M0), which was eligible for surgery. Therefore, the patient requested surgical treatment and underwent VTAS left lower lung lobectomy, lymph node dissection, and pleural adhesion release in March 2021. Postoperative pathology showed focal fibrous tissue hyperplasia with inflammatory cell infiltration in lung tissue, focal bronchial epithelial adenoid formation, and mild alveolar epithelial cell hyperplasia; some multinucleated giant cell reaction was seen; posttreatment changes were considered; no tumor residual was seen; and the status of pathological complete response (pCR) was achieved. 0/10 lymph nodes had tumor involvement, and the postoperative pathological stage was stage 0 (T0N0M0). After surgery, the patient was treated with aumolertinib for adjuvant therapy, and the disease was judged to be stable on the chest CT findings in January 2022.

DISCUSSION

Three patients with unresectable stage III non-small-cell lung cancer were first treated with chemotherapy and were switched to aumolertinib for 2 cycles due to poor efficacy, and all achieved downstaging and met resectability criteria after neoadjuvant aumolertinib treatment. Postoperative pathology showed MPR in the first two cases and pCR in the last case. In addition, the safety profile of aumolertinib in neoadjuvant attempts was satisfactory, with no events that delayed surgery or increased surgical complications. Patient characteristics and treatment response before and after surgery are shown in **Table 1**.

In this case series report, the first patient who received chemotherapy had a more severe rash and pruritus, which the physician judged to be a hypersensitivity reaction caused by carboplatin, which has been shown to predispose patients treated with carboplatin in combination with paclitaxel (15). Symptomatic treatment was replaced with oral aumolertinib

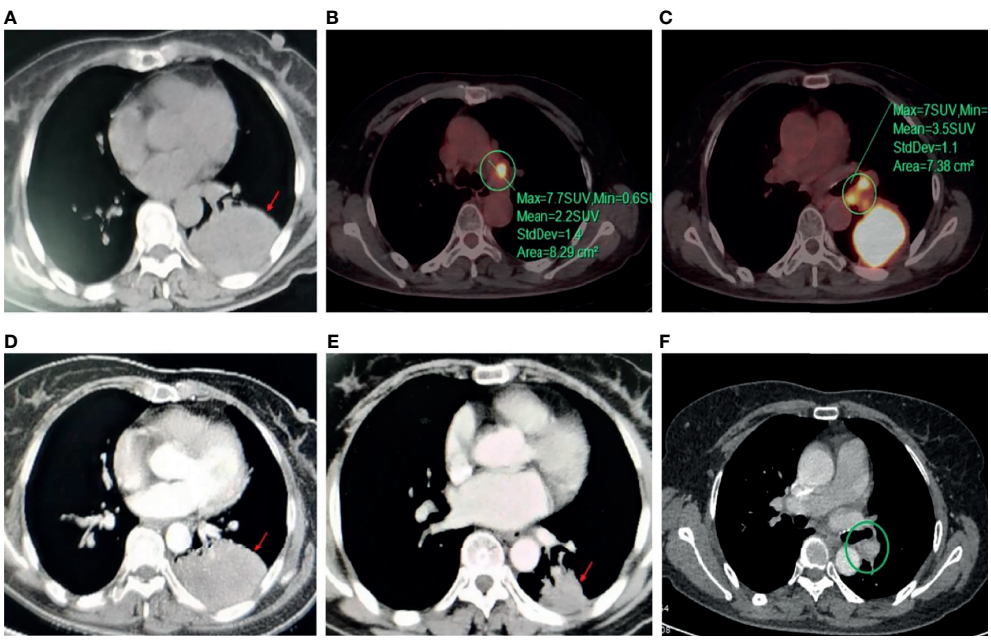

FIGURE 3 | Disease status before and after neoadjuvant chemotherapy and aumolertinib treatment. Computed tomography chest (CT) scan showed that **(A)** before chemotherapy, the size of the left lower lung soft tissue mass was 10.9 × 8.9 × 6.5 cm. PET/CT showed **(B)** mediastinal lymph node enlargement before chemotherapy. **(C)** Multiple lymph node enlargement in the left hilar before chemotherapy. CT scan showed that **(D)** after 2 cycles of chemotherapy, the disease was judged to be stable based on imaging findings. **(E)** After 2 cycles of aumolertinib treatment, the size of the left lower lung soft tissue mass was 3.0 × 4.0 × 4.0 cm, and the tumor achieved partial remission. **(F)** After 2 cycles of aumolertinib treatment, multiple lymph nodes disappeared in the left hilar.

TABLE 1 | Patient characteristics and response to treatment before and after surgery (N = 3).

Patient	Gender/ age	Histological types	EGFR mutation subtypes	PD-LI expression status	First-line treatment		Second-line treatment		Pathologicalassessment
					Treatment	Response	Treatment	Response	
1	M/64	Squamous carcinoma	exon19 deletion	Positive	Chemotherapy	SD	Aumolertinib	PR	MPR
2	M/68	Adenocarcinoma	L858R mutation	Negative	Chemotherapy	SD	Aumolertinib	PR	MPR
3	F/60	Squamous carcinoma	L858R mutation	Negative	Chemotherapy	SD	Aumolertinib	PR	pCR

SD, stable disease; PR, partial response; MPR, major pathological response; pCR, pathological complete response.

and continued for 8 weeks, with imaging showing partial remission and no serious adverse events.

The second and third cases also received 2 cycles of preoperative chemotherapy without significant tumor remission. After chemotherapy and switching to oral aumolertinib treatment for 4–8 weeks with the patient's consent, imaging showed that both patients responded well to treatment, with significant tumor volume reduction to a descending stage that allowed them to undergo surgery.

This may indicate an efficacy advantage of aumolertinib in the neoadjuvant phase of therapy. Similarly, results from the EMERGING-CTONG 1103 study showed a trend toward improved ORR, lymph node step-down, MPR, and R0 resection rates with neoadjuvant erlotinib compared to neoadjuvant chemotherapy for patients with EGFRm NSCLC, and significantly prolonged PFS (16). Evidence from case reports and small non-randomized clinical trials reported suggests that neoadjuvant EGFR-TKI therapy is potentially efficacious in patients with resectable NSCLC (17–21). This suggests that neoadjuvant-targeted therapy modalities are potentially clinically applicable and may be a viable option for patients who are not optimal chemotherapy candidates due to medical comorbidities or who refuse chemotherapy.

Finally, overall oncologic outcomes may be influenced by the timing of surgical intervention after neoadjuvant therapy. A large retrospective study found that in patients with stage IIIa NSCLC, 1- and 3-year survival rates were significantly lower in the short-delayed group after preoperative neoadjuvant therapy compared with the long-delayed group (22). Due to the significant safety advantage of targeted agents over chemotherapy, preoperative EGFR-TKI neoadjuvant therapy may reduce lung injury and make surgery less difficult. Therefore, it may shorten the time between the end of neoadjuvant therapy and surgery, thus affecting prognostic survival. Currently, the optimal duration of preoperative induction-targeted therapy is unclear, and whether 6 weeks of EGFR-TKI induction therapy is sufficient has yet to be verified, so there may be a problem of insufficient induction-targeted therapy.

Aumolertinib has demonstrated excellent tumor remission and a favorable safety profile in the neoadjuvant and adjuvant phases of these three phase III NSCLC cases, which has implications in guiding targeted therapy in the perioperative setting of NSCLC. However, its ability to be a firm choice for neoadjuvant and adjuvant treatment of NSCLC still needs to be validated by large randomized controlled studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the research ethics committee of the Affiliated Brain Hospital of Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed toward data analysis, drafting, and critically revising of the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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REVIEW
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BRAF-Mutated Non-Small Cell Lung Cancer: Current Treatment Status and Future Perspective

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V-Raf murine sarcoma viral oncogene homolog B (*BRAF*) kinase, which was encoded by *BRAF* gene, plays critical roles in cell signaling, growth, and survival. Mutations in *BRAF* gene will lead to cancer development and progression. In non-small cell lung cancer (NSCLC), *BRAF* mutations commonly occur in never-smokers, women, and aggressive histological types and accounts for 1%–2% of adenocarcinoma. Traditional chemotherapy presents limited efficacy in *BRAF*-mutated NSCLC patients. However, the advent of targeted therapy and immune checkpoint inhibitors (ICIs) have greatly altered the treatment pattern of NSCLC. However, ICI monotherapy presents limited activity in *BRAF*-mutated patients. Hence, the current standard treatment of choice for advanced NSCLC with *BRAF* mutations are *BRAF*-targeted therapy. However, intrinsic or extrinsic mechanisms of resistance to *BRAF*-directed tyrosine kinase inhibitors (TKIs) can emerge in patients. Hence, there are still some problems facing us regarding *BRAF*-mutated NSCLC. In this review, we summarized the *BRAF* mutation types, the diagnostic challenges that *BRAF* mutations present, the strategies to treatment for *BRAF*-mutated NSCLC, and resistance mechanisms of *BRAF*-targeted therapy.

Keywords: BRAF, NSCLC, targeted therapy, immune checkpoint inhibitors, tyrosine kinase inhibitors

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INTRODUCTION

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer mortality in China (1). Lung cancer could be divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC); of these, approximately 60% of NSCLC were adenocarcinoma (2). Generally, about 80% of lung adenocarcinoma harbors driver mutations in east Asians (3). Over the past decades, targeted therapies have dramatically revolutionized the treatment pattern of NSCLC and greatly improved the prognosis of NSCLC patients.

V-Raf murine sarcoma viral oncogene homolog B (*BRAF*) gene encodes *BRAF* kinase, a member of mammalian cytosolic serine/threonine kinases, which plays important roles in cell signaling, growth, and survival (4–6). *BRAF* mutations are rare mutations in NSCLC, which account for 2% of lung adenocarcinoma, and more frequently occur in never-smokers, women, and aggressive histological types (micropapillary) (7). Additionally, *BRAF* V600E mutations are mostly mutually exclusive with most druggable abnormalities present in this tumor (8, 9). It should be noted that certain *BRAF* mutations can coexist with *KRAS* mutations (9). However, routine platinum-based chemotherapy presents lower efficacy and is associated with poorer survival (10). Currently, the advent of BRAF inhibitors (BRAFi) and immune checkpoint inhibitors (ICIs) has transformed the

landscape of *BRAF*-mutated NSCLC. In the present review, we will discuss *BRAF* biology within the context of oncogenesis. In addition, we will describe the evolving science of molecularly targeted therapies and ICIs for *BRAF*-dependent cancers.

BRAF MUTATIONS IN CANCER

BRAF is involved in mitogen-activated protein kinase (MAPK) pathway that includes the rat sarcoma (RAS)–rapidly accelerated fibrosarcoma (RAF)–mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase. After activation of epithelial growth factor receptor (EGFR), RAS–RAF–MEK–ERK pathway will be activated and modulate cell proliferation and survival (11) (**Figure 1**). In normal tissue, the *BRAF* kinase is generally silenced *via* negative feedback once the signal has moved on to the next point in the cascade. However, when *BRAF* mutations occur, the activation of the RAS–RAF–MEK–ERK pathway will be sustained and will lead to uncontrolled cell growth and proliferation; this makes *BRAF* mutations potential oncogenic drivers (12, 13). Generally, *BRAF* mutations commonly present in human cancers with an 8% incidence in all human cancers, predominantly in hairy cell leukemia (100%) (14), melanoma tumors (40%–50%) (15–17), thyroid carcinoma (10%–70%, based on the histologic classification) (18, 19), colorectal cancer (10%) (20, 21), and rarely in lung cancer (1%–2%) (11, 22).

BRAF mutations could be divided into three classes based on mutation site. Class I mutants including V600E/K/D/R, which occurs in the valine residue at amino acid position 600 of exon 15, promote constitutive activation of MAPK pathway, causing strong activation of *BRAF* kinase; in addition, this type of

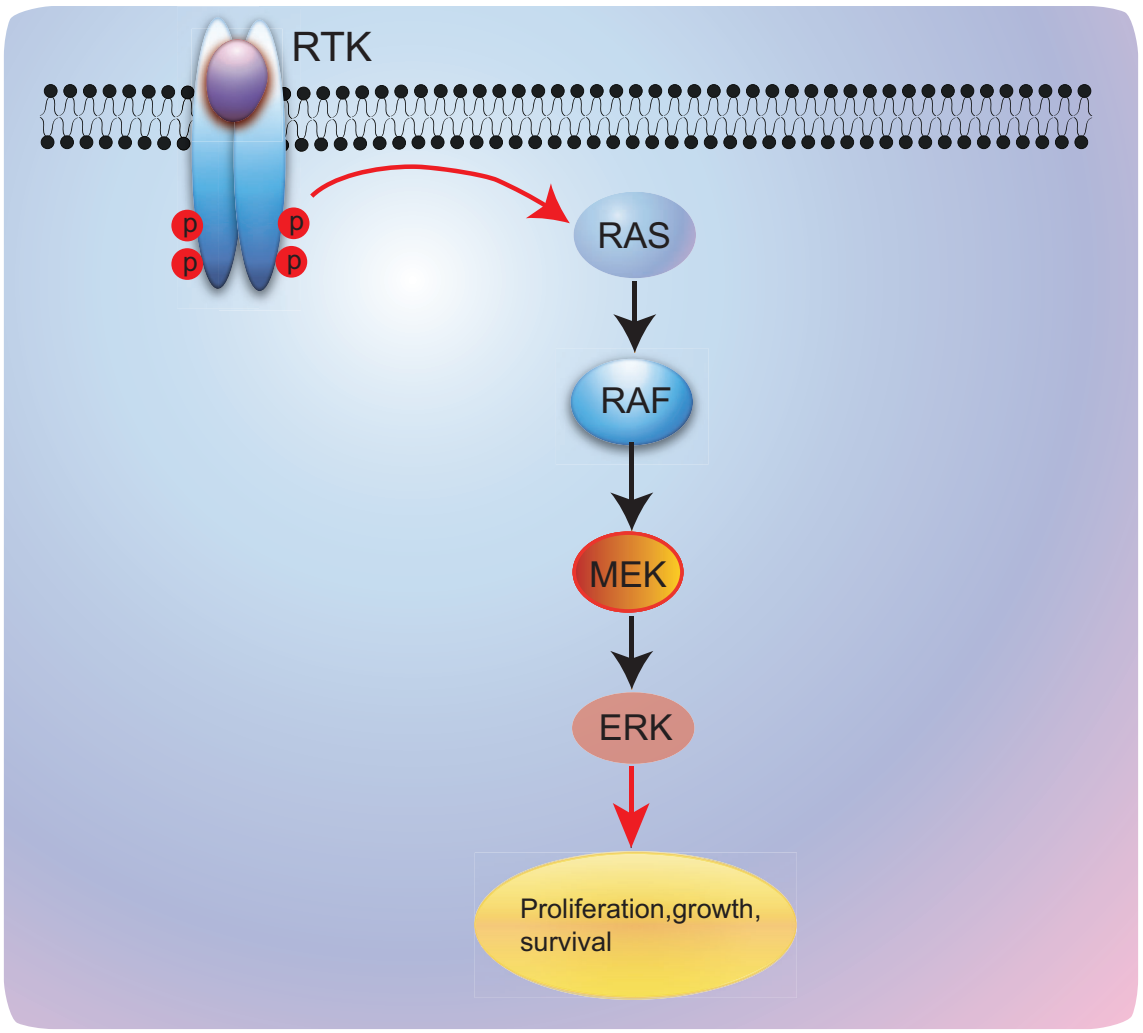

FIGURE 1 | RAS/RAF/MEK/ERK signaling pathway. RTK, receptor tyrosine kinase; RAS, rat sarcoma; RAF, v-raf murine sarcoma viral oncogene; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase.

mutations often presents high sensitivity to *BRAF* and MEK inhibitors (23, 24). Class II mutants, including K601, L597, G464, and G469 mutations, are located in the activation segment or P-loop and signal as RAS-independent dimers (24, 25).

Class III mutants that occur in the P-loop, catalytic loop, or DFG motif have impaired BRAF kinase activity; however, the activity of MAPK pathway signaling is enhanced *via* Raf-1 proto-oncogene CRAF activation (**Figure 2**) (24). All the class II and III mutations are non-V600 mutations, and *BRAF* mutations are usually classified as V600 mutations and non-V600 mutations in routine clinical practice. Actually, approximately 50% of *BRAF* mutations in NSCLC are non-V600 mutations (26–28). In addition, class II and III BRAF mutations are sensitive to current BRAF inhibitors; hence, novel-generation BRAF inhibitors warrant being developed.

BRAF DELETION MUTATIONS

Several previous studies have demonstrated that *BRAF* deletion mutations can occur in melanoma, pancreatic cancer, and thyroid cancer; in addition, activating *BRAF* deletion mutations might serve as a type of resistance mechanism to *BRAF* inhibitors plus MEK inhibitors (29–31). Generally, deletion mutations happen adjacent to the αC helix in the kinase domain of *BRAF*, resulting in enhanced kinase activity by suppressing the αC helix in its active conformation (29). This type of *BRAF* mutation is similar to class I mutants functioning as RAS-independent monomers (31).

BRAF FUSIONS

At least 18 different 5′ fusion partners have been found across different cancer types including NSCLC, and the most common fusion partner is AGK in NSCLC (13, 32). The occurrence rate of *BRAF* fusions is smaller than 1% in NSCLC, and all NSCLCs with *BRAF* fusions were adenocarcinomas or NSCLC with adenocarcinoma features. Most *BRAF* fusion patterns are in-frame with breakpoints on the *BRAF* kinase domain (13, 32). In addition, remarkably, conserved fusions have been reported to occur in 85% of astrocytic pilocytomas (33). Activating *BRAF* fusions occur in truncation of the N-terminal CR1 auto-inhibitory domain, leading to the constitutive activation of *BRAF* pathway that resembles class II BRAF mutants (34). Up to now, limited data have revealed the activities of *BRAF* inhibitors and MEK inhibitors in treating *BRAF* fusion mutations.

DETECTION OF BRAF MUTATIONS

Single-gene assays for *BRAF* mutations are extensively used across other cancer types including melanoma. The most commonly used assay is RT-PCR. So far, the cobas 4800 *BRAF* V600 Mutation Test and THxID-*BRAF* kit are Food and Drug Administration (FDA)-approved companion diagnostic tests (35–37). In addition, laboratory-developed tests also could be applied to test a patient's *BRAF* mutation status, although confirmatory tests *via* other methods are necessary. The major advantages of RT-PCR are faster turnaround time, better reproducibility, higher specificity and sensitivity, and lower cost compared with multiple gene sequencing methods. However, most of these methods are merely for *BRAF* V600E mutation located in exon 15. They lack the ability to detect exon 11 mutations that also are seen in NSCLC (38). Hence, next-generation sequencing (NGS) including a multiple gene panel should be applied to evaluate V600E mutation and non-V600E mutations that could happen in exon 11 and exon 15 (15, 26).

The other kind of single-gene test is immunohistochemistry (IHC) for *BRAF* mutations. However, the only available antibody used in IHC for mutant *BRAF* protein is monoclonal antibody VE1. The advantage of this method is to identify a qualitative change (i.e., the presence or absence of the protein), but the accuracy is limited in quantitating changes in expression than other antibody-based assays, such as the enzyme-linked immunosorbent assay (39). The limitation of this test is similar to RT-PCR that only can test *BRAF*-V600E mutation. In addition, only a few cases of lung cancer have shown that VE1 clone has the potential to stain between 90% and 100% of p.V600E-mutant adenocarcinomas (40). It was previously reported that IHC using VE1 antibody is incapable of testing non-V600E mutation (41). However, another study has demonstrated that 599T insertion mutation in 1/21 cases stained with VE1 is positive for VE1 antibody. Hence, no standard recommendation or consensus was obtained for using *BRAF* p.V600E IHC (VE1) testing in NSCLC; extension validation must be deployed when IHC is used to test *BRAF*-V600E mutation.

NEXT-GENERATION SEQUENCING

As mentioned above, single-gene tests for *BRAF* mutation are unable to identify mutations occurring in exon 11; hence, a multiple-gene panel including *BRAF* mutations is more practical. In addition, with more novel rare driver genes discovered, there is an increased need for multigene testing compared to single-gene

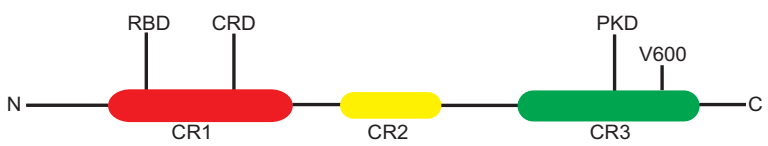

FIGURE 2 | The structure of BRAF gene. N, C, amino and carboxyl end; RBD, Ras-binding domain; CR, conserved region; CRD, cysteine-rich domain; PKD, protein kinase domain; CR1/2/3; conserved region-1/2/3, CR1 contains RBD and CRD, V600E mutation occurs in CR3.

approaches. Current guidelines for gene testing in NSCLC should include *BRAF*, *mesenchymal epithelial transition factor receptor (MET)*, *rearranged during transfection (RET)*, *Human Epidermal Growth Factor Receptor 2 (HER2)*, *neurotrophic tropomyosin receptor kinase (NTRK)*, and *Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS)* for cases in which the common oncogenic drivers (*EGFR*, *anaplastic lymphoma kinase (ALK)*, and *ROS proto-oncogene 1 (ROS1)* are negative and whenever an adequate technique is available (42). The advantages of NGS are as follows: 1) fewer tumor tissue; 2) facilitates testing of multiple biomarkers; 3) includes emerging biomarkers for clinical trial enrollment. Generally, it is more economical than sequential testing (43, 44). However, because of more data, interpreting the NGS reports becomes complex and its availability in the community or rural region is poor. Besides, the turnaround time of NGS is longer than those of RT-PCR and IHC assay. Hence, multiple-gene RT-PCR kit might be a more reasonable choice for gene tests.

CURRENT TREATMENT LANDSCAPE

Chemotherapy

The activities of chemotherapy have been fully explored in patients with *BRAF* V600E mutation advanced NSCLC. Documented studies have revealed that advanced NSCLC patients harboring *BRAF* V600E mutations present poor prognosis when administered with chemotherapy; in addition, patients with *BRAF* V600E mutations appear to be insensitive to platinum-based chemotherapy (45–47). However, several reports showed that NSCLC patients harboring *BRAF* V600E mutations seemed to have extended survival compared with patients without oncogenic drivers (47, 48). Additionally, Claire Tissot et al. (49) have reported that patients' survival is not connected with BRAF mutation status. Another French study also observed similar results that *BRAF* mutation was not prognostic of overall survival (50). In addition, a recent study suggested that class I *BRAF* V600E mutations have the potential to be less aggressive than class II and III non-V600E mutations, which present more possibilities to occur in brain metastases and RAS co-alterations; hence, this specific behavior made non-V600E patients have shorter progression free survival (PFS) and overall survival (OS) to chemotherapy, although the difference might be driven by fewer extrathoracic metastases and higher use of targeted therapies in class I patients (51). However, because of limited cases included in these studies, the results presented here should be interpreted with caution. Hence, future larger randomized trials are urgently warranted.

Immune Checkpoint Inhibitor Monotherapy

Previous retrospective small-sample studies have found that *BRAF*-mutated NSCLC patients tend to display positive programmed cell death ligand 1 (PD-L1) expression (52–56); however, because of limited cases, no clear correlation between PD-L1 and BRAF mutations were found. Recently, a study including 29 NSCLC patients harboring *BRAF* mutations showed us that approximately 69% (20/29) of patients were PD-L1 positive; among them, over 40% (13/29) of patients presented higher PD-L1 expression (PD-L1 ≥50%). In addition, *BRAF*-mutated NSCLC patients were correlated with low/intermediate tumor mutation burden (TMB) and microsatellite-stable status (57). In this study, researchers have reported that patients harboring *BRAF* mutations displayed limited response to ICIs. Additionally, several retrospective studies also observed a similar phenomenon. The objective response rate (ORR) to single anti–PD-(L)1 agent in BRAF-mutant patients is about 10%–30%, with a median PFS of 2–4 months, which is equal to that of a second-line ICI monotherapy in wild-type NSCLC (57–61) (**Table 1**). Combining these data, we can conclude that ORR and PFS of patients with BRAF non-V600E are higher than those in patients harboring BRAF V600E mutations, but OS results seem paradoxical, potential exploration might be that BRAF V600E mutations could benefit from targeted therapy. On the other hand, non-V600E mutations usually happen in smokers, and smoking status was found to be related to response to immunotherapy (62). In summary, these data indicated limited efficacy of ICIs in BRAF-mutant NSCLC. Recently, a case with BRAF V600E mutation presented durable response to ICI combined chemotherapy with PFS of 20 months (63). This is the first evidence of patients with BRAF V600E alteration treated with ICI combination regimens. This case provided evidence that the ICI combined regimens might be a promising choice for BRAF V600E-mutated NSCLC. Further prospective clinical trials are eagerly needed.

Targeted Therapy

Sorafenib, an early-generation BRAF inhibitor, was developed as a targeted therapy against BRAF mutant kinase. Sorafenib is an oral multikinase inhibitor that displays activities to target B/C-RAF,

TABLE 1 | ICI monotherapy for BRAF-mutated NSCLC.

Trial	Mutation type	Numbers	objective response rate (ORR)	progression free survival (PFS)	overall survival (OS)
Immunotarget	V600E	17	NA	1.8	8.2
	Non-V600E	18	NA	4.1	17.2
Memorial Sloan Kettering Cancer center	V600E	10	10	1.4	26
(MSKCC)	Non-V600E	36	22	3.2	24
Isarel lung cancer group (ICLG)	V600E	12	25	3.7	NA
	Non-V600E	10	33	4.1	
Expanded Access Program (EAP) Nivolumab	BRAF	11	9	NA	10.3
French Lung Cancer Group (GFPC) 01-2018	V600E	26	26	5.3	22.5
	Non-V600E	18	35	4.9	12

Vascular Endothelial Growth Factor Receptor (VEGFR2/3), platelet-derived growth factor receptor (PDGFR-β), and c-Kit (64, 65). Preclinical models suggested that sorafenib could suppress various cancer cell proliferation and tumor growth *via* inhibiting MEK and ERK phosphorylation (64). These studies provide a theoretical basis for sorafenib as a BRAF inhibitor. However, a previous study showed that the antitumor activities of sorafenib are correlated with *EGFR* mutation status but not *K-ras* mutation status (67). Carter et al. (68) have demonstrated that concurrent administration of sorafenib with chemotherapeutics could effectively delay tumor growth without increasing toxicity. These data promoted some researchers who have designed clinical trials to testify the value of sorafenib in NSCLC; however, these trials have not tested the patients' *BRAF* mutation status (69, 70). Hence, whether sorafenib could serve as a *BRAF* inhibitor remains to be explored.

Dabrafenib and vemurafenib, novel-generation *BRAF* inhibitors, are ATP-competitive inhibitors of *BRAF* kinase. Both agents are specific in targeting *BRAF* V600E mutations. Vemurafenib was initially tested in a "basket" study including multiple non-melanoma cancers with *BRAF* V600 mutants. In the NSCLC cohort, 20 pretreated NSCLC patients were included and achieved a 42% ORR and 7.3 months of PFS (**Table 2**) (71). Gautschi et al. (72) also found that vemurafenib showed promising antitumor activities in *BRAF* V600-mutated NSCLC patients. Additionally, a recent research revealed that vemurafenib was specifically targeting *BRAF*-V600 mutants but was ineffective in patients with *BRAF* non-V600 mutants (73). Combining these data, current lung cancer guidelines recommended that vemurafenib could serve as an optional regimen in certain circumstances. A prospective trial showed that dabrafenib had clinical activity in *BRAF* V600-mutant NSCLC, and dabrafenib might act as a promising treatment choice for patients harboring *BRAF* V600E-mutant NSCLC, which lacks effective treatment options (74). In addition, a recent study has reported that BGB-283, a novel inhibitor of key RAF family kinases, showed promising antitumor activity with acceptable toxicity in patients with *BRAF* V600-mutated solid tumors including NSCLC (75). However, the activity of single *BRAF* inhibitors is limited; hence, researchers began to explore combination therapy. Several studies are ongoing to investigate the novel *BRAF* inhibitors in *BRAF*-mutated NSCLC patients.

Dabrafenib plus trametinib, a type of MEK inhibitor, was the first explored combination regimen focusing on BRAF pathway inhibition. A previous phase 2, multicohort, multicenter, non-randomized, open-label study included 36 patients harboring *BRAF* V600E mutant who were treated with first-line dabrafenib plus trametinib. The ORR was 64% and PFS was 14.6 months, as assessed by an independent review committee; in addition, an OS of 24.6 months was achieved (76, 77). This study indicated that dual blockade of the BRAF pathway with BRAF inhibitors and MEK inhibitors could produce a much stronger efficacy. Besides, dabrafenib plus trametinib combination as second-line or later setting was also evaluated (76, 77). Surprisingly, dual blockade of the BRAF pathway achieved similar results compared to that in first-line setting, with 63.2% ORR and almost 10 months of PFS. This study further confirmed the survival advantage of dabrafenib plus trametinib combination compared to single agents. Furthermore, LXH254, a novel BRAF/CRAF inhibitor, plus LTT462, an ERK1/2 inhibitor, was explored to evaluate its activity in patients with advanced/metastatic *K-ras*- or *BRAF*-mutant NSCLC in a phase Ib dose escalation study; preliminary analysis showed signs of efficacy in patients with *BRAF*-mutant NSCLC (78). Dose expansion is ongoing, and further efficacy analysis remains to be seen.

Immune Checkpoint Inhibitor Combined Therapy

ICIs have transformed the treatment pattern of advanced NSCLC without oncogenic driver mutations. However, the activity of ICIs in NSCLC with oncogenic driver mutations remains limited. Recently, Lu et al. reported a case diagnosed with stage IV NSCLC with *BRAF* V600E mutation that achieved a longer response after being treated with atezolizumab plus chemotherapy (63). This study suggested that ICI combined therapy might be a promising regimen for NSCLC with *BRAF* V600E mutations. In addition, preclinical data revealed that selumetinib and trametinib could improve T-cell activation and increase CTLA-4 expression. Besides, anti-Cytotoxic T lymphocyte associate protein-4 (CTLA-4) antibody plus selumetinib and trametinib presented a survival benefit in mice bearing tumors with *K-ras* mutation (79, 80). Based on the preclinical data, Hellmann et al. (81) have designed a study to

TABLE 2 | Targeted therapy for BRAF-mutated NSCLC.

Trial	Treatment lines	Agents	ORR	PFS	OS
NCT01524978	≥2	Vemurafenib	42%	7.3	NA
EURAF Cohort	≥2	Vemurafenib, dabrafenib, or sorafenib	53%	5.0	10.8
AcSé	≥2	Vemurafenib	44.9%	5.2	10
NCT01336634	≥2	Dabrafenib	33%	5.5	NA
NCT02610361	≥2	BGB-283	20%	NA	NA
NCT01336634	≥2	Dabrafenib+Trametinib	63%	10.2	18.2
NCT01336634	1	Dabrafenib+Trametinib	64%	10.9	24.6
NCT02974725	≥2	LXH254+LTT462	66.7%	NA	NA

TABLE 3 | Several ongoing trials of ICIs combined with targeted therapy.

Trial	Phase	Treatment lines	Experimental arm	Enrolled population	Status
NCT03600701	II	≥2	Atezolizumab +combimetinib	Metastatic, Recurrent, or Refractory non-small cell lung cancer	Recruiting
NCT03299088	Ib	≥2	Pembrolizumab +trametinib	Stage IV non-small cell lung cancer with *K-ras* gene mutations	Active
NCT03225664	Ib/II	≥2	Pembrolizumab +trametinib	Recurrent non-small cell lung cancer	Active

investigate the safety and clinical activity of combining a MEK inhibitor, cobimetinib, and a PD-L1 inhibitor, atezolizumab, in patients with solid tumors (n = 152). Among them, 28 NSCLC patients were recruited. For NSCLC patients, the median OS was 13.2 months, and the ORR was 18% (81). Additionally, another phase I/II trial was designed to evaluate the safety and efficacy of durvalumab plus tremelimumab with continuous or intermittent administration of selumetinib in advanced NSCLC patients (82) (**Table 3**). Up to now, clinical trials in melanoma have demonstrated the activities of ICI plus BRAF-targeted therapy; notably, the safety profile of this combination regimen warranted more attention. In addition, for NSCLC, data about ICI-combined *BRAF*-targeted therapies remained limited. The safety and clinical efficacy of this pattern warrant further investigation.

Mechanisms of Resistance to BRAF Tyrosine Kinase Inhibitors

Exactly as other targeted therapies in NSCLC, resistance to BRAF pathway inhibitors would inevitably occur, leading to disease progression. However, information about resistance mechanisms of BRAF pathway inhibitors is poorly defined.

Currently, bypass activation is the main cause of secondary resistance of targeted therapy. However, there is limited report thus far that has revealed the resistance mechanisms of BRAF

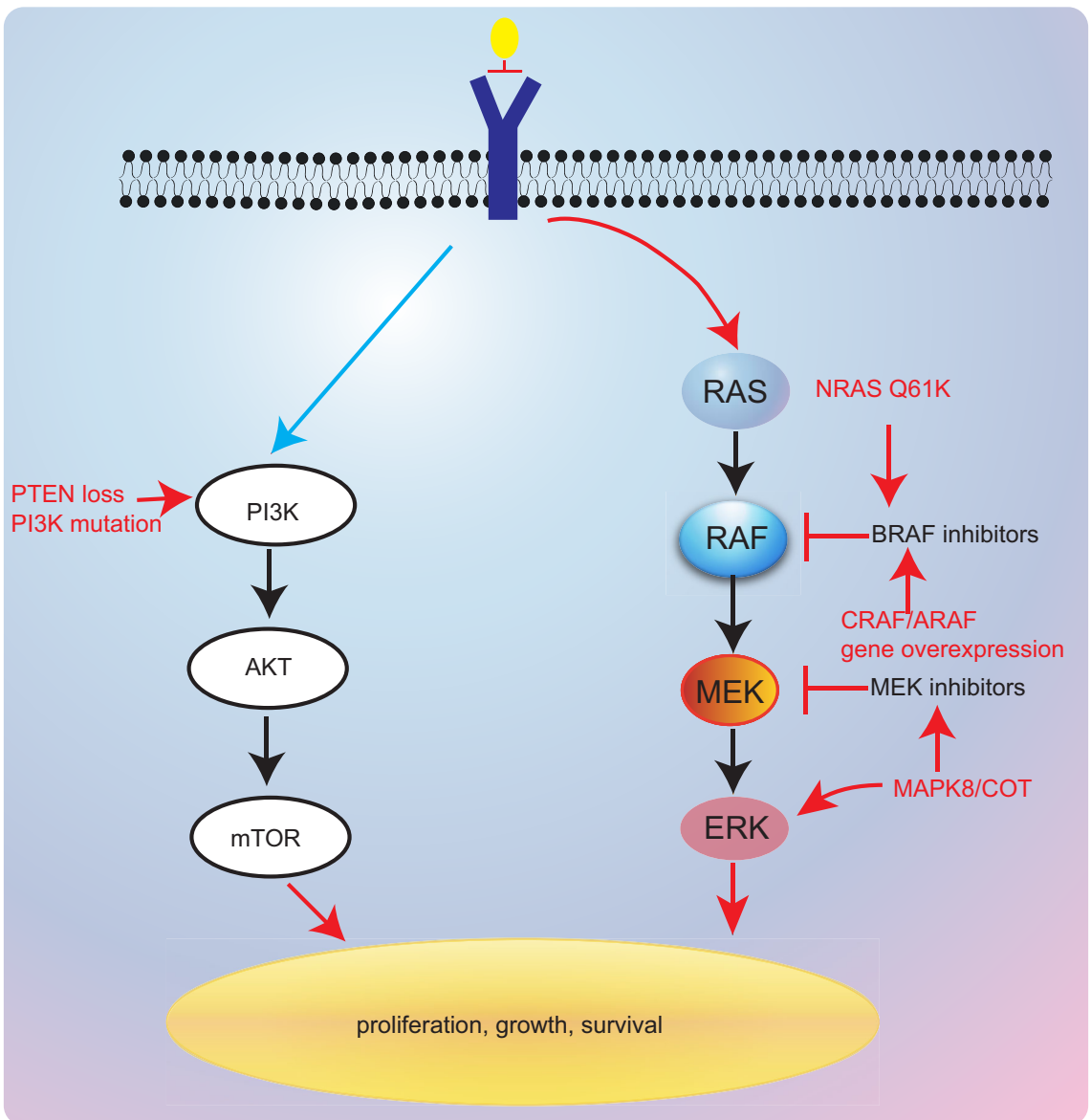

FIGURE 3 | Resistance mechanisms of targeted therapies.

inhibitors in *BRAF* V600E NSCLC. In melanoma, other isoforms of RAF proteins (CRAF and A-Raf proto-oncogene (ARAF)) could also activate the MAPK pathway when *BRAF* was inhibited, which leads to resistance to BRAF pathway inhibitors (83). Several studies have also demonstrated that MAPK pathway stimulation by MAP3K8 or COT is associated with BRAF inhibitor resistance (83, 84) (**Figure 3**). However, the combination of BRAF inhibitors and MEK inhibitors could effectively reverse the resistance to monotherapy in *BRAF*-mutant NSCLC.

Additionally, Rudin et al. (85) have reported that acquired *K-ras* G12D mutation might be contributing to secondary resistance to dabrafenib. Coincidentally, *K-ras* G12V was also considered as mediating resistance to BRAF inhibitors (86). Besides, in a previous case report, researchers presented a case that was treated with dabrafenib and trametinib that developed *N-ras* Q61K mutation (87). These reports revealed that *RAS* gene might a critical gene modulator in resistance mechanisms to BRAF/MEK inhibitors. The last European Society For Medical Oncology (ESMO) congress reported a novel combination of LXH254 and LTT462 that might overcome RAS-related resistance to BRAF/MEK inhibitors (78). This regimen has shown antitumor activity in *BRAF*-mutant and *K-ras*-mutated patients. However, further investigation remains warranted.

Inactivation of phosphatase and tensin homolog (PTEN), a tumor suppressor, was also found to be involved in resistance to BRAF inhibitors in melanoma (88–90). A previous study has suggested that shorter PFS to anti-BRAF drugs was found in *PTEN*-deficient patients, further supporting the role of *PTEN* in resistance to BRAF inhibitors (91). Notably, *PTEN* lack-of-function alterations may be resistant to dabrafenib–trametinib combinations, the current standard of care, which lacks effective resolutions to this resistance.

CONCLUSIONS

Targeted therapy in driver gene-positive NSCLC has obtained significant progress and greatly revolutionized the landscape of NSCLC. However, the current treatment choice for *BRAF*-mutated NSCLC patients is not satisfactory because of lower incidence. Current guidelines recommend dabrafenib plus tremetinib as the only one standard targeted therapy option for BRAF-mutated NSCLC. However, the underlying resistance mechanisms of this combination regimen have not been clearly defined; in addition, current targeted therapy specifically targeted to *BRAF* V600E mutation exhibited poorer efficacy against non-V600E mutation.

Furthermore, clinical investigations will be also confronted with ongoing challenges. Firstly, randomized prospective phase III trials are difficult to conduct owing to the low incidence of *BRAF* mutant-positive NSCLC. Secondly, the utility and ethics of randomizing patients to a control arm with poorer efficacy and shorter survival durations are controversial. In addition, several studies have demonstrated that ICIs could show efficacy in this population; the problem that lies ahead is which regimen should be given first.

In the future, the activity of chemoimmunotherapy and combinations of TKIs with chemotherapy, anti VEGF/VEGFR agents, and/or immunotherapy in patients with *BRAF*-mutated cancers needs to be determined. In addition, the development of agents targeting non-V600E mutations should speed up.

AUTHOR CONTRIBUTIONS

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REVIEW
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Targeting ALK Rearrangements in NSCLC: Current State of the Art

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Anaplastic lymphoma kinase (ALK) alterations in non-small cell lung cancer (NSCLC) can be effectively treated with a variety of ALK-targeted drugs. After the approval of the first-generation ALK inhibitor crizotinib which achieved better results in prolonging the progression-free survival (PFS) compared with chemotherapy, a number of next-generation ALK inhibitors have been developed including ceritinib, alectinib, brigatinib, and ensartinib. Recently, a potent, third-generation ALK inhibitor, lorlatinib, has been approved by the Food and Drug Administration (FDA) for the first-line treatment of ALK-positive (ALK+) NSCLC. These drugs have manageable toxicity profiles. Responses to ALK inhibitors are however often not durable, and acquired resistance can occur as on-target or off-target alterations. Studies are underway to explore the mechanisms of resistance and optimal treatment options beyond progression. Efforts have also been undertaken to develop further generations of ALK inhibitors. This review will summarize the current situation of targeting the ALK signaling pathway.

Keywords: lung cancer, ALK, rearrangement, tyrosine kinase inhibitor, resistance

1 BACKGROUND

1.1 ALK Signaling Pathway

NSCLC accounts for around 80% of lung cancers, with ALK+ NSCLC accounting for 3%–7% of these (1). ALK is a proto-oncogene which encodes anaplastic lymphoma kinase that is primarily expressed in the nervous system. ALK signaling is activated in cancer cells primarily through three mechanisms: gene fusions, gene amplification, and activating point mutations (2). ALK rearrangements were first identified in 2007 in NSCLC, where the 3′ region of the ALK gene was fused with the 5′ sequence of the echinoderm microtubule-associated protein-like 4 (EML4) gene. The rearrangement results in the expression of the EML4-ALK fusion protein (3). Many kinds of ALK fusion genes have been found in multiple cancer types (4). In ALK fusions, the partner drives ALK activity at the level of gene expression and through multimerization of the ALK kinase domain, which is presumed to promote several biological functions including cell differentiation,

proliferation, and anti-apoptosis (5). ALK can activate signaling cascades, such as the mitogen-activated protein kinase (MAPK), (phosphatidylinositol 3–kinase) PI3K/(protein kinase B) AKT, MEK/ERK kinase 2/3 (MEKK2/3), Crk-like/CRK SH3 domain-binding guanine nucleotide-releasing factor (CRKL/C3G), Janus kinase/signal transducer and activator of transcription (JAK/STAT), and mitogen-activated protein kinase kinase 5-extracellular signal-regulated kinase 5 (MEK5-ERK5) pathways (6).

1.2 Diagnosis of ALK Rearrangement

The ALK locus is prone to translocation, and more than 20 different ALK fusion protein partners have been discovered (5). The detection of ALK rearrangements is widely recognized in NSCLC. Different methods are now available, with immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) representing validated diagnostic techniques for the assessment of ALK status (7, 8). As chromogenic *in situ* hybridization (CISH) allows concurrent analysis of histological features and gene rearrangement of the tumors, it is also a useful method in assessing ALK status (9). Next-generation sequencing (NGS) can detect a fusion between any partners, which makes it advantageous. Multiplexed PCR amplicon-based targeted NGS interrogates fusion transcripts involving many known driver genes and partners (10). Furthermore, NGS is able to assess multiple other genes simultaneously with great sensitivity.

Other than identification of ALK rearrangements from tissue biopsy, non-invasive genotyping of circulating tumor nucleic acids has gained attention as an alternative strategy. Compared to mutations and insertions/deletions, ALK rearrangements are more complex as they incorporate diverse breakpoints and multiple fusion partners (11). As DNA shedding in plasma of patients with advanced disease increases, the sensitivity of ALK fusion detection in ctDNA improves at disease progression (12, 13). The longitudinal ctDNA assays for early detection of disease progression in ALK+ patients receiving treatment is under intense investigation.

1.3 Characteristics of ALK+ NSCLC Patients

ALK+ NSCLC patients tend to be younger, with no smoking history, and have adenocarcinoma as the most common histological subtype (14). A recent meta-analysis confirmed that there is an increased incidence of thromboembolism in ALK+ NSCLC patients as compared to non-ALK+ patients (15). Real-world data also suggested an increased risk of venous thromboembolism in ALK-rearranged NSCLC patients (16, 17).

Advanced ALK+ NSCLC has different imaging features of primary tumor and metastatic patterns from those of EGFR+ or wild-type NSCLC (18). ALK+ NSCLC often presents with central tumor location, large pleural effusion, and absence of a pleural tail (19). ALK+ tumors are also prone to nodal metastasis and lymphangitic carcinomatosis. The radiological features can clinically help discriminate ALK+ from ALK- tumors, but genetic evidence is always required.

1.4 ALK Variants and Fusion Partners

ALK variants have been reported to influence the efficacy of ALK TKIs, but results were inconsistent. A prospective study from Camidge et al. did not find that different ALK variants would impact PFS for first-line alectinib or crizotinib (20). In two other studies, ALK V3a/b had a worse OS (21, 22). A recent study also suggested a prognostic role of ALK variants on treatment outcome (23). In that study, 64 ALK variants were identified in 59 patients, with V1 (32.8%) and V3a/b (28.1%) being the most common. Patients with non-V3a/b showed a trend toward longer OS. Meanwhile, although ALK+ NSCLC patients have a high PD-L1 expression rate, there is no significant association with ALK variant subtypes (23). A meta-analysis suggested that there was no significant difference of patients with the V1 variant from non-V1 in terms of PFS and OS, while V3 was associated with shorter OS (24). However, a propensity score analysis did not find a difference of ALK variants regarding clinical features and outcomes (25), which was consistent with sensitivity of ALK variants to alectinib in ALK-transformed cells (26). The molecular link between ALK variants, the differential response to TKIs, and resistance mutations support NGS-based detection of ALK status to guide treatment strategies (27).

Other than ALK variants, other ALK fusion partners include ATIC-ALK, RANBP2-ALK, NPM1-ALK, TFG-ALK, KIF5B-ALK, SQSTM1-ALK, TPM4-ALK, and CLTC-ALK (28). Their responses to ALK TKIs have been reported in several case reports, some of which were associated with better prognosis (29).

The impact of 5′-ALK on the efficacy of crizotinib was reported (30). Compared with 3′-ALK fusion alone, patients with non-reciprocal/reciprocal ALK translocation had a higher incidence of central nervous system (CNS) metastasis at baseline. Harboring non-reciprocal/reciprocal ALK translocation was an independent predictor of worse PFS for crizotinib-treated ALK\+ NSCLC.

1.5 Treatment Modality

As ALK+ NSCLC is a gene fusion-driven cancer, tyrosine kinase inhibitors (TKIs) have been developed to treat this unique disease. Currently, six ALK-target agents have been approved to treat advanced ALK+ NSCLC, including crizotinib, alectinib, ceritinib, ensartinib, brigatinib, and lorlatinib. These targeted agents induce durable responses and improve survival outcomes. Treatment with ALK inhibitors is recognized as the standard of care for advanced ALK+ NSCLC.

2 ALK TARGETED THERAPIES IN NSCLC

2.1 First-Generation ALK TKI

The six currently approved ALK TKIs for advanced ALK+ NSCLC were classified into three generations (**Figure 1**). The drug targets, approved indication by FDA, trial design, and primary endpoint of clinical trials are summarized in **Table 1**, which can help illustrate the currently available ALK-TKIs. The development of crizotinib, a first-in-class and first-generation ALK TKI, revolutionized the treatment of ALK+ NSCLC (52). Crizotinib is a small-molecule

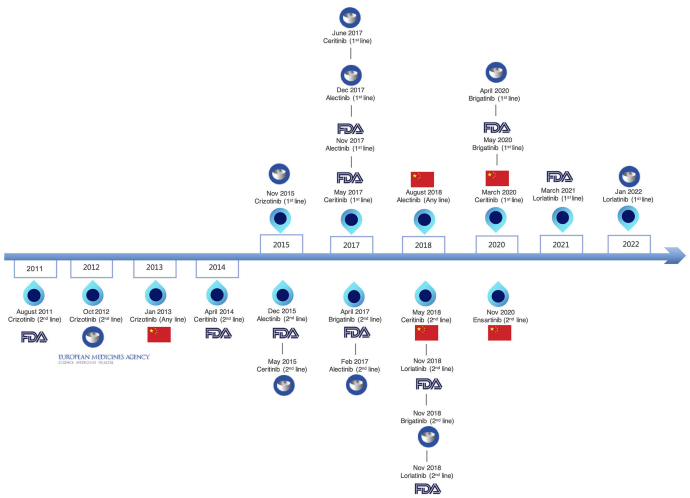

FIGURE 1 | Timeline of approved ALK TKIs.

inhibitor of the receptor tyrosine kinases ALK, ROS1, and c-MET. In phase I and II studies, crizotinib demonstrated durable responses in advanced ALK-positive NSCLC patients (53, 54), leading to the accelerated FDA approval in 2016. In a phase III study PROFILE 1007, crizotinib showed improved PFS compared with chemotherapy in first-line and previously treated patients (34). However, the pharmacokinetic failure to crizotinib is mainly due to its poor blood–brain barrier penetration, and CNS is a common site of progression with crizotinib (55). Crizotinib-treated patients will virtually develop acquired resistance. L1196M, and G1269A, and C1156Y mutations alter the structure of the ATP-binding pocket and thus prevent crizotinib from binding to ALK (56).

2.2 Second-Generation ALK TKIs

The second-generation ALK-TKIs alectinib, ceritinib, ensartinib, and brigatinib were developed to overcome crizotinib resistance, and they exhibited potent activity to crizotinib-resistant ALK+ NSCLC patients.

2.2.1 Alectinib

Alectinib is a next-generation inhibitor that is highly selective for ALK (57). Alectinib, which is not a P-glycoprotein substrate, has a better penetration to the blood–brain barrier compared with crizotinib (58). Alectinib was approved by the FDA for second-line treatment in 2015 based on two single-arm trials (NP28761 and NP28673) including 225 patients treated with alectinib 600 mg orally twice daily (59). The J-ALEX trial was the first study to show that the second-generation ALK inhibitor alectinib provides a PFS advantage and is more tolerable than crizotinib with the dose of 300 mg twice daily (37). Alectinib was approved by the FDA for the first-line treatment of ALK+ NSCLC in 2017 based on the phase III ALEX trial with alectinib 600 mg twice daily (36). In a

TABLE 1 | Pivotal clinical trials of currently approved ALK TKIs in NSCLC.

Drug	Targets	FDA approval	Study	Phase	Trial design	Region	Primary endpoint
Crizotinib	ALK, MET, ROS1 (31)	First line (January 2013)	PROFILE 1014 (32)	3	RCT	Global	PFS
			PROFILE 1029 (33)	3	RCT	Asia	PFS
		Later line (August 2011)	PROFILE 1007 (34)	3	RCT	Global	PFS
Alectinib	ALK, GAK, LTK (35)	First line (November 2017)	ALEX (36)	3	RCT	Global	PFS
			J-ALEX (37)	3	RCT	Japan	PFS
			ALESIA (38)	3	RCT	Asia	PFS
		Later line (June 2013)	ALUR (39)	3	RCT	Global	PFS
Brigatinib	ALK, EGFR, IGFR1 (40)	First line (May 2020)	ALTA-1L (41)	3	RCT	Global	PFS
		Later line (April 2017)	ALTA (42)	2	RCT	Global	ORR
Ceritinib	ALK, IGFR1, InsR, STK22D (43)	First line (May 2017)	ASCEND-4 (44)	3	RCT	Global	PFS
		Later line (April 2014)	ASCEND-5 (45)	3	RCT	Global	PFS
Ensartinib	ALK, ROS1, TRK1/2/3 (46)	First line[a]	eXalt3 (47)	3	RCT	Global	PFS
		Later line[a] (NMPA 2020)	NCT03215693 (48)	2	Single-arm	China	ORR
Lorlatinib	ALK, ROS1 (49)	First line (March 2021)	CROWN (50)	3	RCT	Global	PFS
		Later line (November 2018)	NCT01970865 (51)	2	Single-arm	Global	ORR, iORR

RCT, randomized clinical trial; TKI, tyrosine kinase inhibitor; PFS, progression-free survival; ORR, objective response rate; iORR, intracranial objective response rate; FDA, Food and Drug Administration; NMPA, National Medical Products Administration.

[a]*Not approved by the FDA.*

final analysis of the J-ALEX study, compared to crizotinib, alectinib did not achieve overall survival (OS) benefit (60), reflecting that the crossover to the post first-line treatment might greatly influence OS, especially in ALK+ NSCLC who could get significant benefit from all ALK TKIs. A prospective real-world study investigated the strategy of switching to alectinib in ALK+ NSCLC patients that did not experience disease progression with initial crizotinib (61). The results indicated that an early switch from crizotinib to alectinib might be a viable option and may promote better treatment compliance.

Data from J-ALEX suggested that compared with ALEX wherein 600 mg twice daily was used, alectinib 300 mg twice daily did not produce a markedly different primary outcome of PFS in a Japanese population. Since alectinib 300 mg twice daily will produce fewer adverse events (AEs) and fewer treatment interruptions, the lower dose is therefore an attractive approach in the study population (62). The on-target resistance of the mechanism of alectinib is related with emergence of G1202R and I1171N/S/T mutations (63).

2.2.2 Brigatinib

In preclinical models, brigatinib (AP26113) has been shown to overcome resistance to first- and second-generation ALK TKIs (40). In crizotinib-treated (ALTA trial) and crizotinib-naïve (ALTA-1L trial) patients with ALK+ NSCLC, brigatinib has shown promising antitumor activity, including substantial activity against central nervous system (CNS) metastases (41, 64). In the final analysis of ALTA-1L, brigatinib demonstrated superior efficacy over crizotinib regardless of ALK fusion variant or TP53 mutation status, especially in patients with baseline brain metastases (65). In a network meta-analysis, brigatinib ranked the highest by efficacy in the CNS metastasis subgroup compared with alectinib, while alectinib ranked the highest by efficacy in the overall population (66). In general, brigatinib is well tolerated; however, the early-onset pulmonary toxicity has raised some concerns. The ATOMIC ARI-AT-002 trial (NCT02706626) is ongoing to evaluate the efficacy of brigatinib against ALK-resistant mutations after second-generation ALK inhibitor treatment other than brigatinib in patients with ALK+ NSCLC (67). A phase III ALTA-3 trial (NCT03596866) comparing brigatinib versus alectinib in the first-line ALK+ NSCLC is also ongoing (68).

2.2.3 Ceritinib

Ceritinib obtained FDA approval for the treatment of ALK-positive patients who progressed or were intolerant to crizotinib in 2014, and as a first-line therapy in 2017. Approval was based on the ASCEND-1 (69) and ASCEND-2 studies (70). In the phase II ASCEND-2 study, crizotinib-pretreated ALK+ NSCLC received ceritinib at a standard dose of 750 mg daily and achieved an objective response rate (ORR) of 38.6% (70). A phase I, three-arm ASCEND-8 study demonstrated that ceritinib 450 mg with food showed similar efficacy and less gastrointestinal toxicity compared to 750-mg fasted (71). Two randomized Phase III trials compared ceritinib vs. standard chemotherapy in the first-line (ASCEND-4) (44) or second-line (ASCEND-5) setting (45). However, the toxicity profile of ceritinib from ASCEND-4 and ASCEND-5 indicated a higher frequency of dose interruptions and modifications due to adverse events (AEs) compared to chemotherapy. Real-world data comparing ceritinib versus alectinib in ALK+NSCLC found that alectinib exposure was associated with longer OS compared with ceritinib in ALK+ NSCLC (72). The pharmacokinetic (PK) data from the ASCEND-8 study (71) led to the FDA approval of ceritinib 450 mg QD, administered with food.

2.2.4 Ensartinib

Ensartinib (X-396) is an aminopyridazine-based small molecule that inhibits ALK. Furthermore, ensartinib has reported some activity against ROS1, AXL, and cMET (73). In a phase 1/2 trial, ensartinib has shown promising clinical activity in ALK+ NSCLC (46). A single-arm phase 2 trial investigating ensartinib in second-line ALK+ NSCLC demonstrated an ORR of 52% (48), which led to its approval by the National Medical Products Administration (NMPA) of China. The phase III eXalt3 study comparing ensartinib versus crizotinib for the first-line treatment of ALK+ NSCLC demonstrated that ensartinib is superior to crizotinib in both systemic and intracranial diseases (47). Of note, crossover was not allowed in this trial. A dynamic sequencing of circulating tumor DNA (ctDNA) in ensartinib-resistant ALK+ NSCLC patients revealed that ALK-dependent resistance mechanisms of ensartinib were mainly due to G1269A, G1202R, and E1210K mutations (74).

2.3 Third-Generation ALK TKI

Approximately half of resistance to second-generation ALK-TKIs is associated with secondary mutations in the ALK kinase domain (75). Lorlatinib is a 3^{rdz}-generation ALK TKI and is a small and compact macrocyclic inhibitor. The macrocyclic formation had an improved metabolic stability and a low frequency of P-glycoprotein-mediated efflux *in vitro*. Diverse compound ALK mutations were identified in lorlatinib-resistant cells or patient samples after sequential ALK-TKI treatments (76, 77). Lorlatinib can inhibit G1202R mutation, but not compound mutations (78). Lorlatinib was approved by the FDA in 2018 for the second- or third-line treatment of ALK+ NSCLC (51). The phase III CROWN study comparing lorlatinib versus crizotinib achieved the best-in-class differential PFS benefit of HR 0.28 (50), which led to its first-line approval of the FDA in March 2021. Crossover was not allowed in the CROWN study. This result may redefine the new potential standard of care in the first-line setting. As there are no head-to-head comparisons of lorlatinib to second-generation ALK TKIs, debates were raised regarding whether lorlatinib is the best first-line treatment for ALK+ NSCLC (79, 80). Compared with alectinib, lorlatinib was associated with a higher incidence of grade 3 or higher AEs (81) mostly related to its higher penetration in the CNS.

2.4 Fourth-Generation ALK TKIs Under Investigation

The sequential use of ALK TKIs which is active to ALK "single mutant" will lead to double ALK resistance mutations. Fourth-generation ALK TKIs such as TPX-0131 and NVL-655 have been developed, which are "double mutant active." TPX-0131 is a compact macrocyclic inhibitor, which was designed to fit

completely in the ATP-binding pocket. It may reduce the susceptibility to a variety of ALK TKI-resistant mutations, including solvent front, hinge region, gatekeeper, and compound mutations (82). Other than being sensitive to most single resistant mutations, TPX-0131 is effective for compound mutations such as G1202R+L1198F, G1202R+L1196M, L1196M+ L1198F, and G1202R+C1156F. Another 4th-generation ALK TKI, NVL-655, is a brain-penetrant small-molecule inhibitor with activity against solvent front drug-resistance mutations, such as G1202R, G1202R+L1196M, and G1202R+G1269A (83). Furthermore, NVL-655 displayed brain penetrance to open up the potential to treat brain metastases while avoiding off-target CNS adverse events.

2.5 Other ALK TKIs

Entrectinib is a selective inhibitor of TRKA/B/C, ALK, and ROS1 (84). Combined results from two phase I/II basket trials (ALKA-372-001 and the STARTRK-1 trial) suggested that entrectinib was well tolerated and active against ALK+ NSCLC (85). A phase II basket trial STARTRK-2 (NCT02568267) is currently ongoing to evaluate entrectinib for the treatment of patients with NTRK, ROS1, and ALK gene rearrangements. Repotrectinib (TPX-0005) is a rationally designed macrocyclic TKI developed to inhibit ALK, ROS-1, and TRKA-C (86). It is smaller than lorlatinib and has a high activity in CNS. The TREDENT-1 study (NCT03093116) for repotrectinib showed encouraging data in ALK+ NSCLC patients (87).

Other novel ALK TKIs include TQ-B3139 (88), WX-0593 (89), PLB-1003 (90), SAF-189s (91), and CT-707 (92). Several other ALK TKIs are under preclinical investigation, such as gilteritinib (93) and XMU-MP-5 (94). An ALK proteolysis-targeting chimeric (PROTAC) degrader is also under development. The six different ALK PROTACs are all based on the second-generation ALK-TKIs, including ceritinib-based (95–98), TAE684-based (96), and brigatinib-based ALK PROTACs (99). During this process, kinase mutations and off-target effects may occur, which is a major clinical challenge (100). The ongoing clinical trials investigating novel-generation ALK TKIs in ALK+ NSCLC are summarized in **Table 2** (up to December 18, 2021).

2.6 Treatment Options Other Than ALK TKIs

2.6.1 Chemotherapy

As chemotherapy has limited efficacy in ALK+ NSCLC after failure of a second-generation ALK TKI, combination therapy with ALK TKI and chemotherapy has been proposed in ALK+ NSCLC refractory to at least one second-generation ALK TKI. This strategy has been proved to be a possible choice by several studies. Crizotinib plus pemetrexed in ALK+ NSCLC patients with multiple CNS metastases demonstrated better efficacy than monotherapy (101). Chemotherapy in combination with ALK TKI proved to be of higher efficacy, suggesting a potential role for ongoing ALK inhibition (102).

2.6.2 Anti-Angiogenic Drugs

Anti-angiogenic drugs have also been investigated in ALK+ NSCLC. Vascular endothelial growth factor (VEGFR) expression has been reported to be upregulated in ALK+ NSCLC, which induces resistance to ALK TKIs (103). A single-arm study of involving 12 patients of ALK+ NSCLC demonstrated that crizotinib plus bevacizumab showed benefit in first-line ALK+ NSCLC, with an acceptable safety profile (104). In another phase 1/2 single-arm trial, alectinib plus bevacizumab was also well tolerated (105).

2.6.3 Immune Checkpoint Inhibitors

The PD-L1-positive and strongly positive rates among ALK+ NSCLC patients were 46.7%–50% and 13.3%–16%, respectively (23, 106). Studies have shown that the ALK oncoprotein is able to upregulate PD-L1 expression in lung cancer cells. Upregulation of PD-L1 by EML4-ALK was mediated by activating MEK-ERK and PI3K-AKT signaling pathways in NSCLC, which suggests a link between oncogene and PD-L1 expression (107). The expression of PD-L1 in ALK+ NSCLC has brought immunotherapy drugs such as immune checkpoint inhibitors (ICIs) into consideration for ALK+ NSCLC. A real-world analysis of ICIs in ALK+ NSCLC patients from a Flatiron Health electronic health record demonstrated limited efficacy of ICIs provided either before or after TKIs (108). Recent evidence indicated new roles of ALK and its genetic aberrations in immune evasion and in innate and cell-mediated immunity (109). The tumor microenvironment of ALK + NSCLC suggested a poorly immunogenic "immune desert" of ALK+ NSCLC that also prevents the successful use of immune checkpoint inhibitors (ICI) (110). Furthermore, the toxicity of ICI for ALK+ NSCLC patients was too high. The sequential use of ICIs and crizotinib has also been reported with an increased risk of hepatotoxicity in retrospective studies (111). The challenge to researchers is not only to improve the efficacy of ICI in ALK+ NSCLC but also to find immunotherapeutic drugs that have acceptable toxicity in combination regimens.

2.6.4 Radiotherapy

There are no firm data for concurrent usage of ALK TKIs and radiotherapy. However, radiotherapy acts as a salvage treatment for patients who have oligoprogressive metastatic disease while under targeted therapy (112). In oligoprogressive diseases of ALK+ lung cancer, continuation of ALK TKIs with local ablative therapy should be considered for sustained control, which can potentially eradicate resistant cancer cell clones and confer survival benefit (113). Ablative and hypofractionated radiotherapy is one strategy for ALK+ lung cancer, since many ALK+ NSCLC patients treated with ALK TKIs experienced local disease progression (114). Timing of radiotherapy remains unclear, especially under different clinical settings. Furthermore, the safety of the combination of ALK TKIs and radiotherapy is unclear (115). Case reports using radiotherapy combined with alectinib and lorlatinib presented radiation-induced CNS necrosis, and this toxicity remains long after radiation (116, 117).

3 DISCUSSION

3.1 How to Choose the Optimal First-Line Treatment?

There is a continuous debate regarding the choice of the optimal upfront ALK TKI for the first-line treatment of ALK+ NSCLC, the subsequent sequencing strategies, and whether these considerations

TABLE 2 | Ongoing clinical trials of novel ALK TKIs against ALK-arranged NSCLC.

Clinical trial identifier	Study design	Intervention	Setting	Primary endpoint	Phase	Status
NCT04009317	260 participants Parallel assignment Randomized, open label	TQ-B3139 vs. crizotinib	First line	PFS	3	Recruiting
NCT04632758	330 participants Parallel assignment Randomized, open label	WX-0593 vs. crizotinib	First line	PFS	3	Recruiting
NCT04056572	135 participants Single-group assignment Non-randomized, open label	TQ-B3139	Second line	ORR	2	Recruiting
NCT04641754	176 participants Single-group assignment Non-randomized, open label	WX-0593	Second line	ORR	2	Recruiting
NCT04211922	104 participants Single-group assignment Non-randomized, open label	Alkotinib	Second line	ORR	2	Recruiting
NCT02568267	60 participants (basket) Single-group assignment Non-randomized, open label	Entrectinib (RXDX-101)	Second line	ORR	2	Recruiting
NCT03093116	500 participants (basket) Single-group assignment Non-randomized, open label	Repotrectinib (TPX-0005)	Second line	DLT, RP2D, ORR	1/2	Recruiting
NCT04849273	210 participants Single-group assignment Non-randomized, open label	TPX-0131	Second line	DLT, RP2D, ORR	1/2	Recruiting
NCT04237805	280 participants Single-group assignment Non-randomized, open label	SAF-189s (foritinib)	First/second line	DLT, ORR	1/2	Recruiting
NCT03130881	60 participants Single-group assignment Non-randomized, open label	PLB1003	Second line	DLT	1	Recruiting
NCT03607188	18 participants Single-group assignment Non-randomized, open label	Alkotinib	Second line	DLT	1	Recruiting
NCT05055232	120 participants Single-group assignment Non-randomized, open label	XZP-3621	Second line	Toxicity, DLT, MTD	1	Recruiting
NCT02695550	40 participants Single-group assignment Non-randomized, open label	CT-707	Second line	DLT, toxicity	1	Unknown

PFS, progression-free survival; ORR, objective response rate; DLT, dose-limiting toxicity; RP2D, recommended phase 2 dose; MTD, maximum tolerated dose.

should be based on specific on-target ALK resistance mutations or not. Our recently published Bayesian network meta-analysis has compared the efficacy and safety of 6 ALK TKIs and chemotherapy in the first-line setting (118). Regarding PFS benefit for the first-line setting, lorlatinib ranks first, while the toxicity of lorlatinib needs to be paid attention to. However, the goal of treating advanced ALK+ NSCLC should not just be limited to improve median PFS in the first-line setting. There is no consensus on how to best sequence the ALK TKIs which are "single mutant active." Some advocate using second-generation ALK TKIs due to their favorable toxicity profile, while leaving lorlatinib, the only third-generation ALK TKI, for salvage treatment (**Figure 2**).

ALK+ NSCLC has a high tendency for brain metastases compared to non-oncogene-driven NSCLC subtypes (119). Compared with first-generation ALK TKI, second- and third-generation ALK TKIs have a better efficacy of brain metastases. Ceritinib demonstrated an intracranial ORR of 35%–73% and an intracranial disease control rate (DCR) of 61%–86% in ALK-TKI naïve and -pretreated patients (44, 45, 69, 70). The intracranial ORR and intracranial DCR of alectinib in clinical trials were 54%–81% and 78%–90%, respectively (36, 39, 120, 121). Brigatinib showed an encouraging activity in the CNS, with an intracranial ORR of 42%–73% and an intracranial DCR of 83%–93% (42). A meta-analysis investigated the role of ALK TKIs in the treatment of ALK+ NSCLC patients with brain metastases, who had been pretreated with radiotherapy or not and/or chemotherapy (122). The results also confirmed better intracranial control with second-generation ALK TKIs (alectinib, brigatinib, and ceritinib) compared with crizotinib. Ensartinib demonstrated an intracranial ORR of 63.6%–70% and an intracranial DCR of 98%–100% (47, 48). Lorlatinib had an intracranial ORR of 61%–66% in the first-line setting (50). Lorlatinib also showed substantial intracranial activity in second-generation ALK TKI-pretreated patients, with or without baseline CNS metastases (123, 124). This evidence suggested that withholding brain radiotherapy in patients with asymptomatic brain metastases and use of radiotherapy during progression could be an option. Prospective trials are warranted to confirm the validity of this strategy.

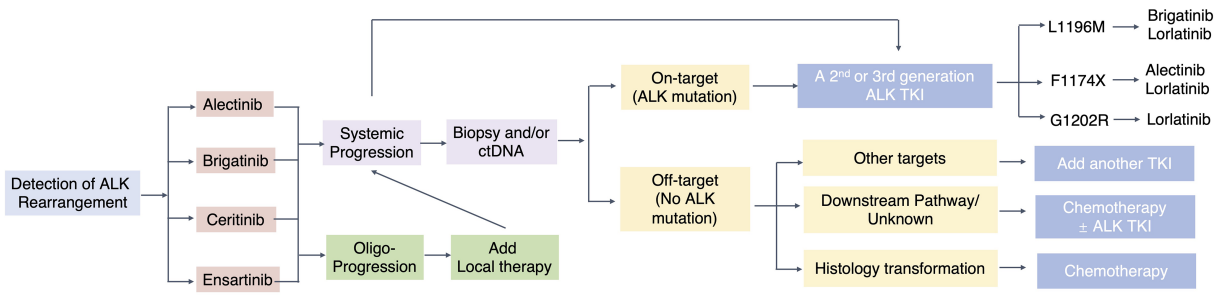

FIGURE 2 | Treatment strategy for ALK+ NSCLC.

3.2 Resistance Mechanism of ALK TKIs

There are two main categories of resistance mechanisms to ALK TKIs, namely, on-target alterations such as ALK mutation/gene amplification and off-target changes such as bypass signaling pathways (75). Substitution with ALK-destabilizing mutations could activate the ALK signaling pathway, which confers drug resistance to inhibitors (125). Inherent ALK resistance mutations are only found in a proportion of patients with acquired resistance to ALK-TKI, for first- and second-generation ALK-TKIs. ALK mutations such as somatic kinase domain mutations are the primary resistant mechanism. Two major ALK mutations after first-generation ALK TKI crizotinib were L1196M (7%) and G1269A (4%) (75), which alter 3D conformation and hinder TKI binding (126). Resistance to second-generation ALK TKIs is associated with specific mutations, such as G1202R, I1171N, S1206Y, and E1201K, for which not all TKIs are equally effective. In patient samples post-ceritinib, secondary mutations were detected in 56% of the cases, with 17% of double mutations: G1202R (21%), F1174 C/L (17%), and C1156Y (8%) (75). Acquired mutations of alectinib have been identified in 53% of the patients: G1202R (29%), I1171T/S (12%), V11180L (6%), and L1196M (6%) (75). Although brigatinib showed activity against G1202R, which is a frequent mutation associated with alectinib-resistant cancer (127), it is worth noting that G1202R has also been detected in brigatinib-resistant samples, raising the question of how clinically useful brigatinib is against this solvent front mutation (128). Of note, G1202R was not the most common ALK mutation in ensartinib-resistant patients, in which G1269A (6.6%) was the more identified than G1202R (2.8%) among 14.2% of the patients with secondary ALK mutations post second-line ensartinib (74). On-target resistance to the third-generation ALK inhibitor lorlatinib is primarily mediated by compound ALK mutations (129). Interestingly, some compound mutations that lead to lorlatinib resistance result in re-sensitization to first- or second-generation ALK TKIs, such as I1171N + L1256F, and C1156Y + L1198F which lead to re-sensitization to alectinib and crizotinib, respectively (76, 130). Patients with secondary ALK mutations refractory to the previous ALK TKI can be treated with other ALK TKIs. This re-sensitization phenomenon supported the sequential and possibly alternating use of different ALK TKIs.

ALK-independent mechanisms are only partially understood and particularly challenging, as they may result in refractoriness to further ALK inhibition. ALK-independent resistance mechanisms involve bypass pathways, such as EGFR, cMET, and AXL, or histological transformation into small cell lung cancer (SCLC) (131–133). Mechanisms of resistance to novel generation ALK TKIs are complex and diverse, reflecting the selective genetic pressure of drugs (134). In a prospective MATCH-R study, adaptive mechanisms driving resistance to lorlatinib were explored by a longitudinal assessment of tumor biopsies and ctDNA and the development of patient-derived xenograft (PDX) and cell lines (135). Epithelial–mesenchymal transition (EMT) mediated resistance in two patient-derived cell lines, and a novel bypass mechanism of resistance caused by NF2 loss-of-function mutations was described.

3.3 Toxicity Considerations

Clinical trials have established that ALK TKIs are generally safe and well tolerated. First-generation crizotinib has demonstrated a spectrum of toxicities, such as visual disorders (diplopia, photopsia, blurred vision), as well as QTc prolongation and bradycardia, while most of the AEs are grades 1–2 (136). Gastrointestinal toxicities were associated with different ALK TKIs, such as vomiting, nausea, and diarrhea. Brigatinib was characterized by a peculiar and early-onset interstitial lung toxicity (137). The most common AEs of lorlatinib were notably hypercholesterolemia (81%) and hypertriglyceridemia (60%), with cases of grade 3–4 toxicities occurring in 16% of patients. Special AEs of lorlatinib include CNS effects such as changes in mood, mental status, and peripheral neuropathy (138). Although different ALK TKIs share some common AEs, they have some unique toxicities, which should be taken into account to identify the right drug for the right patient. Finding ways to tackle these toxicities will play an essential role in drug strategies for ALK+ NSCLC patients.

A list of different parameters could potentially affect the interpretation of toxicity (139). Among them, the drug dose is one of the reasons which influence the tolerability and toxicity of ALK TKIs. As toxicity is related to drug dose, fewer toxicities were noted with the 300-mg dose than with the 600-mg dose of alectinib (62). Exposure-response analyses indicated that a lower dose of alectinib and crizotinib could result in diminishing treatment efficacy (140). Therefore, monitoring drug dose and toxicity might influence the treatment outcome of patients receiving ALK TKIs.

TABLE 3 | Clinical trials using ALK TKIs in neoadjuvant and adjuvant settings of ALK-arranged NSCLC.

Clinical trial identifier	Study design	Intervention	Setting	Primary endpoint	Phase	Status
NCT03456076	255 participants Parallel assignment Randomized, open label	Alectinib vs. chemotherapy	Adjuvant	DFS	3	Recruiting
NCT02201992	168 participants Parallel assignment Randomized, open label	Crizotinib vs. observation	Adjuvant	OS	3	Recruiting
NCT04302025	60 participants Single group assignment Non-randomized, open label	Alectinib	Neoadjuvant	MPR	2	Recruiting
NCT05015010	33 participants Single group assignment Non-randomized, open label	Alectinib	Neoadjuvant	MPR	2	Recruiting
NCT03088930	3 participants Single group assignment Non-randomized, open label	Crizotinib	Neoadjuvant	ORR	2	Completed

DFS, disease-free survival; MPR, major pathological response; OS, overall survival; ORR, objective response rate.

3.4 Beyond Advanced NSCLC

The treatment strategy of advanced ALK+ NSCLC has brought ALK-targeted therapy into early and locoregional (N2) stages. As acquired resistance of targeting ALK in the advanced stage setting emerges inevitably, TKIs are able to inhibit cancer cell proliferation, hinder tumor growth, and control cancer metastasis, but not to eradicate or cure the disease. There are no clear data regarding the frequency in early-stage or locoregional disease (141). Inhibiting the ALK signaling pathway at earlier stages still faces many challenges. Neoadjuvant and adjuvant ALK TKIs in ALK+ NSCLC have yielded mixed results (142). **Table 3** shows the clinical trials of ALK TKIs in neoadjuvant and adjuvant settings (up to December 18, 2021).

4 CONCLUSIONS

In this "precision medicine" era, although the detection of oncogenes is common practice and the administration of targeted agents is a recognized option, molecular results should be interpreted with caution. The integration of the roles including pathologists, molecular biologists, and clinicians is needed. The treatment algorithm of ALK+ NSCLC is becoming more complex. New-generation TKIs have better CNS penetration across the blood–brain barrier, resulting in superior intracranial response rates and preventing brain metastases. A head-to-head comparison between all ALK TKIs is still lacking, but novel ALK TKIs are being developed to overcome resistance to currently available ALK TKIs, hypothesizing a defined sequential ALK TKI strategy in this disease. After failure of targeted therapies, chemotherapy might still be a valid option, while the role of immunotherapy is yet to be clarified. Overcoming the challenges for the development of more potent drugs will be essential to improving the survival rate of ALK+ NSCLC in the future.

AUTHOR CONTRIBUTIONS

Conceptualization, LP and YZ. Writing—original draft preparation, LP and ZY. Writing—review and editing, LP, JS, GS, and YS. Supervision, JS and ZY. All authors contributed to the article and approved the submitted version.

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The Treatment Status of Patients in NSCLC With RET Fusion Under the Prelude of Selective RET-TKI Application in China: A Multicenter Retrospective Research

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Background: Rearranged during transfection (RET) fusion is a kind of uncommon mutation (about 1%) in non-small cell lung cancer (NSCLC). Although selective tyrosine kinase inhibitors (TKI) (selpercatinib and pralsetinib) have been available, there are no real-world data about the difference in the efficacy between RET-TKI and other regimens in China.

Methods: We conducted a multicenter retrospective analysis of 49 patients with RET-fusion-positive NSCLC. The characteristics and the clinical outcomes with RET-TKI, multi-kinase inhibitor (MKI), systematic chemotherapy, and immune-checkpoint inhibitor (ICI)-based regimens were evaluated.

Results: Of the 92 treatments in patients included, RET-TKI was administered 24 times (26.1%), systematic chemotherapy was 35 times (38.0%), ICI-based regimens was 26 times (28.3%), and MKI was 7 times (7.6%). RET-TKI had a higher objective response rate than the chemotherapy and ICI-based regimens (63.6% vs. 14.3% vs. 21.0%, $p < 0.001$). The median progress-free survival (mPFS) of RET-TKI, chemotherapy, immunotherapy, and MKI was 16.9 (95% CI: 1.8–32.0) months, 11.9 (95% CI: 7.7–16.1) months, 6.7 (95% CI: 2.9–10.5) months, and 2.8 (95% CI: 1.1–4.4) months, respectively. The mPFS of RET-TKI was longer than MKI and immunotherapy ($p < 0.001$), while without difference with chemotherapy ($p = 0.096$). Moreover, chemotherapy had longer mPFS than MKI ($p < 0.001$). In subgroup analysis, patients with brain metastases in RET-TKI treatment had worse mPFS than the one of patients without brain metastases (6.1 (95% CI: 0.0–13.9) months and 8.5 (95% CI: 6.3–10.6) months, $p = 0.012$). For patients having chemotherapy with or without angiogenesis inhibitors, the mPFS was 12.0 (95% CI: 11.05–13.02) months and 9.1 (95% CI: 8.31–9.89) months ($p = 0.468$). In the group of ICI-based regimens, the expression level of PD-L1 did not affect the mPFS of ICI [PD-L1 (+) vs. PD-L1 (–): 4.7 (95%

CI: 1.8–9.0) months vs. 7.6 (95% CI: 1.1–14.0) months, p = 0.910]. For overall patients, ECOG PS score, therapy lines, and therapeutic regimens were the independent factors affecting the prognosis.

Conclusions: In RET-fusion-positive NSCLC, RET-TKI is the best choice for a better response rate and PFS. In addition, chemotherapy which may bring a good PFS, is still a good choice for this group of patients.

Keywords: RET, NSCLC, tyrosine kinase inhibitors, survival, risk factor

INTRODUCTION

Rearranged during transfection (RET) is a kind of transmembrane receptor tyrosine kinase, which plays an important role in the early development of kidneys and the enteric nervous system. With proto-oncogene properties, RET associates with cell proliferation, growth, differentiation, and survival through activation of downstream signaling pathways such as RAS/MAPK, PI3K/AKT, and JAK/STAT (1, 2). RET aberration mainly has two forms of mutation, namely, point mutations and fusions. The former is more related to the occurrence and development of medullary thyroid cancer, while the latter is more related to papillary thyroid cancer and non-small cell lung cancer (NSCLC) (3).

First found in 2012, RET fusion is one of the rare gene mutations in NSCLC (about 1%–2%) (4, 5). Although the incidence is low, basing on the huge base of the NSCLC population, it is worth to study the characteristics, prognosis, and the treatments, which could bring better efficacy of this group of patients. Over the past decade, the treatment of RET-fusion-positive NSCLC patients has evolved from chemotherapy alone to multi-kinase inhibitor (MKI) to selective RET (tyrosine kinase inhibitors) (RET-TKI) nowadays. In particular, due to the excellent efficacy results of RET-TKIs, they were quickly approved for indications in just 2 years and became the first-line treatment recommendation for patients with RET-fusion-positive NSCLC in the National Comprehensive Cancer Network guidelines. Chinese Society of Clinical Oncology guidelines also list selpercatinib as a level III recommendation for RET-fusion-positive patients no matter in any treatment line; pralsetinib as a level II recommendation for subsequent-line treatment.

Although the efficacy of the regimens except RET-TKIs is limited, they are still a reasonable choice for NSCLC patients with RET fusion, especially under the prelude of the RET-TKI application, meaning that issues such as not only price but also accessibility may turn many patients away (6). According to previous data, other treatments such as chemotherapy can also bring efficacy and can still be the treatment options for this population. However, there is lack of study to directly compare the efficacy of different regimens in the real world.

In order to bring more data support for the better choice of regimens for NSCLC patients with RET fusion, we study the efficacy of RET-TKI in the real world and explore the difference between other treatment options and RET-TKI, including MKI, chemotherapy, and immune-checkpoint inhibitor (ICI)-based regimens.

MATERIALS AND METHODS

Study Design and Patients

We conducted a retrospective study of all patients with RET+ NSCLC in three centers (center 1: Hainan Cancer Hospital, center 2: the First Affiliated Hospital of Guangzhou Medical University & State Key Laboratory of Respiratory Disease, center 3: Shanghai Pulmonary Hospital & Thoracic Cancer Institute) from January 2015 to December 2021. The selected patients must be NSCLC patients with RET rearrangement at the time of initial diagnosis and had a specific treatment history (including regimens, the time of start of use, the time of end of use, and the reason of discontinuation). The patients who acquired other treatable mutations such as EGFR mutation were excluded due to the concern of the potential prognostic impact of other TKI administration.

Data Collection

The baseline information at the time of diagnosis of included patients was collected including age, sex, smoking history, history of lung disease, Eastern Cooperative Oncology Group Performance Status score (ECOG PS) score, histologic types, tumor node metastasis (TNM) stage, with or without brain metastases, RET fusion partner, and the expression of programmed cell death ligand 1 (PD-L1) of patients using ICIs. The histologic type was based on the fifth edition of the WHO classification of lung tumors. The TNM stage was classified according to the eighth edition of the TNM Classification for Lung Cancer (6). RET fusion was detected locally at each center and collected retrospectively. Detection methods include next-generation sequencing and reverse transcription-polymerase chain reaction. The expression of PD-L1 was assessed by immunohistochemistry using the 22C3 antibody. When the expression <1%, it was recorded as negative PD-L1 expression [PD-L1 (–)]; when the expression ≥1%, a positive PD-L1 expression [PD-L1 (+)] was recorded.

Tumor Response Assessment

The information of treatment for each patient was recorded including treatment line, treatment regimen, efficacy, date of treatment beginning, progression or loss to follow-up or latest follow-up, and survival status. The specific treatment regimens were divided into four cohorts, namely, RET-TKI, MKI, chemotherapy, and ICI-based regimens, and their objective response rate (ORR) and progression-free survival (PFS) were set as the main outcomes. Since median overall survival time has not been reached, it was not included as one of the outcomes of

this study. We performed further subgroup analyses of the efficacy of different treatments in first-line or subsequent-line treatments. Besides, in order to figure out the influence of brain metastasis on the efficacy of RET-TKI, a subgroup analysis of RET-TKI in patients with or without brain metastasis was performed. For the reason that the addition of angiogenesis inhibitors may affect the efficacy, we performed subgroup analyses of the efficacy of chemotherapy with or without angiogenesis inhibitors. For ICI-based regimens, whether the expression of PD-L1 would affect the efficacy was analyzed.

The efficacy assessment was based on the Response Evaluation Criteria in Solid Tumors (RECIST version 1.1), and the tumor response included complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) (7). ORR was defined as the proportion of patients with tumor response in CR or PR to the total population. PFS was defined as the time from the beginning of treatment to disease progression or death.

Statistical Analysis

Continuous variables are described by median (minimum to maximum), and categorical variables were described by frequencies (percentages). Differences in ORR between groups were achieved by the chi-square test, and the Z test was used for pairwise comparisons. The median PFS and its 95% confidence interval (CI) were obtained through the Kaplan–Meier method, and the log-rank test and Breslow test were used to compare survival curves.

Age, sex, smoking history, ECOG PS score, brain metastasis, RET fusion partner, treatment regimens, and treatment line were included in the risk factor analysis of PFS. We used the Cox regression model to do the univariate survival analysis and multivariable survival analysis. If the p-value is less than 0.1 in the univariate survival analysis, the factors would be included in the multivariable survival analysis. A hazard ratio (HR) with 95% CI and its p-value was used to describe the results. Except for special instruction, a two-sided p value of less than 0.05 ($p < 0.05$) was considered statistically significant. Bonferroni correction was used if there were more than two groups needed to be compared. Statistical analyses were conducted by SPSS version 25.0 (IBM Corporation, Armonk, NY, USA), while data were visualized with GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Patient Characteristics

A total of 49 patients with RET-rearranged NSCLC were included in this study (15 in center 1, 10 in center 2, 24 in center 3). The median age of the included patients was 56 (range: 26–77). The distribution of men (26, 53.1%) and women (23, 46.9%) was almost equal. A percentage of 69.4% of patients never smoked. Except one who had sarcomatoid carcinoma, all patients were diagnosed with adenocarcinoma. A percentage of 20.8% of patients had brain metastases at initial diagnosis. As for the gene-fusion spectrum, KIF5B was the most common RET fusion partner, while others included CCDC6, NCOA4, and TXNDC11. The specific information of patient characteristics is shown in **Table 1**.

Efficacy of Overall Patients

Of the 92 treatments in 49 patients, 24 had received RET-TKI, 7 for MKI, 35 for chemotherapy, and 26 for ICI-based regimens (**Figure 1A**). The specific regimens are shown in **Table 2**. The median follow-up time was 9.4 (95% CI: 6.8–12.0) months.

A total of 22 patients with RET-TKI, 1 with MKI, 28 with chemotherapy, and 19 with ICI-based regimens had information to assess the best tumor response. The ORRs of RET-TKI, chemotherapy, and immunotherapy-based regimens were 63.6% (95% CI: 41.8–85.5), 14.3% (95% CI: 0.5–28.1), and 21.0% (95% CI: 0.9–41.2), respectively ($p < 0.001$) (**Figure 2A**). As for pairwise comparison, the ORR of RET-TKI was better than that of chemotherapy or ICI-based regimens ($p < 0.05$), but there was no statistically significant difference between chemotherapy and ICI-based regimens.

All patients in any treatment regimen participated in the PFS analysis. RET-TKI had the longest median PFS (mPFS) (16.9 [95% CI: 1.8–32.0) months]. This was followed by chemotherapy [11.9 (7.7–16.1) months], ICI-based therapy [6.7 (95% CI: 2.9–10.5) months], and MKI [2.8 (95% CI: 1.1–4.4) months]. No matter in the log-rank test or Breslow test, the difference in mPFS of RET-TKI compared with ICI-based regimens or MKI and the difference

TABLE 1 | The baseline information of patients at the time of diagnosis.

Characteristics	Patients (n = 49)
Age—y	56
Median	26–77
Range	
Male/female—no. (%)	26 (53.1%)/23 (46.9%)
Smoking status—no. (%)	
Former/current	15 (30.6%)
Never	34 (69.4%)
Histologic types—no. (%)	
Adenocarcinoma	48 (98.0%)
Sarcomatoid carcinoma	1 (2.0%)
TNM stages—no. (%)	
IIIA	1 (2.0%)
IIIB	1 (2.0%)
IIIC	2 (4.2%)
IVA	23 (46.9%)
IVB	22 (44.9%)
ECOG PS	
0	1 (2.0%)
1	40 (81.6%)
2	8 (16.3%)
Brain metastasis	
Yes	11 (22.4%)
No	37 (75.5%)
Unknown	1 (2.0%)
RET fusion	
KIF5B	13 (26.5%)
CCDC6	6 (12.2%)
NCOA4	1 (2.1%)
TXNDC11	1 (2.1%)
Unknown	28 (57.1%)

y, years old; TNM, tumor node metastasis; ECOG PS, Eastern Cooperative Oncology Group Performance Status; RET, rearranged during transfection.

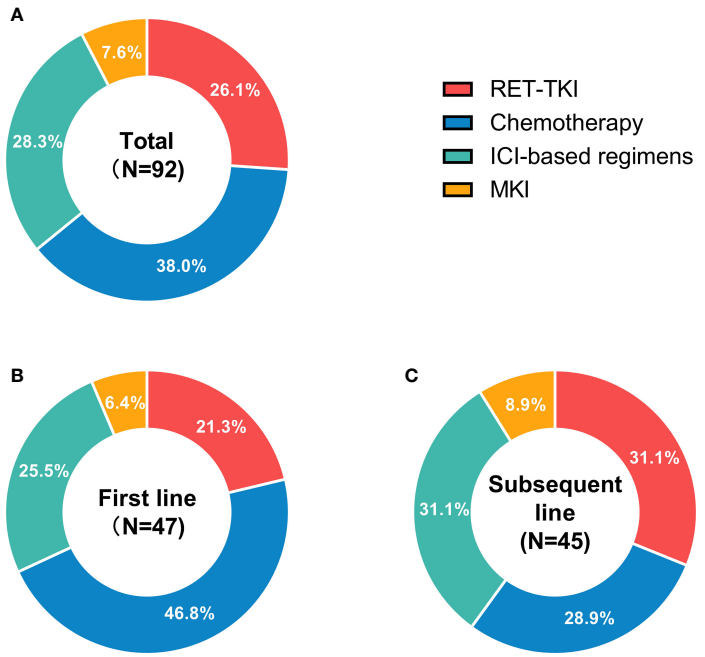

FIGURE 1 | Status of different treatments for RET-fusion non-small cell lung cancer patients in different treatment-lines. **(A)** The usage status of different treatments in any treatment line. **(B)** The usage status of different treatments in first-line treatments. **(C)** The usage status of different treatments in subsequent-line treatments. N, usage count. RET-TKI, rearranged during transfection-tyrosine kinase inhibitors. ICI, immune-checkpoint inhibitor; MKI, multi-kinase inhibitor.

between mPFS of chemotherapy and the one of MKI were statistically significant. In the comparison of chemotherapy and ICI-based regimens, the statistical difference existed only in the Breslow test, suggesting that there was a significant difference at the beginning, but the difference is no longer significant as time when on. The difference in median PFS between RET-TKI and chemotherapy was not statistically significant (**Figure 2B** and **Table S1**).

Efficacy in Different Treatment Lines

Different regimens were used at different frequencies in each treatment line. In the first line, there were more patients used chemotherapy (46.8%), while in subsequent-line treatments, ICI-based regimens and RET-TKI were the main choices (**Figures 1B, C**).

For the reason that the number of patients using MKI in each subgroup was too small be analyzed, the subgroup analysis did not include it. The ORR of RET-TKI, chemotherapy, and ICI-based regimens had a significant difference in first-line treatments (70% (95% CI: 34.8–93.3) vs. 11.1% (95% CI: 1.4–34.7) vs. 20.0% (95% CI: 2.5–55.6), p = 0.005) (**Figure 3A**). In pairwise comparisons, the difference only existed between RET-TKI and chemotherapy. Although a numerical difference is shown among different regimens in subsequent-line treatments [58.3% (95% CI: 27.7–84.8) for RET-TKI, 20.0% (95% CI: 2.5–55.6) for chemotherapy, 22.2% (95% CI: 2.8–60.0) for ICI-based regimens] (**Figure 3B**), there was no significant difference for these subgroup analyses (p = 0.146).

The mPFS of RET-TKI in first-line treatments had not been achieved, and its median follow-up time was 7.6 (95% CI: 5.6–9.6) months. For chemotherapy and ICI-based regimens, the median PFS in the first-line treatment was 11.9 (95% CI: 7.2–16.6) months and 11.4 (95% CI: 3.1–19.7) months, respectively. However, there was no significant difference among these three regimens (p = 0.527) (**Figure 4A**; **Table S2**). In subsequent-line treatments, RET-TKI [16.9 (95% CI: 7.9–32.4) months] and chemotherapy [8.6 (95% CI: 3.6–13.6) months] had longer mPFS than ICI-based regimens [3.0 (95% CI: 0.0–9.0) months] (p = 0.001 and p = 0.004) (**Figure 4B**; **Table S2**).

Subgroup Analysis for Different Regimens

In a subgroup analysis for the RET-TKI group, six patients had brain metastases. The ORR and intracranial ORR were 50.0% (95% CI: 11.8%–88.2%) and 33.3% (95% CI: 4.3%–77.7%). However, there was no statistical difference in ORR with or without brain metastases (50.0% vs. 68.8%, p = 0.624) (**Figure 3C**). For chemotherapy, 16 patients used it with angiogenesis inhibitors (C+A) and 19 patients without (C-A). Although the ORR of C+A was numerically higher [21.4% (95% CI: 4.7%–50.8%) vs. 7.1% (95% CI: 0.2%–33.9%)], the difference was not statistically significant (p = 0.596) (**Figure 3D**). In the group of ICI-based regimens, a total of 13 patients had evaluated the expression of PD-L1, among which the ORR of patients with a negative PD-L1 expression was 11.1% (95% CI: 0.3%–48.2%), and the ORR of patients with a positive PD-L1 expression was 25.0% (95% CI: 0.6%–80.6%) (p = 1.000) (**Figures 3E**).

The mPFS of patients with brain metastases in the RET-TKI group was 6.1 (95% CI: 0.0–13.9) months; the mPFS of patients without brain metastases was not reached [the median follow-up

TABLE 2 | Specific treatment regimens of included patients.

Treatment	Patients n (%)
RET-TKI	24
Selpercatinib	9 (37.5%)
Pralsetinib	15 (62.5%)
Chemotherapy	35
Pemetrexed-based regimens	25 (71.4%)
Paclitaxel-based regimens	7 (20.0%)
Docetaxel	2 (5.7%)
Platinum	1 (2.9%)
ICI-based regimens	26
Anti-PD-1	20 (76.9%)
Anti-PD-L1	2 (7.7%)
Unknown	4 (15.4%)
MKI	7
Cabozantinib	4 (57.1%)
Alectinib	2 (28.6%)
Lenvatinib	1 (14.3%)

RET-TKI, rearranged during transfection-tyrosine kinase inhibitors; ICI, immune-checkpoint inhibitor; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; MKI, multi-kinase inhibitor.

time was 8.5 (95% CI: 6.3–10.6) months] (p = 0.012) (**Figure 4C**). The mPFS was 12.0 (95% CI: 11.1–13.0) months for C+A and 9.1 (95% CI: 8.3–9.9) months for C-A, but the difference was not significant (p = 0.468) (**Figure 4D**). For ICI-based regimens, the expression of PD-L1 did not bring a statistically significant difference for mPFS [4.7 (95% CI: 1.8–9.0) months vs. 7.6 (95% CI: 1.1–14.0) months, p = 0.910] (**Figure 4E**).

Risk Factor Analysis

In the overall population, age<60, ECOG PS score ≥2, brain metastases, subsequent-line treatment, and chemotherapy, immunotherapy, and MKI compared with RET-TKI were associated with worse PFS in the univariate analysis. In the multivariate analysis, the p-value of ECOG PS score ≥2 [HR: 2.672 (95% CI: 1.224–5.834)], subsequent line treatment [HR: 2.42 (95% CI: 1.29–4.57)], and treatments other than RET-TKI (chemotherapy (HR: 3.48 (95% CI: 1.23–9.84), ICI-based regimens [HR: 7.20 (95% CI: 2.55–20.34), MKI (HR: 17.63 (95% CI: 4.87–63.87)] were less than 0.05, suggesting that the above factors were independent risk factors for poor PFS (**Figure 5**; **Table 3**).

DISCUSSION

In this multicenter retrospective research of RET-fusion-positive NSCLC patients, we described the clinical characteristics and compared the efficacy among the latest treatment regimens including RET-TKI, chemotherapy, ICI-based regimens, and MKI. The results support that RET-TKI is the first choice of NSCLC patients with RET fusion, while chemotherapy especially with angiogenesis inhibitors is still a good choice. Similar with the former studies, the efficacy brought by ICI and MKI was limited. In our knowledge, this is a comparative efficacy study to date that includes the latest and most comprehensive treatment options for patients with RET-fusion-positive NSCLC in the real world. Moreover, the findings from this study can give advices for the better clinical decision making.

TKI of other gene mutations such as EGFR and ALK has brought long survival to patients harboring corresponding mutations. However, patients with a RET-fusion mutation have not been able to obtain a good prognosis until the emergence of two RET-TKIs, selpercatinib and pralsetinib, in 2018 (8). The phase 1–2 clinical trial LIBRETTO-001 of selpercatinib showed that the objective response (OR) was 64% for patients previously receiving at least platinum-based chemotherapy and 85% for treatment-naïve patients, while the median PFS was 16.5 (95% CI: 13.7 to NE) months and not reached, retrospectively (9). For the Chinese population, a further phase 2 study LIBRETTO-321 was conducted by Lu et al. Similar to LIBRETTO-001, the ORR was 66.0% with 96.8% of responses ongoing at a median follow-up of 10.3 months, further demonstrating the stable efficacy of selpercatinib (10). Another phase 1–2 clinical trial ARROW of pralsetinib also achieved a good outcome, showing 61% OR for patients treated in subsequent lines and 70% for patients treated in the first line; the mPFS was 16.5 months for patients after treatment and 13.0 months for treatment-naïve patients (11). Based on far better efficacy and safety than other previous treatments, the FDA approved

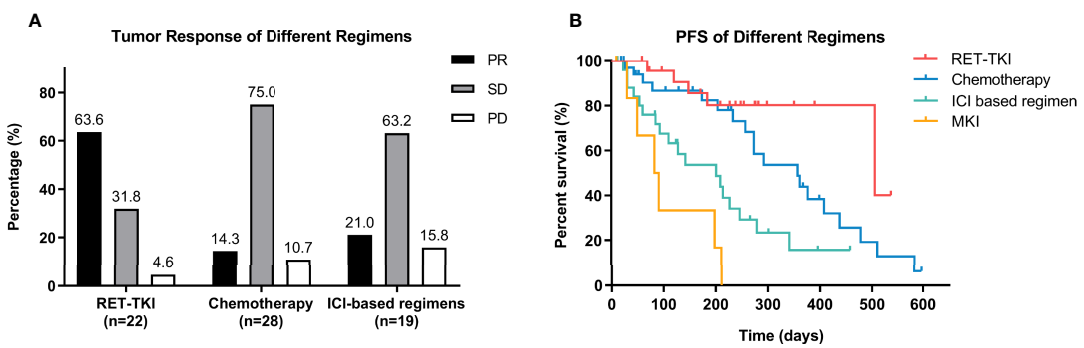

FIGURE 2 | Efficacy analysis of different regimens in overall patients. **(A)** Tumor response of different regimens including RET-tyrosine kinase inhibitors (RET-TKI) or chemotherapy or immune-checkpoint inhibitor (ICI)-based regimens or multi-kinase inhibitor (MKI). **(B)** Progression-free survival (PFS) of patients treated with RET-TKI or chemotherapy or ICI-based regimens or MKI. PR, partial response; SD, stable disease; PD, progressive disease.

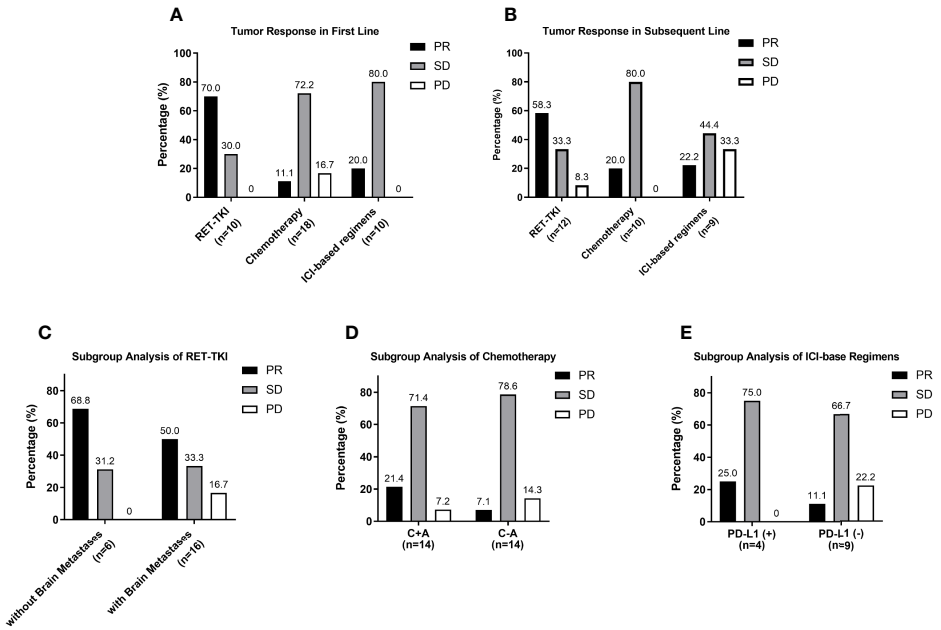

FIGURE 3 | Subgroup analysis of tumor response. **(A)** Analysis of tumor response of different regimens in first-line treatment. **(B)** Analysis of tumor response of different regimens in subsequent-line treatment. **(C)** Analysis of tumor response in group of patients treated with RET-TKI with brain metastases or without. **(D)** Analysis of tumor response in group of patients treated with chemotherapy with or without angiogenesis inhibitor. **(E)** Analysis of tumor response in group of patients treated with ICI-based regimens with negative PD-L1 or positive PD-L1. PR, partial respons;. SD, stable disease; PD, progressive disease.

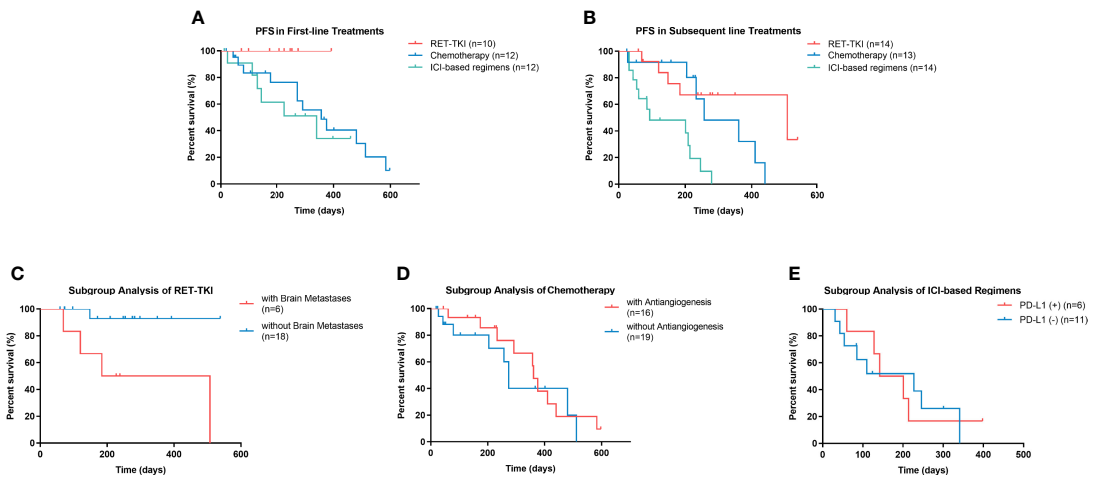

FIGURE 4 | Progression-free survival (PFS) of patients with rearranged during transfection rearrangements (RET) in different conditions. **(A)** PFS of patients with RET-TKI, chemotherapy, or ICI-based regimens in first-line treatments. **(B)** PFS of patients RET-TKI, chemotherapy, or ICI-based regimens in subsequent-line treatments. **(C)** PFS of patients treated with RET-TKI with or without brain metastases. **(D)** PFS of patients treated with chemotherapy with or without angiogenesis inhibitors. **(E)** PFS of patients treated with ICI-based regimens with negative PD-L1 expression or positive one. PFS, progression-free survival, RET-TKI, rearranged during transfection-tyrosine kinase inhibitors. ICI, immune-checkpoint inhibitor. MKI, multi-kinase inhibitor; PD-L1, programmed death-ligand 1.

selpercatinib and pralsetinib for adult patients with metastatic RET fusion-positive NSCLC in 2020, while the National Medical Products Administration of China granted accelerated approval for pralsetinib in 2021. The overall results of our study also showed a good efficacy of RET-TKI with 63.6% ORR and a 16.9-month mPFS. The ORR of RET-TKI was 58% in subsequent-line treatments and 70% for treatment-naïve patients. The mPFS was 16.9 months for patients after treatment, and the one for patients in first-line treatments was not

reached. These data are very close to the results of clinical trials. However, the difference between RET-TKI with chemotherapy or ICI-based regimens seems to just exist in subsequent-line treatments. As the follow-up time of patients with RET-TKI was not long enough, and the number of patients was limited, the results still need further follow-up data to certify.

For the reason that RET mutation is the risk factor for brain metastasis (12), and the incidence of brain metastasis during the lifetime of patients with RET fusion is nearly 50% (13), the intracranial response of RET-TKIs is one of the focuses. Both selpercatinib and pralsetinib showed good intracranial efficacy in clinical trials. In LIBRETTO-001, 22 patients with measurable intracranial disease at baseline achieved 82% ORR including 23% with CR. In overall patients, the median intracranial PFS was 13.7 months (14). In the ARROW study, four in eight patients with brain metastases at the time of diagnosis obtained OR, with two CRs (11). All these results show that both two RET-TKIs can cross the blood–brain barrier and bring good efficacy. Among 24 patients treated with RET-TKI in this study, 25% patients had brain metastases when diagnosed. According to the mPFS of subgroup analysis, brain metastases were an independent risk factor for a shorter time to RET-TKI benefit although the mPFS still had more than 6 months. Among the six patients, the intracranial ORR was 33.3% (all had CR), which seems lower than the results of clinical trials. This bias may be related to the heterogeneity caused by the small number of patients. At the same time, different RET-TKIs may also affect the results as the intracranial efficacy of selpercatinib seems to be better as seen in clinical trials. However, there will not be any head-to-head comparison, and a meta-analysis may be helpful to find out the detailed differences between the two RET-TKIs when clinical trial data gradually increase in the future. Besides, a real-world study with larger numbers of patients with brain metastases is needed to further evaluate the intracranial efficacy of RET-TKIs.

Before the development of RET-TKIs, chemotherapy was the recommendation of first-line therapy. A retrospective study from Drilon et al. showed that the mPFS of 18 RET-rearranged lung cancer patients was 19 months (15), proving that RET-rearranged patients could also benefit from pemetrexed-based systemic therapies. Besides, a study from China also proved that pemetrexed-based chemotherapy is better than other chemotherapy regimens (mPFS: 9.2 vs. 5.2 months) (16). In our study, the mPFS of chemotherapy is similar to the former studies. Although it was shorter than the one of RET-TKI numerically, the difference did not have a statistical significance. We also try to figure out whether the addition of angiogenesis inhibitors will bring better efficacy. Unfortunately, there was no statistically significant difference. As the numerical difference of mPFS between chemotherapies with or without angiogenesis exists, further studies may be of implementation value.

Different from chemotherapy, patients with RET fusion NSCLC have been unable to benefit well from MKI and ICI, and the same outcomes were shown in this study, though MKIs including cabozantinib and vandetanib were recommended in clinical guidelines (17–20). The expressions of PD-L1 in patients with

TABLE 3 | Univariate and multivariate Cox regression analyses for PFS in overall patients.

Factors	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age (years)				
<60	1			
≥60	0.457 (0.228–0.916)	0.027		0.384
Sex				
Male	1			
Female	0.713 (0.392–1.297)	0.267		
Smoking history				
No	1			
Yes	0.708 (0.366–1.367)	0.304		
ECOG PS				
0–1	1		1	
2	3.543 (1.734–7.239)	0.001	2.672 (1.224–5.834)	0.014
Brain metastases				
No	1			
Yes	1.763 (0.977–3.181)	0.060		0.779
RET fusion partner				
KIF5B	1			
Others	1.097 (0.431-2.793)	0.846		
Treatment line				
First	1		1	
Subsequent	1.809 (1.009–3.244)	0.047	2.423 (1.286–4.567)	0.006
Treatment regimens				
RET-TKI	1		1	
Chemotherapy	2.362 (0.872–6.393)	0.091	3.478 (1.232–9.842)	0.019
ICI-based regimens	5.581 (2.050–15.196)	0.001	7.198 (2.547–20.340)	<0.001
MKI	15.054 (4.401–51.495)	<0.001	17.628 (4.865–63.873)	<0.001

HR, hazard ratio; ECOG PS, Eastern Cooperative Oncology Group Performance Status score; RET-TKI, rearranged during transfection-tyrosine kinase inhibitors; ICI, immune-checkpoint inhibitor; MKI, multi-kinase inhibitor.

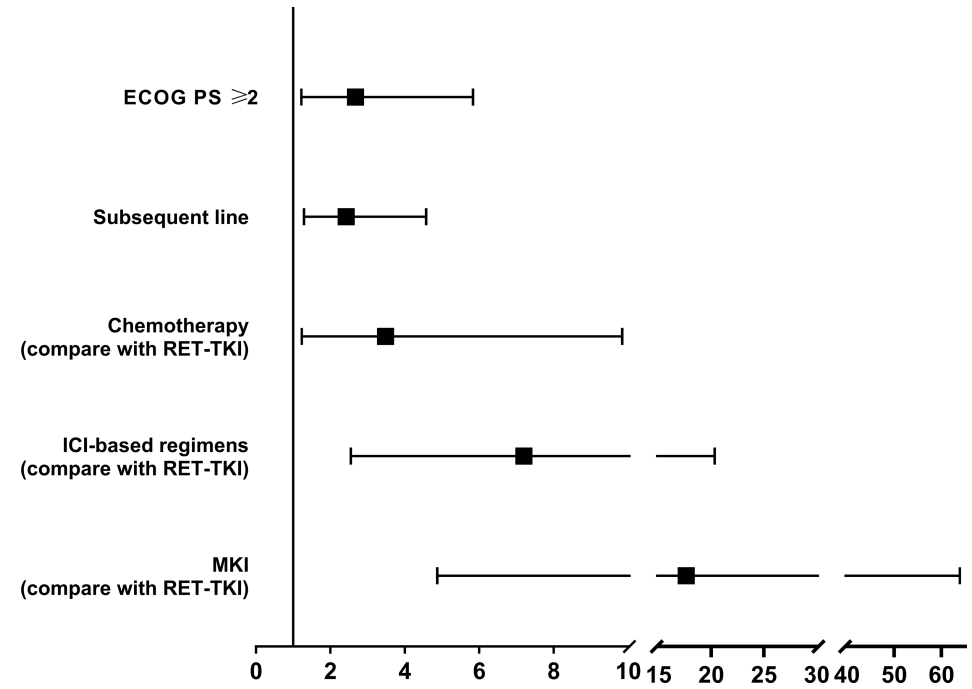

FIGURE 5 | The forest plot of the risk factors associated with the efficacy of patients with RET-fusion positive non-small cell lung cancer. RET-TKI, rearranged during transfection-tyrosine kinase inhibitors. ICI, immune-checkpoint inhibitor; MKI, multi-kinase inhibitor; PD-L1, programmed death-ligand 1.

RET fusion are heterogeneous, but most patients had a negative PD-L1 expression (21). The former study has proved that the expression of PD-L1 cannot affect the treatment efficacy in this group of patients (22). The same as the former study, the PD-L1 expression level did not correlate with the efficacy of ICI in patients with RET-fusion NSCLC in our result. Besides, the mPFS of ICI-based regimens was 6.7 months which seems longer than the one in a former study (17). This result may be caused by the use of immune-combination therapy in most patients, which may bring better efficacy than ICI monotherapy, and its efficacy is not directly correlated with the expression of PD-L1. In former studies, the mPFS of MKI in patients with RET-fusion NSCLC ranged from 3.4 to 7.3 months with poor tolerability due to off-targeted activity (23). Although safety analysis was not performed in this study, the efficacy of MKI was also poor as in previous studies, further suggesting the importance of precise targeting.

There are still some limitations of this study. First, this is a multicenter retrospective study, which means that bias was inevitable in the data collection process and some data were missing. Second, the number of patients in this study is not large enough, which prevented some more detailed subgroup analyses from being completed. Moreover, for the reason that we lack the records of adverse events, the safety analysis among different regimens cannot be achieved.

In conclusion, RET-TKI is the best choice for patients with RET-fusion-positive NSCLC nowadays, and chemotherapy is still a good choice. Besides, ICI-based regimens and MKI should not be recommended for this group of patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

YM, YY, and YF designed the study. XL, XX, HD, JW, MZ, and NS collected the patients' data. YM, YY, YF, ZX, ML, and MO analyzed the data. YY, YF, YQ, CS, and MZ drafted and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.864367/full#supplementary-material

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REVIEW
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The Evolution of BRAF Activation in Non-Small-Cell Lung Cancer

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Non-small-cell lung cancer (NSCLC) is the most common subtype of lung cancer, of which approximate 4% had BRAF activation, with an option for targeted therapy. BRAF activation comprises of V600 and non-V600 mutations, fusion, rearrangement, in-frame deletions, insertions, and co-mutations. In addition, BRAF primary activation and secondary activation presents with different biological phenotypes, medical senses and subsequent treatments. BRAF primary activation plays a critical role in proliferation and metastasis as a driver gene of NSCLC, while secondary activation mediates acquired resistance to other targeted therapy, especially for epidermal growth factor tyrosine kinase inhibitor (EGFR-TKI). Treatment options for different activation of BRAF are diverse. Targeted therapy, especially two-drug combination therapy, is an important option. Besides, immune checkpoint inhibitors (ICIs) would be another option since BRAF activation would be a positive biomarker of tumor response of ICIs therapy. To date, no high level evidences support targeted therapy or immunotherapy as prioritized recommendation. After targeted therapy, the evolution of BRAF includes the activation of the upstream, downstream and bypass pathways of BRAF. In this review, therapeutic modalities and post-therapeutic evolutionary pathways of BRAF are discussed, and future research directions are also provided.

Keywords: BRAF activation, EGFR mutation, non-small cell lung cancer, targeted therapy, acquired resistance, immune checkpoint inhibitors

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INTRODUCTION

Lung cancer is the leading reason of cancer death worldwide, accounting for 18% of all and non-small-cell lung cancer (NSCLC) is the most common subtype of lung cancer (1). With the development of precision medicine, especially next-generation sequencing (NGS) technology and circulating tumor DNA (ctDNA) technology, targeted therapy has replaced platinum-based chemotherapy as the first-line treatment for NSCLC patients with driver gene mutations (2, 3). More and more driver genes have been found in NSCLC, among which activated BRAF proto-oncogene accounts for approximate 4% (4, 5). BRAF mutant tumors are characterized as an aggressive histologic pattern with micropapillary features, and indicates a poor prognosis.

This review provides a comprehensive overview of characteristics, treatment modalities, and outcomes for NSCLC patients with different BRAF mutations. The pathways of activation and evolution of BRAF are divided into primary and secondary mutations. And different BRAF types have different clinical, biological and pathological features. The mechanism of acquired resistance and subsequent evolution of BRAF activation and the strategies after resistance are also discussed.

A BRIEF HISTORY OF BRAF SIGNALING

The RAF kinase has been closely and inextricably linked to cancer since 1983, when v-raf was first described by Ulf Rapp et al. (6) This is a murine retroviral oncogene with a mammalian cell homolog, called CRAF. And in 1984-1985, two CRAF-related genes were identified in studies in mice and humans: ARAF and BRAF (7, 8). In 2002, following the pioneering work of Davies et al. (9) on the BRAF gene, a number of studies have clarified the specific implications of BRAF mutations in lung cancer (10, 11). In 2011, following the results of a phase III trial (BRIM-3), the FDA approved the first drug targeting BRAF-mutated cancers, PLX4032 (vemurafenib) (12). Two years later, based on the results of the Phase III trial (NCT01227889), another targeted agent against BRAF mutations, Dabrafenib (GSK21188436), was also approved by the FDA for the treatment of advanced melanoma (13). In the same year, Trametinib (GSK1120212) was also approved for the treatment of patients with advanced melanoma with the BRAF V600E mutation (14). In 2017, dabrafenib and trametinib received FDA approval for the treatment of metastatic non-small cell lung cancer carrying the BRAF V600E mutation (15). The next year, the FDA approved encorafenib in combination with binimetinib which is an anti-MEK1/2 protein kinase inhibitor for the treatment of unresectable or metastatic melanoma patients with mutations in BRAF V600E or BRAF V600K based on a Phase III randomized, active-controlled, open-label, multicenter trial (COLUMBUS) (16). (**Figure 1**).

THE ACTIVATION OF BRAF

The Primary Activation of BRAF

BRAF is a mammalian cytosolic serine/threonine kinase that belongs to the rapidly accelerated fibrosarcoma (RAF) kinase family (ARAF, BRAF, CRAF), which uses the mitogen-activated protein kinase (MAPK) pathway to transmit signals downstream of RAS (17). The primary activation of BRAF comprises of BRAF classic mutations, BRAF rare mutations, BRAF fusion and amino acid insertion, etc. (17, 18). Different types of BRAF classical mutations have different clinical, pathological and biological characteristics. Studies have found that the occurrences of BRAF V600E mutations were not associated with age, tumor size, lymph node status, tumor stage and BRAF V600E mutation is more common in female lung adenocarcinoma, but very rare in male or squamous cell carcinoma (19). Besides, BRAF non-V600E mutations were prone to be found in smokers. Their occurrences were not associated with clinicopathological parameters or had no impact on prognosis. When treated with platinum-based chemotherapy, NSCLC patients with BRAF V600E mutation had a tendency of shorter progression-free survival (PFS) than those with BRAF non-V600E mutations, and the clinical outcomes between patients with BRAF mutation-positive and wild-types were similar, suggesting that BRAF mutations were not sensitive to chemotherapy.

The BRAF primary classic mutations process that leads to tumors can be broadly divided into three categories. Compared to wild-type, Class I (BRAF V600 mutation) increases 500-700 times kinase activity and activates downstream MAPK cascade pathways through activating monomers in a non-RAS dependent form and transcription factors. Class II and class III are mainly BRAF non-V600 mutations. Class II mutants have moderate kinase activity and can transmit signals through RAS independent constituent dimer to activate MEK1, which in turn activate ERK1/2, ultimately promote cell growth and infinite reproduction. Unlike class II mutants, class III mutants have no or little kinase activity, relying on RAS generates upstream signals that induce class III mutants to signal in the form of dimers (17, 20, 21). In general, class I and class II BRAF mutants can be independent of RAS signals and inhibit the negative feedback of ERK signals. In addition, class I BRAF mutants transmit signals in the form of active monomer, while class II

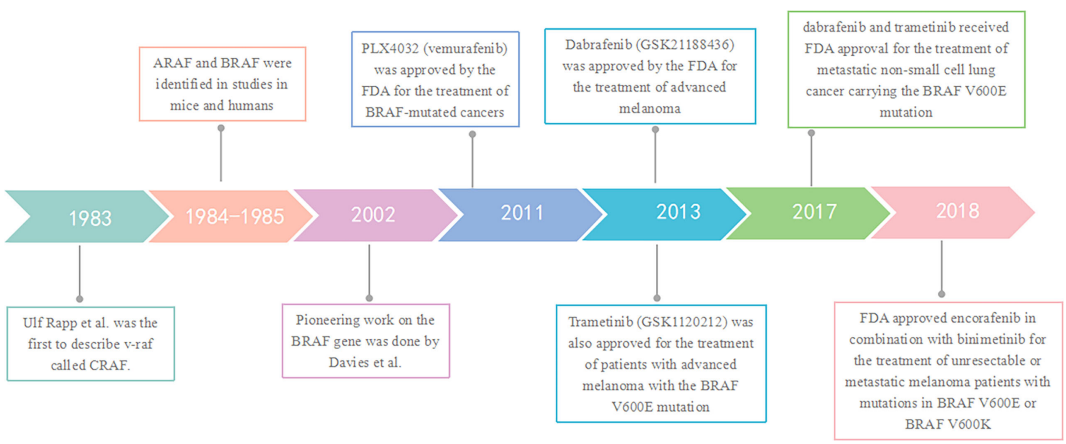

FIGURE 1 | Timeline of key events in BRAF signaling research.

and III BRAF mutants in the form of dimer, and the final signal transduction leads to the continuous activation of MAPK.

In addition to the three types of BRAF mutations, there are other forms of mutations that lead to over-activation of the pathway and ultimately the development of tumors. For example, BRAF in-frame deletions are mutually exclusive with RAS mutations, and these mutations can continuously transmit signals to activate the MAPK pathway by forming BRAF homologous dimers (22). Another study has also demonstrated that BRAF internal deletion is a mechanism of acquired drug resistance to BRAF/MEK inhibitors (23).

BRAF fusion has also been reported to be associated with tumorigenesis and progress. It was reported that BRAF fusion could cause the deletion of n-terminal inhibitory domain and activate downstream MAP kinase signal through recruiting CRAF protein to form a dimer (24, 25). In addition, a study reported that two melanoma cases whose pathogenesis was similar to BRAF fusion leading to tumorigenesis, but different from BRAF fusion, these two cases leaded to the over-activation of the pathway through the loss of BRAF inhibitory domain caused by chromosomal translocations of BRAF oncogene (26).

Amino acid insertions were found at position 599 of the BRAF codon, which is rare in the BRAF primary gene alteration. It is speculated that this may be related to the increase of kinase activity caused by changes in the spatial structure of the P ring (18).

NGS technology has revealed better comprehensive understanding of the gene mutations in various tumors. For BRAF, more and more co-mutations have been found between BRAF and other genes, which also indicates that the branching cloning process occurs at the early stage of tumor evolution, which leads to the generation of BRAF co-mutations. According to literature reports, BRAF can co-occur with KRAS mutation (27, 28), NRAS mutation (29), PTEN mutation (30, 31), MEK2 mutation (32), PIK3CA mutation (33) and other gene mutations. And most of these BRAF co-mutations occur in melanoma and lung cancer, but also found in other tumors.

The Secondary Activation of BRAF

The secondary activation of BRAF includes BRAF classic mutations, BRAF fusion and rearrangement, which are mainly acquired resistance to EGFR-TKI (25). Osimertinib has been prior recommended to the resistance caused by first- and second-generation EGFR-TKIs (34). But it will inevitably cause acquired resistance. It has been reported that the underlying mechanisms of acquired resistance to third-generation EGFR-TKIs include activation of parallel pathways, such as mutations of BRAF or other genes, and rearrangement of resistant genes, such as fusions of BRAF or other genes (25). The mutation and fusion mechanism of BRAF induced by EGFR-TKI resistance constitute an important part of BRAF gene evolution, and different treatment schemes have been explored for different types of BRAF evolution. BRAF rearrangement accounts for 4.4% of BRAF changes in NSCLC, and BRAF fusion is a form of BRAF rearrangement. Four cases were reported that BRAF fusion was a mechanism of EGFR-TKI acquired resistance in EGFR mutant lung adenocarcinoma (25).

Three cases revealed BRAF V600E mutation may be the mechanism for acquired crizotinib resistance after ROS1 rearrangement in NSCLC, two of them had acquired ROS1 rearrangement co-existing with BRAF V600E (35, 36). In another patient, ROS1 rearrangement was lost during treatment, leaving only the BRAF V600E mutation (37). Through single circulating tumor cell (CTC) sequencing, researchers found that patients with ALK mutation developed acquired drug resistance after ALK-TKIs therapy (27). And the mechanism of ALK-TKIs resistance mainly included mutations of RTK-KRAS pathway and TP53 pathway independent of ALK pathway. In the RTK-KRAS pathway, BRAF mutations accounted for 6.2% of the RTK-KRAS pathway (38). In addition, studies showed that BRAF mutation and BRAF fusion were secondary to adagrasib therapy in patients with KRAS G12C mutation (39).

THE THERAPY OF BRAF ACTIVATION

The treatment of BRAF mutation is mainly divided into two types, one is BRAF V600 mutation and the other is BRAF non-V600 mutation. BRAF V600 accounts for approximately 50% of BRAF mutation, and is more aggressive, and it occurs by mutation of glutamate into valine at position 600 of exon 15 (40). BRAF V600 develops by the previously described Class I mutation that activates the pathway in RAS independent monomer form. The other type of BRAF non-V600 mutation is mainly the previously described Class II and III BRAF mutations, which develop from signaling to downstream molecules in the form of dimers (17). The class II BRAF mutant is divided into class IIa within the activation segment and class IIb within the glycine-rich p-loop (20). The different structure, mechanism of occurrence and development leads to different treatment modalities for BRAF V600 and BRAF non-V600 mutations. On the other hand, current targeted drugs are mainly targeted at BRAF V600E, while there is no specific treatment modality for BRAF non-V600E.

Targeting BRAF V600 Mutation

BRAF V600 mutations including V600E, V600K, V600D and other subtypes, among which V600E is the most common subtype. The initial treatment for BRAF V600 mutation was monotherapy and FDA approved the first successful therapy targeting BRAF mutant melanoma called vemurafenib, an oral small molecule inhibitor of BRAF V600 mutations in 2011 (41). The evidence came from a histology-independent, flexible, early phase II "basket" study of vemurafenib in patients with non-melanoma cancers harboring BRAF (42). In this study, the objective response rate (ORR) was 42% (95% confidence interval [CI], 20 to 67%) and the median PFS was 7.3 months (95% CI, 3.5 to 10.8 months). The 12-month rate of PFS was 23% (95% CI, 6 to 46%) and the preliminary 12-month overall

survival (OS) rate was 66% (95% CI, 36 to 85%). The most common adverse event was nausea. Vivek Subbiah et al. (43) explored whether BRAF V600E mutations in NSCLC were sensitive to vemurafenib or not. The results turned out that among sixty-two NSCLC patients with BRAF V600 mutation, the overall ORR was 37.1% (95% CI, 25.2 to 50.3%), and 37.5% (95% CI, 8.5 to 75.5%) in previously untreated patients, and 37.0% (95% CI, 24.3 to 51.3%) in previously treated patients. The median PFS was 6.5 months (95% CI, 5.2 to 9.0 months), and the median OS was 15.4 months (95% CI, 9.6 to 22.8 months). Vemurafenib had a similar safety profile in studies focused on melanoma patients. Furthermore, the French National Cancer Institute (INCA) conducted a trial to assess the efficacy and safety of vemurafenib in cancers with various BRAF mutations (44). Among 118 NSCLC patients, 101 of them presented with a BRAF V600E mutation and 17 with BRAF non-V600 mutations. In the BRAF V600 cohort, the ORR was 44.9%, the median PFS was 5.2monthes (95% CI: 3.8 to 6.8%), and the OS was 10 months (95% CI, 6.8 to 15.7 months). The results indicated that vemurafenib is beneficial to NSCLC patients with BRAF V600E mutation.

By inhibiting BRAF V600E kinase activity, dabrafenib resulted in decreased phosphorylation of MEK and ERK, inhibition of cell proliferation, and ultimately G1 cell cycle arrest and cell death (45). In a phase II, multicenter, nonrandomized, open-label study, 84 advanced NSCLC patients with BRAF V600E mutation showed dabrafenib had some active killing effect, though the effect was limited. The adverse events were mainly skin-related, but these adverse events were tolerable (46).

A study had demonstrated that the acquire resistance to BRAF inhibitors was largely caused by reactivating the MAPK signaling pathway (47). Trametinib is a MEK1/2 inhibitor which blocks MEK1/2 kinase activity and prevents RAF-dependent MEK phosphorylation (48). A phase II, multicenter, non-randomized, open-label study assessed the efficacy of the combination of trametinib and dabrafenib, among previously treated or untreated metastatic NSCLC patients with BRAF V600E mutation. All patients were divided into three cohorts. In cohort B, 57 patients were enrolled and resulted in an ORR of 63.2%, disease control rate (DCR) of 79%, median PFS of 9.7 months (95%CI: 6.9-19.6) and 37 patients (65% [95% CI 51–76]) achieved 6-month PFS and median duration of response was 9·0 months ([95% CI 6·9–18·3]. The median OS data are immature, but 47 (82%) of 57 patients were alive at 6 months. The most common adverse event is pyrexia in 26 patients (46%) (49). The results of cohort C of this phase II study demonstrated promising results with ORR of 64% and DCR of 75%,the median PFS of OS 10.9 months and OS of 24.6 months, which was slightly better than in the previously treated cohort (cohort B) of this trial (50). In addition, the side effect profile was mostly similar to that of cohort B, BRAF-MEK combination therapy (dabrafenib plus trametinib) demonstrated tolerability and efficacy in a recent phase II clinical trial and in light of these promising results, combination dabrafenib and trametinib was approved by the US FDA for patients with metastatic melanoma and BRAF V600E mutation. Moreover, a real-life cohort of patients with BRAF mutant advanced NSCLC shows that treatment with BRAF inhibitors and MEK inhibitors in BRAF V600E tumors is associated with ORR of 67%, median PFS of 5.5 months, and median OS since treatment initiation of 9.5 months, which indicate the combination of BRAF inhibitors and MEK inhibitors is clearly superior to monotherapy with a BRAF inhibitors (51). In addition, the incidences of pyrexia and myelosuppression are higher with combination therapy than with monotherapy.

NSCLC patients with EGFR mutation could develop BRAF V600E mutation after acquiring resistance to targeted therapy. Given the secondary activation of BRAF, Huang et al. (52) proposed a strategy of combination of dabrafenib, trametinib and osimertinib, and the patient achieved long-term control of the disease. Another study also demonstrated that the combination of dabrafenib, trametinib and osimertinib was effective to NSCLC patient who developed a BRAF V600 mutation after EGFR-TKI resistance. In addition, the adverse events could be controlled by reducing the dose (53). In another experiment, the treatment of patient also demonstrated that the BRAF inhibitor encorafenib inhibited MEK signaling but had no significant effect on ERK phosphorylation, while the combination of encorafenib and osimertinib significantly reduced MEK and ERK phosphorylation and cell growth (54). In addition, in the review of 7 additional patients who were also reported to be treated with combined therapy of dabrafenib, trametinib and osimertinib, all patients obtained extended PFS and clinical benefit (54–57). In summary, NSCLC patients harbored secondary BRAF V600E mutations because of acquired resistance to EGFR-TKI could benefit from the combination with EGFR-TKI (e.g., osimertinib) and FDA-approved two-drug therapy (e.g., dabrafenib, trametinib).

In 3 patients with secondary activation of BRAF V600E, two patients had both ROS1 rearrangement and BRAF V600E mutations and one of them died 15 days after taking dabrafenib, while the other one died 11 days after taking dabrafenib and trametinib (35, 36). The third patient who developed BRAF V600E secondary to ROS1 rearrangement loss on crizotinib received a partial response of more than 6 months with dabrafenib and trametinib (37).

To date, two-drug therapy is only approved in NSCLC with BRAF V600E for FDA indication and recommended by the NCCN guideline. However, some studies showed that the treatment mode and clinical characteristics of BRAF V600E were similar with other subtypes, such as BRAF V600K (58). In light of guidelines for BRAF V600 mutated melanoma, dual-targeted therapy is also recommended. Therefore, this review recommends that dual-targeted therapy (dabrafenib and trametinib) could be initiated in BRAF V600 mutated patients, as a congener disease as well. Now that we know the treatment for BRAF V600, and then we talk about how to treat BRAF non-V600?

Targeting BRAF Non-V600

The most patients of BRAF non-V600 mutation has less aggressive phenotype and significantly superior survival compared to those

with BRAF V600 mutation, suggesting the potential need of different therapeutic strategies (59). A retrospective multicenter cohort study concluded that patients with BRAF non-V600E mutations located outside of the activation segment of the BRAF kinase domain were resistant to BRAF therapy (60). Another trial recommended chemotherapy as the dominant strategy for non-V600 mutation patients in the first-line treatment (61).

Recent experiences *in vitro* and *in vivo* show that class IIa BRAF mutant cells were sensitive to single-agent BRAF inhibitors, whereas class IIb BRAF mutant cells were not (62). Moreover, dual MAPK pathway inhibition (dMAPKi) effectively impaired the growth of subsets of non-V600 (62). *In vitro*, other trials have also demonstrated that BRAF non-V600 (L597, K601E) had significant response to MEK inhibitors (63).

Instead, research about class III mutant that have impaired kinase activity or are kinase-dead and linked with high RAS levels suggest Class III BRAF mutants may be treated with MEK inhibitors which co-existing with mutations in *RAS* and NF1 in melanomas, but in epithelial tumors, the great majority of class III mutations are not associated with RAS/NF1 alterations and may be treated with receptor tyrosine kinase (RTK) inhibitors that block the RAS pathway (20). Another study came to similar conclusions (64). A case report have also shown that dMPAKi is also benefit for patients harboring a dual G469A and W604C BRAF mutations and the response is more than 15 months (65). However, other studies found vemurafenib is not effective in NSCLC patients with BRAF non-V600 mutation (44, 66).

There is no evidence for patients resisted to EGFR-TKI yet, which could result in BRAF non-V600 mutations. A basic research demonstrated that, in osimertinib resistant PC9 cells transfected with BRAF G469A mutant plasmid, the combination of osimertinib, selumetinib (MEK 1/2 inhibitor) and trametinib (MEK 1/2 inhibitor) or dabrafenib reversed osimertinib resistance (67). Except for the two classical mutations of BRAF V600 and non-V600, there are also co-mutations of BRAF, and we will continue to discuss the treatment of BRAF co-mutations.

Treatment Recommendations for BRAF Co-Mutations

There is no high-level clinical trial for primary BRAF co-mutations to date. Since BRAF co-mutations were a clinical problem, we provide some recommendations for reference. Based on the studies on EGFR/ALK co-mutations, the phosphorylation level of the mutant genes would be a rational treatment option, and the abundance of gene mutations was also a positive biomarker for clinical decision (68). Besides, the treatment of primary BRAF co-mutation can refer to the treatment of secondary BRAF activation with EGFR/ALK/ROS1 mutation and adopt double or triple targeted therapy (53, 69). Considering the cost effectiveness and adverse events, immunotherapy, specifically immune checkpoint inhibitors (ICIs) would be a choice for BRAF co-mutations, which is introduced in detail as follows. We have solutions for all three of BRAF mutation patterns. And in recent years, the rise of immunotherapy has also brought new solutions to BRAF mutations.

Immunotherapy

Studies have reported BRAF mutant NSCLC patients have high expression of programmed cell death ligand 1 (PD-L1), which means that patients with BRAF mutation have great potential for ICIs (4). A retrospective cohort study conducted in 31 NSCLC patients with BRAF mutations showed that there was no statistically significant difference in OS among BRAF classic mutant patients who received first-line chemotherapy or immunotherapy (70). In a multi-institution retrospective chart review of 39 patients with BRAF mutated NSCLC, 22 of whom received ICIs, the ORR for V600E and non-V600E were 25% and 33%, respectively (P =1.0); PFS was similar in patients received ICIs treatment; median OS was equal for patients who received or did not receive ICIs (71). Another study collecting 4178 patients and 4462 samples from a cBioPortal database showed that BRAF wild-type mutants had a longer OS than BRAF mutants. Unlike previous study, this study showed that non-V600E had a longer OS than V600E under ICIs treatment (72). A BRAF G469A mutant NSCLC case obtained a deep and durable response after ICIs treatment, which suggested that BRAF non-V600 mutation may benefit more from immunotherapy than EGFR/ALK-driven mutation in NSCLC (73). However, in a retrospective, multicenter and real world analysis, 44 of 107 patients with BRAF mutations (V600:26, non-V600:18) received ICIs, with the response rates of 26% in BRAF V600 cohort and 35% in the non-V600 cohort. Besides, BRAF V600 cohort have longer PFS and OS than non-V600 cohort (74).

The above studies demonstrate the survival in various types of BRAF mutations treated with ICIs immunotherapy or targeted therapy are different. However, there is a lack of high-level evidence to prove which is better. Further prospective clinical trials are necessary to prove which is the optimal first line strategy. We have solutions for all three of BRAF mutation patterns. In recent years, the rise of immunotherapy has also brought new solutions to BRAF mutations.

Strategies for Resistance to BRAF Inhibitors

BRAF mutant tumors might initially respond to treatment with BRAF inhibitors, but eventually developed drug resistance. For acquired resistance to BRAF inhibitors caused by BRAF fusion, clinical trials have demonstrated the efficacy of pan-RAF inhibitors in patients with BRAF fusion (75). Evidence suggests that BRAF proteins undergo homodimerization and heterodimerization, therefore BRAF rearrangement is very insensitive to BRAF inhibitors. And RAF inhibitors could bind and inhibit all RAF isomers, so they are effective for BRAF fusion (76, 77). Evidence is also provided that a combination of MEK inhibitors and EGFR inhibitors is effective in patients with BRAF fusion (25, 77). As for acquired drug resistance, a series of post-resistance measures were reported. Intermittent dosing would be a choice. A melanoma case with vemurafenib showed the accelerated growth of RAS-mutant leukemia, and intermittent dosing of vemurafenib relieved the disease and reduced the disease burden (78). Subsequent studies showed that

intermittent dosing of BRAF inhibitors and RAF inhibitors may delay the progression of resistant tumors and make it sensitive to inhibitors again (79, 80). An international team of 180 scientists proposed the concept of a low toxicity "broad-spectrum" treatment based on the sequencing of the cancer's genome, which targeted multiple key tumorigenesis pathways and mechanisms to prevent cancer growth (81). What's more, for resistant mutations at different gene target, different drug combinations could be adopt. For example, FDA has approved the combination of BRAF inhibitors (Vemurafenib and Dabrafenib) and MEK inhibitor trametinib for the treatment of BRAF inhibition resistance. And clinical studies of PI3K/AKT inhibitor plus MAPK inhibitor, everolimus (RAD001) plus bevacizumab, everolimus (RAD001) plus temozolomide (TMZ), and targeted therapy plus immunotherapy have also been conducted, but there is a lack of more data to support these therapies, so further exploration is needed (82). The strategies for resistance to BRAF inhibitors were listed in **Table 1**.

THE EVOLUTION OF BRAF ACTIVATION

The Pathways of BRAF Evolution

As reported, the evolution of BRAF comes from the changes of various genes mainly in the following three ways after targeted therapy (**Figure 2**). First, the changes of BRAF itself and BRAF downstream molecules which lead to resistance to BRAF targeted inhibitors mainly come from the following aspects. BRAF splice variants are the most common situation. Studies has demonstrated that AGK-BRAF fusion leads to loss of the CR1 region of BRAF, thereby eliminating the inhibitory RAS-binding domain, and results in RAS-independent constitutive activation of the kinase (83). A research has found that the loss of the inhibitory RAS binding domain resulting from the loss of the internal BRAF leads to the reactivation of RAS-RAF-MEK-ERK signaling and mediates resistance to BRAF inhibitors (23). Besides, BRAF copy number amplification also can lead to resistance to BRAF targeted inhibitors. Hubing Shi et al. (84) proved that BRAF V600E amplification was the mechanism of acquired resistance of BRAF inhibitors, providing evidence for drug target changes leading to clinical relapse. Moreover, Montagut et al. (85) found that CRAF overexpression to increased ERK1/2 level indicating some BRAF mutant tumor cells were primary insensitive to RAF inhibition in the experiment, which was related to a switch from BRAF to CRAF dependency in tumor cells. And Lu et al. (86) found p21-activated kinases phosphorylate CRAF and MEK to reactivate ERK, which drive acquired drug resistance to MAPK inhibitors in BRAF mutants. Furthermore, MEK1 mutation can also lead to reactivation of the MAPK pathway. MEK is downstream of RAS signaling MEK reactivation caused by MEK mutation does not require stimulation of BRAF signaling, so BRAF inhibitors are ineffective against MEK1/2 mutation. Therefore, MEK1 mutation can promotes ERK phosphorylation, and MEK2 can also heterodimerize with MEK1, ultimately leading to the reactivation of EKR (87).

Second, changes in upstream molecules of BRAF lead to the evolution of BRAF mainly from the following aspects. First of all, studies have shown that NRAS upregulation is another resistance mechanism of BRAF inhibitors and NRAS upregulation may promote the dimerization of RAF, which will cause insensitivity of ERK signaling to drugs, leading to tumor drug resistance (88–90). And the mutation of RAS gene may lead to the reactivation of MAPK pathway. On the one hand, the mutant RAS protein will not dissociate after binding to GTP but become permanently activated. On the other hand, overactivated RAS may lead to overactivation of ARAF and CRAF, and thus cell proliferation. These two aspects jointly promote signal transduction of MAPK pathway (89, 91, 92). And ERK protein is a negative regulator of RAS protein, BRAF inhibitors can inhibit ERK pathway, thereby inducing part of RAS activity and leading to the activation of MAPK pathway (93, 94). And as well as RTKs alteration, overexpression of platelet derived growth factor receptor (PDGFR)-β or siRNA knockdown of PDGFRβ demonstrates the potential role of PDGFRβ signaling in drug resistance, and the introduction of PDGFRβ into untreated cells reduces sensitivity to vemurafenib (89). In addition, up-regulation of EGFR expression was found in BRAF inhibitor resistant cell lines and resistant tumor biopsies (95). EGFR activation binds to specific tyrosine residues on the receptor and results in a conformation change of Sos protein, thereby recruiting and activating RAS-GDP, and finally ERK activation induces cell proliferation (96). Besides, the upregulation of IGF1R/IR in BRAF and MEK inhibitor resistant cells and the maintenance of P-ERK and P-Akt suggest that IGF1R/IR may mediate resistance to inhibitors through the reactivation of MAPK (97).

Third, activation of bypass pathways leads to overactivation of the BRAF signaling pathway mainly come from the following aspects. At first, elevate expression of COT, like CRAF, activates ERK through MEK-dependent mechanisms that do not require RAF signals, thus driving resistance to RAF inhibition (98). Besides, studies have shown that loss of STAG2 or STAG3 inhibits CCCTC-binding factor (CTCF) mediated dual

TABLE 1 | Strategies for resistance to BRAF inhibitors.

Situation	Strategies	Ref.
BRAF fusion	pan-RAF inhibitors	(75)
	BRAF inhibitors and RAF inhibitors	(76, 77)
	combination of MEK inhibitors and EGFR inhibitors	(25, 77)
Acquired drug resistance to vemurafenib	intermittent dosing	(78–80)
Changes in multiple key tumorigenesis pathways and mechanisms	low toxicity "broad-spectrum" treatment	(81)
Resistant mutations at different gene target	different drug combinations	(82)

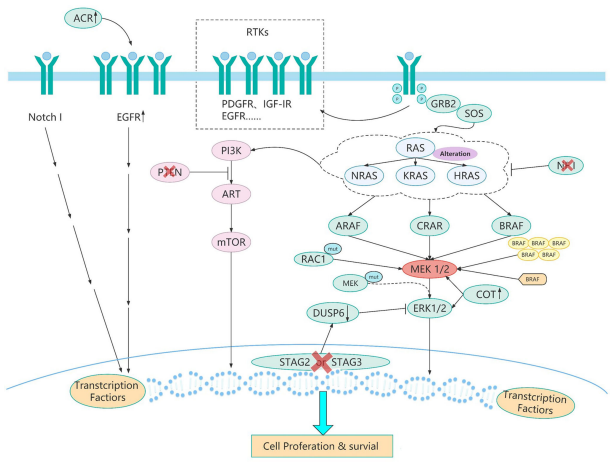

FIGURE 2 | The resistance pathways of BRAF inhibitor. BRAF mutated tumor cells evolve different drug resistance pathways to maintain cell growth after chronic inhibition by BRAF inhibitors. These evolutionary mechanisms (**Table 2**) include BRAF splice variants, BRAF copy number amplification, CRAF overexpression, MEK1 mutations, and other mechanisms. Different pathways of BRAF evolution can tell us how to overcome the problem of resistance to BRAF inhibitors and how to develop more rational protocols to address the resistance problem.

specificity phosphatase 6 (DUSP6) expression, leading to a significant decrease in DUSP6 protein levels and ultimately reactivation of MEK-ERK signaling in BRAF-inhibitor treated melanoma cells (99). What's more, RAC1 is a GTP-binding protein that modulates cytoskeletal rearrangement by signaling g-protein-coupled receptors and other molecules and RAC1 P29S mutations may mediate resistance to vemurafenib and dabrafenib by maintaining MAPK signaling (100). Additionally, NF1 is a tumor suppressor that inhibits RAS activity. Experiments have proved that loss of NF1 can re-drive MAPK pathway by activating RAS activity and increasing CRAF, thus mediating resistance to RAF inhibitors (101). Moreover, studies have found that ACK1 can inhibit the expression of EGFR, so the loss of ACK1 induces the increase of EGFR protein, thus increasing cell signal transduction to mediate the generation of drug resistance (102). Furthermore, it has been confirmed that increased Notch signaling results in increased expression of markers associated with cell dedifferentiation and increased cell migration and does not reactivate ERK in the presence of drug therapy to mediate acquired resistance to MAPK inhibitors (103). Finally, the study of Hubing Shi et al. (56) named the PI3K-PTEN-AKT pathway as the second core resistance pathway, and it has been reported that upregulation of the PI3K pathway accounts for approximately 22% of BRAF inhibitor acquired resistance melanoma. PTEN is an important tumor suppressor, which acts to counteract the effect of PI3K and when PTEN is lost, mutant or methylated, the activity of PI3K pathway will increase, and cells can finally survive by adopting PI3K signal (104). The evolution and pathways of BRAF activation were listed in **Table 2** and **Figure 2**. We have collected several paths of BRAF evolution. In addition, we hope to find certain rules from the evolutionary pathway, so we need to study the pathway of BRAF evolution through some methodologies.

Methodology to Track the BRAF Evolution

Due to the diversity and randomness of gene evolution, we need to use various emerging technologies and methods to find certain rules from dynamic evolution, so as to obtain certain therapeutic effects, and also to find effective therapeutic strategies. With the advent of cancer genomics and the development of multi-region sequencing, single-cell correlation sequencing and cloning techniques, it has become possible to describe gene phylogeny and evolution (105–107). In recent years, studies have been carried out on clonal phylogeny using single time point snapshot, multi-region sampling and spatio-temporal modeling to analyze diseases. In addition, mathematical models and other methods can be used to explore new evolutionary methods. At the same time, it also puts forward the direction and challenge to bioinformatics and computer science (108). In addition, it has been proposed that the development of single-cell multi-omics technology is crucial for a comprehensive understanding of the evolutionary mechanism. For example, multiple sampling methods can be used to analyze the evolutionary mechanism of tumors by different sampling methods (such as multiple regions or multiple times). And examples include *in vivo* and *in vitro* modeling of tumor evolution through optical or sequencing barcodes (109). Furthermore, deep sequencing of multiple regions of a tumor directly to detect evolutionary mutations is another way (110).

UNDERGOING STUDIES FOR BRAF ACTIVATION

The efficacy and safety of BRAF inhibitors are being explored in several clinical studies (e.g., NCT03915951, NCT04543188 etc.). In addition, more treatment options for patients with BRAF mutations can be explored, for example, BRAF inhibitors as adjuvant/neoadjuvant therapy for patients with NSCLC; BRAF inhibitor combined with MEK inhibitor and EGFR-TKI as three-target combination therapy; BRAF inhibitors combined with immunotherapy, anti-angiogenic drugs and other drug combinations. With the success of the ADAURA study, a new direction of targeted therapy in the adjuvant treatment for NSCLC patients has been opened. Therefore, we believe that the use of dabrafenib in combination with trametinib in neoadjuvant/adjuvant therapy for early-stage NSCLC patients is worthy of further exploration (111). Besides, for patients with acquired resistance to BRAF inhibitors, re-biopsy and NGS test to find new targeted drugs or new combination therapy are necessary. Finally, we still want to know if the strategy of dual-targeted or triple-targeted therapy could be re-challenged. Small-sample case reports suggest that sequential therapy with targeted therapy and immunotherapy, combined with the "rechallenge"

TABLE 2 | The evolution and pathways of BRAF activation.

Cancer types	Evolutionary types	Evolutionary pathways	Ref.
Melanoma	changes in BRAF itself	BRAF splice variants	(23, 83)
Melanoma	-	BRAF copy number amplification	(84)
Melanoma	downstream of the BRAF	CRAF overexpression	(85, 86)
Melanoma	-	MEK1 mutations	(87)
Melanoma	upstream of the BRAF	RAS alteration	(88, 94)
Melanoma	–	RTKs alteration	(89, 95, 97)
Melanoma	activation of bypass pathways	Elevated expression levels of COT	(98)
Melanoma	–	Loss of stromal antigen 2 (STAG2) or STAG3	(99)
Melanoma	–	RAC1 mutation	(100)
Melanoma	–	Loss of NF1	(101)
Melanoma	–	Loss of ACK1	(102)
Breast cancer and melanoma	–	Activation of the Notch1 pathway	(103)
Melanoma	–	Phosphoinositide 3-kinase (PI3K)/AKT pathway dysregulation	(56, 104)

TABLE 3 | Selected ongoing trials with BRAF Inhibitors for NSCLC.

Clinical Trial Identifier	Study Design	Intervention/s	Setting	Primary Endpoint	Phase	Status
NCT03915951	90 participants Open-label, Multicenter, Non-randomized, Phase 2 study	Encorafenib plus Binimetinib	First line	ORR	Phase 2	Recruiting
NCT04543188	225 participants two-part, phase 1A/B, open-label, multicenter trial evaluating pharmacokinetics	PF-07284890 plus Binimetinib plus Midazolam	First line	DLTs, AEs, Overall response	Phase 1	Recruiting
NCT04526782	119 participants Open-label, Multicenter, multi-cohort Phase 2 study	encorafenib plus binimetinib	First line	ORR	Phase 2	Recruiting
NCT05003622	6 participants Multicenter, Open-label, Phase 1 Study	Encorafenib	First line	DLTs	Phase 1	Active, not recruiting
NCT05065398	20 participants Open Label, Multicenter Phase II Clinical Trial	HLX208	First line	ORR	Phase 2	Recruiting
NCT05275374	221 participants Dose-escalation and Expansion Phase I/IIa Study	XP-102 or XP-102 plus Trametinib or	First line	Characterize the safety of XP-102, Evaluate the pharmacokinetics of XP-102, Establish maximum tolerated dose of XP-102	Phase 1 Phase 2	Not yet recruiting
NCT05195632	55 participants Multicenter, Open-label, phase 2 study	Encorafenib plus Binimetinib	First line	DLTs, ORR	Phase 2	Not yet recruiting
NCT02974725	331 participants Phase Ib, Open-label, Multicenter Study	LXH254 plus LTT462 or LXH254 plus Trametinib or LXH254 plus Ribociclib	First line	DLTs, AEs, Tolerability	Phase 1	Recruiting
NCT04620330	100 participants Multicenter, Non-randomized, Open-label Phase 1b/2 study	VS-6766 or VS-6766 plus Defactinib	First line	the optimal regimen, the efficacy of the optimal regimen	Phase 2	Recruiting

of dabrafenib and trametinib, may benefit patients with V600E mutation and positive PD-L1 (112). Among the 2 patients in the prospective match-R study, 1 patient was switched to chemotherapy and then dual-target therapy after double-target drug resistance. Another patient, after double-target drug resistance, was first switched to immunotherapy, followed by chemotherapy, and then sequential double-target therapy, all of which achieved disease stability in the "re-challenge" of double-target therapy (113). These explorations are expected to become hot research directions in the future, and we eagerly look forward to more effective drugs or treatments. The undergoing studies for BRAF activation were listed in **Table 3**.

CONCLUSIONS

There are many forms of activation of BRAF in primary and secondary activation, including classical mutations (BRAF V600 and non-V600), other mutations, BRAF fusion, rearrangement, in-frame deletions, insertions, co-mutations, etc. with different

biological phenotypes, medical senses and different subsequent treatments. Currently, the FDA recommends dual targeted drug combination for BRAF V600, while there is no unified treatment regimen for other types of BRAF mutations. As for the treatment of primary BRAF co-mutations, it could base on a comprehensive consideration of the phosphorylation level and abundance of the mutant genes, cost effectiveness and adverse events of combined targeted therapy. Immunotherapy can also benefit for patients with BRAF mutations with high PD-L1 expression in small sample size studies. After resistance of BRAF inhibitors, the evolution of BRAF mainly evolves through activation of upstream, downstream and bypass pathways of BRAF. The evolutionary pathway can be tracked by various emerging technologies including genomics, next-generation sequencing, single-cell sequencing and cloning techniques, which may find a solution for the resistance of BRAF inhibitors.

In the future, it's necessary to explore head to head clinical trials to compare targeted therapy with immunotherapy, to develop drugs for other BRAF mutations except V600, to find new strategies for the resistance of BRAF inhibitors. Furthermore, whether BRAF inhibitors can be used as adjuvant/neoadjuvant therapy or re-challenged treatment are likely to be hot topics.

AUTHOR CONTRIBUTIONS

LYZ and JS researched the data, wrote the review, and designed the figure. JGS, LPZ, and QY reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: Two Patients With EGFR Exon 20 Insertion Mutanted Non-Small Cell Lung Cancer Precision Treatment Using Patient-Derived Xenografts in Zebrafish Embryos

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Background: Epidermal growth factor receptor (EGFR) exon 20 insertion mutations are uncommon EGFR mutations and generally resistant to first- and second-generation EGFR-tyrosine kinase inhibitors (TKIs). In precision oncology, treatment regimens are tested for improving the clinical outcomes. Zebrafish embryo tumor transplant models are used in cancer research.

Methods: We report two Chinese females who were diagnosed with stage IV lung adenocarcinoma and shown to harbor EGFR exon 20 insertion mutations by next-generation sequencing (NGS). Then, we established lung cancer patient-derived xenografts using a zebrafish model. The tumor cells were isolated from the patient. For case one, tumor cells were collected from lymph node biopsy, while the tumor cells were obtained from the pleural effusion. Zebrafish were inoculated with tumor cells and placed in the culture medium containing the third-generation EGFR-TKI, osimertinib. Fluorescence microscope photographs were used to record the red fluorescence area, which represented the proliferation and migration of tumor cells in the zebrafish.

Results: Case one was diagnosed with lung adenocarcinoma (cT4N3M1b, stage IVB) and had an EGFR exon 20 mutation (p. N771delinsHH [abundance 14.08%]). Tumor cell proliferation and migration were significantly reduced in the osimertinib group compared with the control group. The patient received first-line osimertinib (160 mg). According to RECIST v1.1, she achieved a partial response. Case two had stage IVA lung adenocarcinoma with a pleural effusion. The pleural effusion sample was selected to obtain tumor cells for injection, and the zebrafish lung cancer model was established. The

proliferation of tumor cells in the osimertinib group was significantly reduced compared to the control group. The migration of tumor cells was not significantly reduced compared to the control group. The patient also received first-line osimertinib (160 mg). The lung lesions were stable, but the pleural effusion was poorly controlled.

Conclusion: Our study demonstrates the applicability of a zebrafish embryos model as an innovative platform to targeted drug testing. More precise methods are needed to select treatment options in the future.

Keywords: non-small cell lung cancer, zebrafish, EGFR mutation, xenograft, screening

1 INTRODUCTION

The emergence of targetable oncogenic driver alterations has transformed treatment models of non-small cell lung cancer (NSCLC) by incorporating tumor genotyping into therapeutic strategies. Specifically, epidermal growth factor receptor (EGFR)-activating mutations have resulted in routine use of EGFR tyrosine kinase inhibitors (TKIs) (1, 2). EGFR mutations mainly occur between exons 18 and 21 in NSCLC; common mutations are an EGFR 19 deletion and EGFR mutations in exon 21. Drugs against cancers harboring common EGFR mutations have a response rate of 60%–70%, with a median progression-free survival (mPFS) of 9.2–18.9 months (1, 3–6).

An EGFR exon 20 insertion mutation is an uncommon subtype of EGFR mutation that accounts for 4%–10% of all EGFR mutations (6). Exon 20 insertion mutations include A767-V769dup and D770-N771ins NPG, which are also associated with a lack of sensitivity to first- or second-generation EGFR-TKIs (6, 7). Therefore, the standard treatment for patients with EGFR exon 20 insertions is a chemotherapy-based treatment regimen (8). The development of novel targeted drugs, such as poziotinib (9), mobocertinib (TAK-788) (10), and amivantamab (JNJ-61186372) (11), have shown better efficacy in the treatment of EGFR exon 20 insertion mutations. Poziotinib, is an irreversible pan-HER TKI, initially being investigated in the Asian population, shown a slightly better ORR/DCR and PFS in EGFR exon 20 insertion mutations. Mobocertinib inhibited viability of various EGFRex20ins-driven cell lines more potently than approved EGFR TKIs and demonstrated *in vivo* antitumor efficacy in patient-derived xenografts and murine orthotopic models. Amivantamab inhibited proliferation by effectively downmodulating EGFR-MET levels and inducing immune-directed antitumor activity with increased IFN γ secretion in various models. However, these targeted drugs are not currently available in China.

Osimertinib is an irreversible, selective EGFR TKI that is indicated for sensitizing EGFR and EGFR T790M resistance mutations. Preclinical studies have reported that osimertinib is active in EGFR exon 20 insertion mutant cell lines (12, 13) and some clinical trials have also demonstrated clinical activity in EGFR 20 insertion mutant NSCLC (14–16); however, *in vitro* evidence demonstrated 20 insertion mutation cell lines that responded poorly to osimertinib (12, 13, 17). Several studies have reported that the overall response rate (ORR) and progression-free survival (PFS) differed among patients with 20 insertion mutations (15, 16, 18).

Currently, patient-derived xenografts (PDXs) or patient-derived organoids (PDOs) are used in tumor preclinical models in which the genetic mutation map of tumor characteristics is highly consistent with the original tumor tissue with respect to morphology and genetic characteristics (19, 20). Tumor cell behavior in zebrafish xenografts correlates with human cancers as follows: similar growth kinetics; histology; and proliferation and apoptosis rates (21). Herein, to give patients more precision treatment options, we studied two NSCLC patients with EGFR exon 20 insertion mutations who were the source of PDXs used in zebrafish embryos as *in vitro* tumor models for therapeutic screening. In addition, we assessed the antitumor activity of osimertinib in the two NSCLC patients with EGFR exon 20 insertion mutations.

2 METHODS

2.1 Lung Cancer PDXs Using a Zebrafish Model

In this model, 48 hpf AB wild-type zebrafish embryos were selected for microinjection of tumor cells, with approximately 800 cells/embryo, to establish a PDX model of zebrafish lung cancer. The 48 hpf AB wild-type zebrafish embryos were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). The zebrafish embryos were maintained at 28.5°C in a 14:10 h light/dark cycle. Embryos were obtained by mixing two males and two females in a water tank equipped with a grill to avoid the introduction of new eggs. The fish were mated and spawned at the beginning of the light period. Embryos were collected and placed in petri dishes containing an embryo medium (0.2 g/L of Instant Ocean® Salt in distilled water) at 28.5°C. Whole embryos were pooled and counted, and the malformed embryos were discarded. The age of the embryos is represented by hours post-fertilization (HPF). After removal of the chorionic villi, the embryos were immersed in an embryo medium containing 0.2 mM 1-phenyl-2-thiourea after 24h of incubation at 28.5°C. At 48 HPF, the embryos were anesthetized with 0.0003% tetracaine (Sigma-Aldrich) and placed on a wet agarose pad with the right side up. Approximately 200 cells were injected into the yolk sac of the embryos using a microinjector (im-31; Narishige, Japan). The

zebrafish were placed in an incubator at 28°C for 3 h. The zebrafish that were successfully transplanted with tumor cells and relatively uniform in size were selected using a stereoscopic fluorescence microscope for follow-up observation of tumor migration and angiogenesis. At least 10 fish were subjected to each treatment. The images of the sub-intestinal venous plexus (SIV) were obtained using a Leica MZ10 F fluorescence microscope. For tumor proliferation, a group of 10 embryos was selected and dissociated into a single cell suspension.

One day after tumor cell inoculation (1 dpi), zebrafish embryos of uniform tumor size were screened and randomly divided into control and experimental groups (soaked drugs [grouped by different drugs]). Zebrafish inoculated with tumor cells for 1 day (1 dpi) were placed in the culture medium containing drugs, and the fresh culture medium containing drugs was replaced every 24 h for 3 consecutive days. The red fluorescence area (representing zebrafish with proliferation and migration of tumor cells in the body) were recorded in control and experimental groups with fluorescence microscope photos after 3 consecutive days.

2.2 Follow-Up Data

The patients were followed by one year or until death.

2.3 DNA Extraction and Next-Generation Sequencing

Two paraffin blocks of formalin fixed tissue or mass cells were collected from department of pathology from Affiliated Hospital of Nanjing University of Chinese Medicine. The DNA extraction and the next generation sequencing was conducted by Burning Rock Company (Guangzhou, China).

2.4 Image and Data Analysis

Image J software was used for image processing. Graphpad Prism 8 software was used for statistical analysis. All statistical tests were two-sided, and a P value <0.05 was considered statistically significant.

3 CASE REPORT

The study was approved by the Institutional Review Board of Jiangsu Province Hospital of Chinese Medicine and by the Ethics Committee of Jiangsu Province Hospital of Chinese Medicine. Informed consent was obtained from both patients.

3.1 Case One

A 73-year-old non-smoking female was admitted to the hospital in September 2020 for evaluation of a cough. A computed tomography (CT) scan showed a right middle lobe mass, multiple mediastinal lymph node metastases, metastatic supraclavicular lymph nodes, multiple solid nodules in both lungs with bilateral pleural effusions, and a pericardial effusion. A brain MRI showed no metastases and bone imaging revealed bone destruction in the third thoracic vertebra with bone metastases. Cytology of the pleural fluid showed a profiled epithelial cell mass that was suspected to be adenocarcinoma. A puncture biopsy of the right mediastinal lymph node suggested lung adenocarcinoma (**Figure 1**). The patient was subsequently diagnosed with lung adenocarcinoma (cT4N3M1b, stage IVB). DNA was extracted from the cell block of the pleural effusion for next-generation sequencing (NGS). Genetic testing showed that the patient had an EGFR exon 20 mutation (p.N771delinsHH [abundance 14.08%]).

A lymph node biopsy tissue sample of the patient was selected to obtain tumor cells for injection. A zebrafish lung cancer model was established with the above method for therapeutic screening. Tumor cell proliferation and migration were significantly reduced in the osimertinib group compared with the control group (**Figures 2A, B**).

Therefore, due to the patient's advanced age and PS score of 3, chemotherapy was not acceptable. The patient received first-line osimertinib (160 mg) in October. According to RECIST v1.1, she achieved a partial response (**Figure 3**) after 1 month of treatment, then unfortunately developed interstitial pneumonia. She discontinued osimertinib and received steroid treatment. After 2 weeks, a CT showed interstitial pneumonia that progressed in December. Beginning in December 2020 the patient received anlotinib as second-line treatment; however, the disease progressed and she died on 31 December 2020.

3.2 Case Two

A 52-year-old non-smoking female was admitted to the hospital in October 2020 due to chest pain that persisted > 1 month. A CT scan showed that the right lung had bilateral pleural effusions and pulmonary atelectasis. A brain MRI and bone imaging

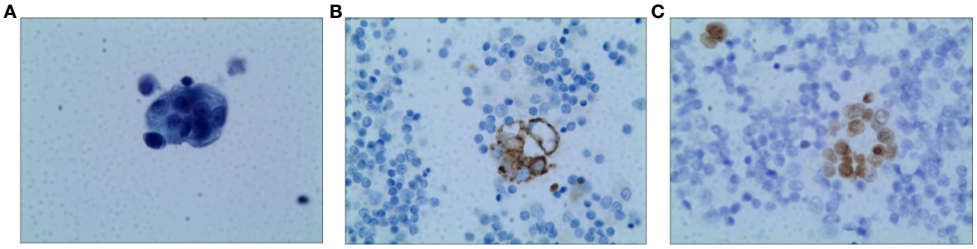

FIGURE 1 | Pathology of Case one patient **(A)** H&E staining suggested lung adenocarcinoma (X400) **(B)** The result of IHC-Napsin A was positive (X400). **(C)** The result of IHC-TTF-1 was positive (X400).

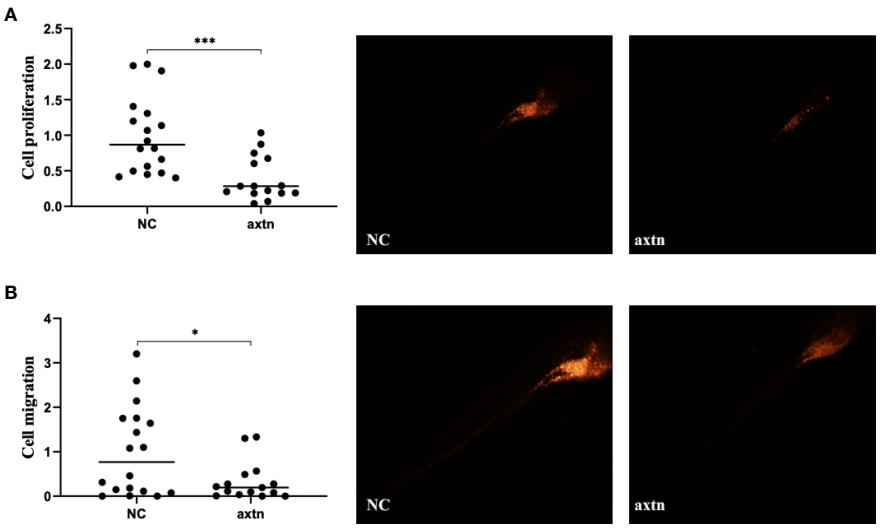

FIGURE 2 | Zebrafish lung cancer model was established **(A)** Tumor cell proliferation was significantly reduced in the osimertinib group compared with the control group **(B)** Migration were significantly reduced in the osimertinib group compared with the control group. Whole-body image of the zebrafish embryo at 4 dpi (25× magnification). *(P<0.05), ***(P<0.001).

showed no metastases. Cytologic evaluation of the pleural fluid was consistent with lung adenocarcinoma (**Figure 4**). The patient was diagnosed with lung adenocarcinoma (cT4N0M1a, stage IVA). DNA was extracted from the cell block of pleural effusion for NGS. Genetic testing showed the patient had an EGFR exon 20 mutation (p.H773_V774insPHPH).

A pleural effusion sample from the patient was selected to obtain tumor cells for injection, and the zebrafish lung cancer model was established with the above method for therapeutic screening. The proliferation of tumor cells in the osimertinib group was significantly reduced compared with the control group, and the difference was statistically significant (**Figure 5A**). The migration of tumor cells in the osimertinib group was not significantly reduced compared with the control group (**Figure 5B**), suggesting that osimertinib could be used as a treatment choice for this patient.

Chemotherapy was not acceptable, thus the patient received first-line osimertinib (160 mg) in October. According to RECIST

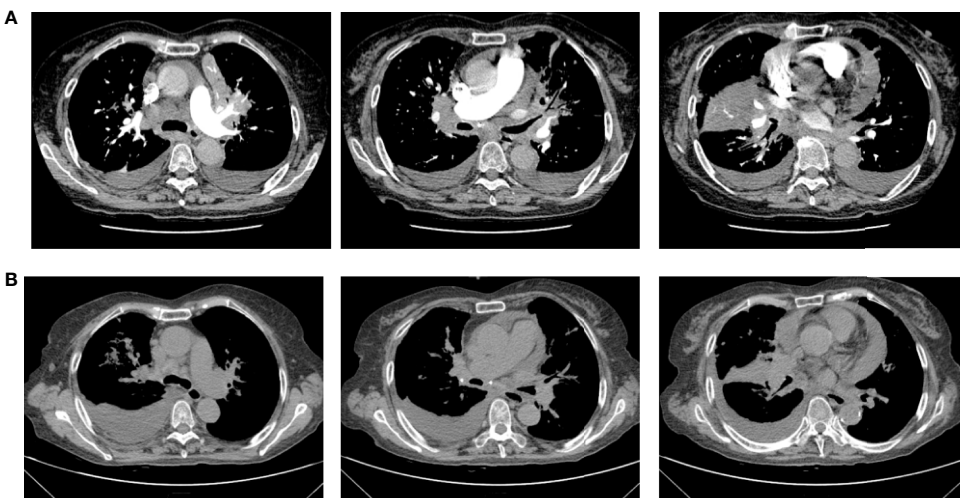

FIGURE 3 | Chest computerized tomography (CT) scan images of patient one before and after one month of osimertinib treatment. **(A)** The images were before osimertinib treatment. **(B)** The images of were receiving osimertinib treatment after one month.

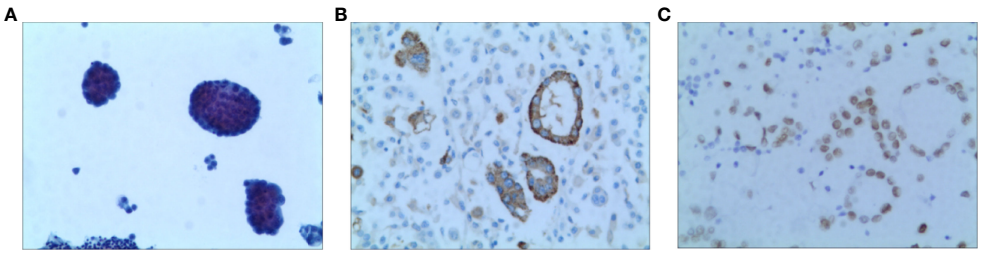

FIGURE 4 | Pathology of Case two patient **(A)** H&E staining suggested lung adenocarcinoma (X100) **(B)** The result of IHC-Napsin A was positive (X400). **(C)** The result of IHC-TTF-1 was positive (X400).

v1.1, she achieved stable disease (**Figure 6**) and the pleural effusion was not controlled. The pleural fluid was repeatedly drained. Osimertinib treatment was continued for 3 months.

4 DISCUSSION

The results of these two cases indicated that third-generation EGFR-TKIs could be used as a therapeutic choice for patients with an EGFR 20 exon insertion mutation, and patient-derived zebrafish embryo xenotransplantation was used as an *in vitro* tumor model for therapeutic screening with accurate prediction and guidance for precise clinical treatment.

Innate immunity in zebrafish plays a key role in engraftment success of implanted cancer cells that is cell line-dependent (22). Currently, > 50 zebrafish models of human cancer have been established in which the histologic and/or genomic levels were closely human counterparts (23). Zebrafish in cancer models have promoted the exploration of new mechanisms and identified new drugs (24). The zebrafish embryos have a short generation time and the transparency which enables non-invasive imaging could facilitate visualization of tumor cell behavior (25, 26). Recently, research illustrated that the zebrafish tumor xenograft platform provide a fast, accurate, and clinically relevant system for evaluation of treatment outcome and invasion/dissemination of PDX models, providing an attractive platform for combined mouse-zebrafish PDX trials and personalized medicine (27). In the current study we collected pleural effusion samples from both patients with lung adenocarcinoma, which successfully established the zebrafish embryo PDX model. This method greatly reduces the time of establishing the PDX model and has the advantage of more rapid screening of effective treatment in the clinic setting.

NSCLC with EGFR exon 20 insertion mutations represent a unique subset of advanced NSCLC patients, in whom target therapy has demonstrated little efficacy and the standard first-line therapy is the same as EGFR-negative patients (28). In recent

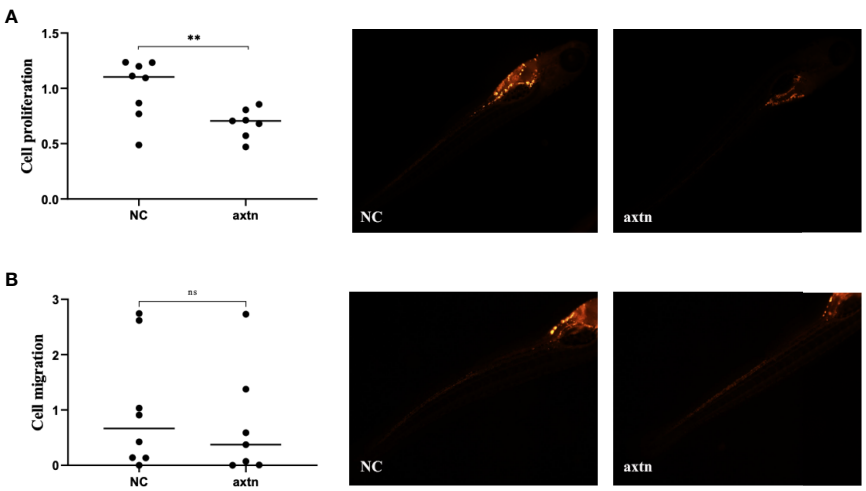

FIGURE 5 | Zebrafish lung cancer model was established **(A)** The proliferation of tumor cells were significantly reduced in the osimertinib group compared with the control group **(B)** The migration of tumor cells was not significantly reduced compared with the control group. Whole-body image of the zebrafish embryo at 4 dpi (25× magnification). **(P<0.01), ns: no significance.

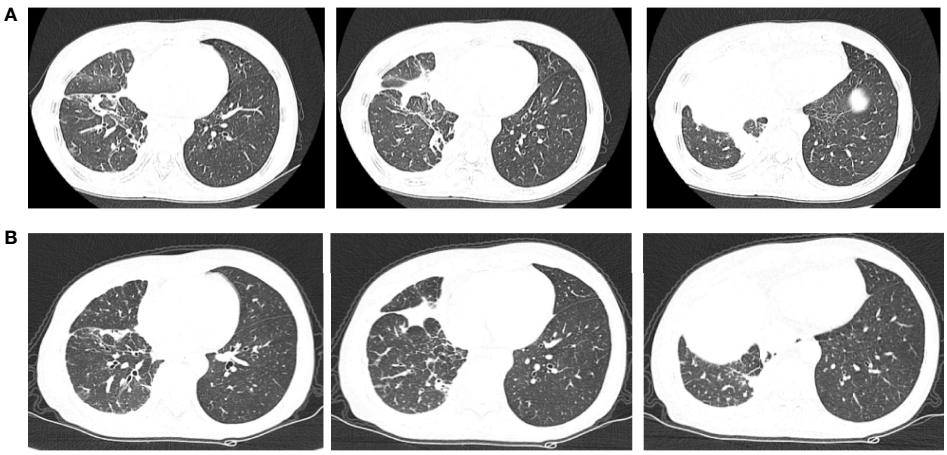

FIGURE 6 | Chest computerized tomography (CT) scan images of patient two before and after one month of osimertinib treatment. **(A)** The images were before osimertinib treatment. **(B)** The images were receiving osimertinib treatment after one month.

years, targeted therapies for EGFR 20 insertion mutations have been explored; however, because of the availability of the drugs and the demonstration in several preclinical studies that osimertinib is active in cell lines with EGFR exon 20 insertion mutations, osimertinib is a preferred treatment option. In some studies, the efficacy of osimertinib has been controversial. Fang et al. (15) reported a mPFS of 6.2 months in 6 Chinese EGFR exon 20 insertion-mutated NSCLC patients. Although there are some studies addressing the poor activity of osimertinib for EGFR 20 insertion mutations. Yang and colleagues (28) showed that the mPFS in 62 patients was 2.3 months and the A763_Y764insFQEA and D770delinsGY might respond better to osimertinib than the other exon 20 insertion subtypes (18). Therefore, we reasoned that different EGFR 20 insertion mutation subtypes have different efficacies for osimertinib treatment. We are of the opinion that the PDX model is a promising platform to perform preclinical drug screening. In case one, drug screening *in vitro* showed that osimertinib significantly inhibited the proliferation and migration of tumor cells. The tumor was significantly reduced and anastomosed after treatment. In case two, drug screening *in vitro* showed that osimertinib inhibited the proliferation of tumor cells, but had no significant effect on migration. The primary tumor of the patient was well-controlled after osimertinib treatment, however, metastatic lesions were not well-controlled, which was consistent with the results of a zebrafish model. Moreover, zebrafish can be used to demonstrate the heterogeneity of tumors. Therefore, two patients received precision treatment through PDXs in zebrafish embryos as *in vitro* cancer models for therapeutic screening.

CONCLUSIONS

In conclusion, we showed the applicability of PDXs in zebrafish embryos model as an innovative platform to targeted drug screening. Our future studies with a larger sample size will focus on more accurate localization of beneficial populations of NSCLC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Written informed consent was obtained from the participant for the publication of this case report.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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