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Advances in the use of EGFR TKIs in the treatment of NSCLC

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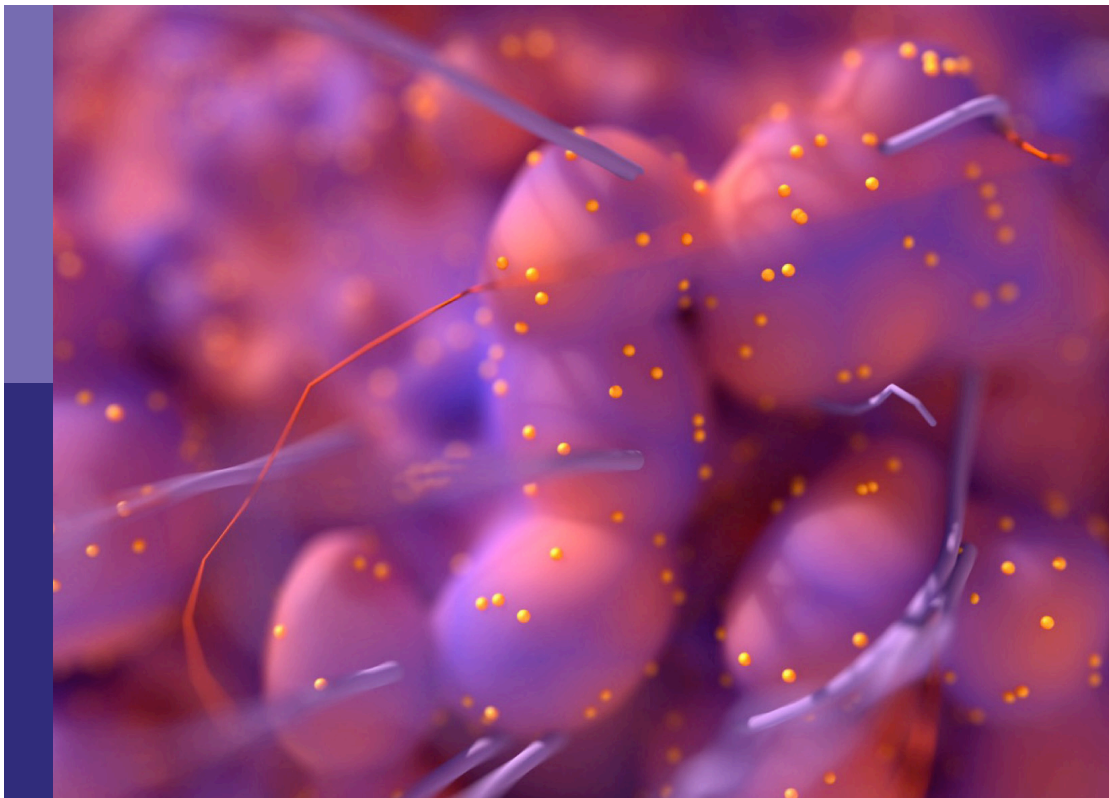

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Advances in the use of EGFR TKIs in the treatment of NSCLC

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Editorial: Advances in the use of EGFR TKIs in the treatment of NSCLC

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KEYWORDS

EGFR, TKI, lung cancer, liquid biopsy, resistance

Editorial on the Research Topic
Advances in the use of EGFR TKIs in the treatment of NSCLC

Lung cancer is the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for approximately 85% of all cases. Over the last decade, there have been significant advances in the treatment of NSCLC, particularly with the use of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs). EGFR is a key driver of NSCLC, and EGFR TKIs have shown remarkable clinical activity in patients with EGFR-mutant NSCLC.

Despite these advances, however, there are still many unanswered questions regarding the optimal use of EGFR TKIs in the treatment of NSCLC. To improve patient outcomes, further research into the use of EGFR TKIs in lung cancer therapy is vital.

This Research Topic collected 20 publications: nine original articles, seven case reports, two reviews, one meta-analysis and one leader opinion.

Several aspects of EGFR TKI treatment have been discussed.

The issue of TKIs resistance has been treated in some papers. In their Opinion, Bronte et al. discussed the topic of Osimertinib resistance and the possible combination strategies or the use of fourth generation EGFR-TKIs to overcome it. They supposed that in the next future oncologists will be able to address each patients who experience resistance to upfront osimertinib toward a different treatment strategy on the basis of molecular alterations highlighted by liquid biopsy or tumor biopsy. In this line, Carlo Bao et al. presented two clinical cases harboring concomitant EGFR and BRAF alterations and treated with Osimertinib. Both cases were responsive to treatment and liquid biopsy results were in accordance with the clinical behavior. Patients with advanced NSCLC with secondary T790M can benefit from Osimertinib, but the role of this drug in patients who exhibit resistance without T790M or with T790M unknown status is not well established. From the systematic review and meta-analysis performed by Yi et al. emerged that patients who undergo progression with brain metastasis on first or second generation TKIs can benefit from subsequent Osimertinib regardless T790M status. This represent an important finding that could orientate clinical decision management after Osimertinib resistance.

Other types of resistance mechanisms are known, and the role of tumor-associated macrophages (TAMs) is also emerging. Previous studies have demonstrated that high infiltration of TAMs is significantly associated with an unfavorable prognosis in NSCLC patients treated with EGFR-TKIs (1). So, the inhibition of TAMs may a potential approach to improving resistance to EGFR-TKIs. In the review reported by Cheng et al., a number of preclinical studies have been discussed, analyzing the combination of EGFR-TKIs with several compound directed versus TAMs. In particular, inhibiting mTOR, AKT and STAT3 pathways, such as the lipid metabolic pathway, could all be possible potential strategies to overcome EGFR-TKI resistance.

The addition of antiangiogenic treatments to EGFR-TKis was also studied. The systematic review and meta-analysis by Zheng et al. highlighted that the addition of bevacizumab to EGFR-TKI provide significant better PFS and OS, and the benefit is more evident for patients who have ever smoked, aged <75 years and of Asian population. Hsu et al. reported the results of a study in which first-line bevacizumab was combined with erlotinib or afatinib in EGFR-mutated NSCLC, demonstrating the development of T790M as resistance mechanism in 57.9% of cases. The addition of anti-angiogenic treatment in patients with an acquired resistance to Osimertinib was also demonstrated in a clinical case described by He et al. In particular, a NSCLC patients harboring acquired EGFR 19Del/T790M/cis-C797S mutation resistance was treated with sintilimab, an anti-VEGF drug and chemotherapy. The patient remained progression-free for 15 months and the regimen was well tolerated. The addition of immunotherapy in EGFR-mutated patients is a controversial topic (2). Microsatellite instability is a rare event in NSCLC, but Yang et al. reported a case of a patient with a rare pulmonary enteric adenocarcinoma with EGFR mutation and MSI-H, who receive benefit from a combination approach of EGFR-TKI plus immune check point inhibitor.

Rare EGFR mutations are another important issue object of several studies in the last years. It was reported that afatinib, an irreversible ErbB family inhibitor, is more efficacious in treating patients with uncommon EGFR mutations (3).

Dong et al. performed a literature search on studies evaluating the efficacy of afatinib in any line of treatment in NSCLC patients with uncommon EGFR mutations. Their conclusion was that afatinib seems to be effective in patients with the more frequent uncommon EGFR mutations, whereas inconsistent data are present with regard to the other uncommon EGFR alterations, due to the high heterogeneity of them. Christopoulos et al. described seven clinical cases of patients carrying different uncommon EGFR mutations treated with afatinib. Overall, patients responded to afatinib, with six partial response and three durable responses. Interestingly, co-mutations did not preclude sensitivity to the drug, with durable response observed also in patients exhibiting co-occurring TP53 or CDKN2A mutations. Another case report was reported by Wang et al., describing a patient carrying two exon 18 mutations and who acquired an EGFR amplification after treatment with osimertinib. The patient showed a partial response to neratinib. Also, a further case report of a patient carrying an EGFR kinase domain duplication was reported by Lin et al. The patient received the 3rd generation EGFR-TKI furmonertinib and obtained a partial response in primary tumor and in central nervous system metastases. The efficacy of Furmonertinib was also studied in a real word setting in the study of Yan et al. The authors analyzed a cohort of EGFR mutated patients treated with furmonertinib as first line treatment. They observed a median PFS of 19.5 months without significant correlations with ECOG, presence of brain or livera metastasis, sex, age EGFR status or number of metastatic sites. The author suggested that the use of furmonertinib could be a valid option for the first line treatment of EGFR mutated patients, also considering the manageable nature of adverse events. Furmonertinib was also studied specifically in EGFR exon 20 insertion positive NSCLC in the study of Hu et al. Patients were treated in the first line setting and the median PFS observed was of 7.2 months, with a good safety profile.

Although adenocarcinoma is the predominat NSCLC histotype carrying EGFR mutation, also adenosquamous cell carcinoma, a low incidence histotype, could in some cases carry an EGFR mutation (4). Xia et al. explored the efficacy of EGFR-TKis. They observed an efficacy of EGFR-TKIs similar to that observed in adenocarcinoma.

EGFR-TKIs are also used in the non-metastatic disease. In the neo-adjuvant setting Shao et al. conducted a small study with the aim to compare the efficacy of neoadjuvant targeted therapy versus targeted combined with chemotherapy in operable EGFR-mutated NSCLC, concluding that targeted therapy alone was equally effective and more safety with respect to the combination regimen. In the adjuvant setting, there is a debatable question about the treatment of patients at recurrence after adjuvant osimertinib. An international Delphi consensus report was published in the paper by Mirza et al., reporting the conclusions of a panel of experts after discussing of this topics. Consensus was reached on six statements describing treatment considerations for the specific NSCLC recurrence scenarios, and agreed that more clinical trials are required before precise recommendations for specific patient populations can be made.

From a biological point of view, the identification of biomarkers able to give prognostic and predictive indications represent a crucial point in cancer. EGFR-mutated NSCLC could have different prognosis and different response to targeted treatment. Secretome is represented by proteins released from tumor cells that could regulate several pathways involved in cancer proliferation. In NSCLC recent studies have identified specific proteins affecting TKI resistance. Luu et al. Performed a mass spectrometry analysis on the secretome of EGFR mutated cells representing different stages of NSCC transformation, and identified three candidates (MDK, GDF15, SPINT2) associated with a poor survival.

Discovery biological studies are needed to identify novel potential biomarkers in NSCLC. Abudereheman et al. reported a study in which RNASequencing and Whole Exome Sequencing results were examined in patients with tuberculosis, with the aim to examine and identify crucial genes implicated in NSCLC genesis. They identified four genes (EGFR, CECR2, LAMA3 and HSPA2)

that play an important role in lung cancer tumorigenesis.

Other than EGFR-TKIs, other targeted treatment are both approved in the clinical practice and also under investigation. A clinical case of a patient with adenocarcinoma and malignant pleural effusion, carrying a ROS1 rearrangement, was reported by Tian et al. The patient was treated with Crizotinib and Anlotinib, with a significant reduction and even disappearance of the malignant effusion, suggesting that this drug combination could be a promising strategy for the treatment of ROS1 rearranged tumors.

Author contributions

PU: Writing – original draft, Writing – review & editing, Conceptualization, Supervision, Validation, Visualization.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SYSTEMATIC REVIEW
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Efficacy of Osimertinib in EGFR-Mutated Advanced Non-small-Cell Lung Cancer With Different T790M Status Following Resistance to Prior EGFR-TKIs: A Systematic Review and Meta-analysis

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Purpose: Epidermal growth factor receptor (EGFR) T790M-negative/unknown advanced non-small cell lung cancer (NSCLC) patients lack subsequent approved targeted therapies. This meta-analysis aimed to assess the efficacy of osimertinib in advanced NSCLC patients with different T790M status after resistance to prior first- or second-generation EGFR-tyrosine kinase inhibitors (EGFR-TKIs) and to predict the subgroups that may benefit beside T790M-positive disease.

Methods: PubMed, Embase, Web of Science, and Cochrane Library databases were searched for relevant trials. Meeting abstracts were also reviewed to identify appropriate studies. Studies evaluating the efficacy and/or survival outcomes of osimertinib in patients with different T790M status (positive, negative, or unknown) after resistance to prior first- or second-generation EGFR-TKIs were enrolled, and data were pooled to assess hazard ratios (HRs) or relative risk ratios (RRs) in terms of overall survival (OS), progression-free survival (PFS), and objective response rate (ORR).

Results: A total of 1,313 EGFR-mutated NSCLC patients from 10 retrospective and one prospective studies treated with osimertinib after resistance to first- or second-generation EGFR-TKIs were included. In overall groups, T790M-positive patients showed an improved OS (HR=0.574, p=0.015), PFS (HR = 0.476, p = 0.017), and ORR (RR = 2.025, p = 0.000) compared with T790M-negative patients. In the brain metastases subgroup, no significant difference in OS was observed between T790M-positive and T790M-negative patients (HR = 0.75, p = 0.449) or between T790M-positive and T790M-unknown patients (HR = 0.90, p = 0.673). In the plasma genotyping subgroup, PFS was similar between T790M-positive and T790M-negative patients (HR = 1.033, p = 0.959).

Conclusion: Patients with progressive brain metastases on first- or second-generation EGFR-TKIs can benefit from subsequent osimertinib therapy regardless of T790M status. Patients with plasma T790M-negative status and lack of tissue genotyping should be allowed to receive osimertinib treatment.

Keywords: non-small cell lung cancer, epidermal growth factor receptor, osimertinib, T790M mutation, brain metastases

INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality, and the most common type is non-small-cell lung cancer (NSCLC), accounting for 85% (1). Because of the high incidence rate and poor prognosis of advanced NSCLC, effective treatment strategies are urgently needed. Activating mutations in the tyrosine kinase domain of epidermal growth factor receptor (EGFR) as one of the significant drivers are mainly found in NSCLC patients; these mutations have motivated the emergency of targeted therapy, which has notably improved the survival of NSCLC patients. For treatment-naive advanced NSCLC patients with EGFR-sensitizing mutations, first-line EGFR-TKIs including first-generation gefitinib, erlotinib, and icotinib, second-generation afatinib and dacomitinib, and third-generation osimertinib and almonertinib have replaced traditional platinum-based chemotherapy as the current therapeutic standard, with a progression-free survival (PFS) range of 9–19.3 months (2–8). Although osimertinib, an irreversible third-generation EGFR-TKI, has been recommended by the National Comprehensive Cancer Network guidelines as a preferred first-line treatment for patients with EGFR-sensitizing mutation advanced NSCLC, first- or second-generation EGFR-TKIs are still an important first-line choice in some parts of the world due to cost and lower overall survival (OS) benefit of osimertinib in subgroups of the Asian population or patients with the 21L858R point mutation compared to first-generation EGFR-TKIs gefitinib and erlotinib observed in the FLAURA study (7).

The most common acquired resistance mechanism to first- or second-generation EGFR-TKIs is a threonine-to-methionine substitution at amino acid position 790 in exon 20 (i.e., T790M mutation), accounting for 49%–73% of the cases of resistance (9–11). Patients with acquired T790M will benefit from subsequent treatment with osimertinib that selectively targets both EGFR-sensitizing mutations and the T790M mutation (12, 13). However, only 50%–60% of resistant patients can undergo tissue rebiopsy to test for T790M (14–16). Plasma circulating tumor DNA (ctDNA), a type of liquid biopsy, is often used as an alternative for genotyping. However, it only exhibits 30%–70% sensitivity for detection of T790M compared with tissue genotyping using next-generation sequencing (NGS) or polymerase chain reaction (PCR)-based detection (17–19). As a result, <30% (14%–27.2%) of patients after resistance to prior EGFR-TKIs can be subsequently treated with osimertinib, and some patients who would likely benefit from osimertinib will go untreated due to a lack of detection or false-negative report of T790M by ctDNA detection (10, 20).

Osimertinib has also been shown to exhibit clinically significant activity for some T790M-negative patients after resistance to first- or second-generation EGFR-TKIs, especially in patients with brain metastasis (BM) (21, 22). Therefore, this meta-analysis aimed to assess the efficacy of osimertinib in advanced NSCLC patients with different T790M status after resistance to prior first- or second-generation EGFR-TKI treatment and to predict the subgroups that may benefit.

METHODS

Search Strategy

PubMed, Embase, Web of Science, and Cochrane Library databases were searched using the following search terms: ("non-small cell lung cancer" OR "NSCLC") AND ("osimertinib" OR "AZD9291" OR "third-generation EGFR-TKI") AND (("EGFR" AND "mutation") OR ("epidermal growth factor receptor" AND "mutation")) to find relevant articles. In addition, abstracts from the American Society of Clinical Oncology (ASCO), European Society of Medical Oncology (ESMO), and World Conference on Lung Cancer were reviewed. Finally, the reference lists of the eligible articles were manually checked to ensure all relevant literature was retrieved. The search end date was October 26, 2021. The article search was performed separately by two investigators.

Eligibility Criteria

Studies that met the following criteria were included (1): advanced EGFR-mutant NSCLC patients treated with third-generation EGFR-TKIs after resistance to first- or second-generation EGFR-TKIs (2); evaluation of the efficacy and/or survival outcomes of different T790M statuses (positive, negative, or unknown); and (3) outcomes including at least one of the following endpoints, namely, overall survival (OS), PFS, ORR, and duration of response (DOR). The selection of articles was separately performed by two investigators based on a common set of criteria. Differences in opinion were settled through discussion.

Data Extraction

The extractable data included authors, year of publication, number of patients, gene detection information (T790M positive, negative, or unknown) after resistance to prior-generation EGFR-TKIs, BM status, genotyping sample types, OS, PFS, and hazard ratios (HRs) with 95% confidence interval (CI) for OS and/or PFS, ORR, DOR. Data extraction was performed separately by two investigators.

Statistical Analysis

All statistical analyses were performed with STATA 14.0 (StataCorp, College Station, TX, USA). The primary endpoints

were OS and PFS, and the secondary endpoints were ORR and DOR. The effects of all outcomes were presented with HRs or relative risk ratios (RRs), 95% CIs, and p-values. Subgroup analyses were performed on BM and genotyping samples. HRs and 95% CIs were estimated using the procedures described by Tierney et al. if not reported in a study (23). Kaplan–Meier curve data were recovered *via* Engauge Digitizer version 11.1. This process was repeated two times to reduce variability. The I^2 statistic was applied to evaluate heterogeneity. The random effect models were chosen if I^2 was >50% or the p-value was <0.05, implying obvious heterogeneity; otherwise, fixed-effects models were applied. Two-sided $p < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the Included Studies

A total of 1,313 EGFR-mutated NSCLC patients from 10 retrospective and one prospective study (18, 21, 22, 24–31) treated with osimertinib after resistance to first- or second-generation EGFR-TKIs were included in the meta-analysis **(Table 1)**. A Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) flow diagram of the retrieval process is presented in **Figure 1**. Eight studies compared outcomes between T790M-positive and T790M-negative patients, one compared outcomes between T790M-positive and T790M-unknown patients, and two provided survival outcomes of BM subgroups among T790M-positive, T790M-negative, and T790M-unknown patients. The percentages of T790M-positive, T790M-negative, and T790M-unknown patients were 65.80%, 26.70%, and 7.50%, respectively **(Figure 2)**.

Comparison Between T790M-Positive and T790M-Negative Patients

Overall Group

As shown in **Table 2**, overall OS in osimertinib-treated patients was 18.53 months (95% CI, 16.48–20.59) vs. 13.90 months (95% CI, 11.95–15.85) in T790M-positive and T790M-negative groups, respectively, with an HR of 0.57 (95% CI, 0.37–0.90; p = 0.015) (**Figure 3A**). Overall PFS for T790M-positive vs. T790M-negative groups was 9.14 months (95% CI, 8.22–10.06) vs. 3.96 months (95% CI, 3.07–4.85), with an HR of 0.58 (95% CI, 0.36–0.91; p = 0.017) (**Figure 3B**). Overall ORR for T790M-positive vs. T790M-negative groups was 58.41% (95% CI, 52.82–63.99) vs. 24.20% (95% CI, 16.22–32.17), with an RR of 2.03 (95% CI, 1.59–2.58, p < 0.001).

Subgroup of Plasma Detection

PFS was not different between plasma detection T790M-positive and T790M-negative subgroups: 9.09 months (95% CI, 8.16–10.02) vs. 9.84 months (95% CI, 8.00–11.69), respectively, with an HR of 1.02 (95% CI, 0.44–2.35) (p = 0.959). ORRs in T790M-positive and T790M-negative subgroups were 63% (95% CI, 55.50–70.50) and 46% (95% CI, 36–56), respectively, with an RR of 1.36 (95% CI, 1.07–1.73; p < 0.001). Tissue genotyping outcomes were extracted from one study with PFS of 9.70 vs. 3.40 months, respectively, in T790M-positive and T790M-negative patients (HR, 0.36; 95% CI, 0.26–0.49) (18). Results are summarized in **Table 3**.

Comparison Among BM Patients With Different T790M Mutation Status

T790M-Positive vs. T790M-Negative Groups

Pooled results of the subgroup with regard to BM demonstrated that there was no significant difference in OS between the T790-positive and T790-negative groups. OS in T790M-positive and T790M-negative patients was 16.28 months (95% CI, 13.62–18.94) and 17.50 months (95% CI, 14.61–20.39), respectively, with an HR of 0.75 (95% CI, 0.36–1.58; p = 0.449) (**Figure 4A**). PFS data were only available in one trial: 8.80 vs. 10.80 months, respectively, in T790M-positive and T790M-negative patients (26). These results are summarized in **Table 4**.

T790M-Positive vs. T790M-Unknown Groups

Three studies reported OS in BM patients with T790M-positive and T790-unknown statuses. Pooled OS results in T790M-positive and T790-unknown groups were 20.78 and 22.98 months, respectively (these were calculated using a weighted average of single study medians because of insufficient data of the 95% CI values) (32), with an HR of 0.90 (95% CI, 0.55–1.47; p = 0.673) **(Table 4; Figure 4B)**.

T790M-Positive vs. T790M-Negative vs. T790M-Unknown Groups

A direct comparison of BM patients with the three T790M statuses was also performed in two studies. OS was 22.59, 21.17, and 24.86 months in T790M-positive, T790M-negative, and T790M-unknown groups, respectively; these were calculated using a weighted average of single study medians because of insufficient data of the 95% CI values (32) (**Table 4**).

DISCUSSION

Patients with advanced NSCLC harboring a secondary EGFR T790M mutation following treatment with first- or second-generation EGFR-TKIs can benefit from subsequent treatment with osimertinib. However, other patients exhibiting resistance with T790M-negative/T790M-unknown statuses lack subsequent approved targeted therapies, and the efficacy of osimertinib in these patients remains unclear. Therefore, it is necessary to explore other subgroups of patients who may benefit from osimertinib treatment to expand its scope of application. Our meta-analysis showed that patients with plasma T790M-negative status or BM patients with T790M-negative or T790M-unknown statuses had similar efficacy to that of T790M-positive patients when treated with osimertinib, suggesting that patients with BM progression with first- or second-generation EGFR-TKIs can benefit from subsequent osimertinib therapy regardless of T790M status, and patients with plasma T790M test-negative status and lack of tissue rebiopsy and genotyping should be

TABLE 1 | Study characteristics.

Study(year)	Study arms	No. of patients (n)	No. of BM patients (n)	Genotypingsamples	Study endpoints	Results (T790M+ vs. T790M–/unk)
Xu H[24] (2021) (26)	T790M+	16	16	Plasma/tissue/CSF	OS/PFS	mOS[a,d]: 11.40 vs. 17.20 mos
	T790M–	24	24			mPFS[a,d]: 8.80 vs. 10.80 mos
						mPFS[b]: 8.60 vs. 11.10 mos
Zhang M[22] (2021) (24)	T790M+	28	7	Plasma/tissue/CSF	OS	mOS[a]: 15.92 vs. 9.00 mos
	T790M–	16	16			mOS[d]: 22.15 vs. 13.39 mos
Yu X[23] (2021) (25)	T790M+	80	80	Plasma/tissue	OS	mOS[a,d]: 27.00 vs. 27.00 vs. 27.00 mos
	T790M–	15	15			
	T790M unk	64	64			
Lee J[25] (2020) (27)	T790M+	60	60	Plasma/tissue/CSF	OS	mOS[a,d]: 16.70 vs. 18.80 vs. 14.30 mos
	T790M–	37	37			
	T790M unk	13	13			
Eide IJZ[20] (2019) (22)	T790M+	120	–	Plasma/tissue	OS/PFS/ORR/ DoR	mOS[a]: 22.50 vs. 13.40 mos
	T790M–	52	–			mPFS[a]: 10.80 vs. 5.10 mos
						ORR[a]: 60 vs. 28%
						DOR[a]: 11.80 vs. 10.70 mos
Yang JCH[19] (2019) (21)	T790M+	20	20	Plasma/tissue	OS/PFS/ORR/ DoR	mOS[a,d]: 8.10 vs. 16.60 mos
	T790M unk	21	21			mPFS[a,d]: 8.00 vs. 12.30 mos
						ORR[a,d]: 45 vs. 38%
						DOR[a,d]: 7.00 vs. 15.10 mos
Mehlman C[27] (2019) (29)	T790M+	184	–	Plasma/tissue	OS/PFS/ORR	mOS[a]: 27.00 vs. 14.20 mos
	T790M–	35	–			mPFS[a]: 11.50 vs. 6.00 mos
						ORR[a]: 54 vs. 40%
Mu Y[26] (2019) (28)	T790M+	77	–	Plasma/tissue	PFS/ORR	mPFS[a]: 8.60 vs. 3.20 mos
	T790M–	15	–			ORR[a]: 51.40 vs. 26.70%
Saboundji K[28] (2018) (30)	T790M+	13	–	Plasma/tissue	PFS/ORR	mPFS[a]: 49.57 vs. 58.36 mos
	T790M–	7	–			ORR[a]: 100 vs. 54%
Oxnard GR[16] (2016) (18)	T790M+	164	–	Plasma/tissue	PFS/ORR	mPFS[b]: 9.70 vs. 8.20 mos
	T790M–	102	–			ORR[b]: 63 vs. 46%
						mPFS[c]: 9.70 vs. 3.40 mos
						ORR[c]: 62 vs. 26%
Jänne PA[29] (2015) (31)	T790M+	138	–	Plasma/tissue	PFS/ORR	mPFS[a]: 9.60 vs. 2.80 mos
	T790M–	62	–			ORR[a]: 61 vs. 21%

BM, brain metastases; CSF, cerebrospinal fluid; DOR, duration of response; mos, months; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; T790M+, T790M-positive; T790M–, T790M-negative; T790M unk, T790M-unknown.

[a]*Overall.*

[b]*Plasma genotyping group.*

[c]*Tissue genotyping group.*

[d]*Brain metastases group.*

allowed to receive osimertinib treatment, especially in the absence of later standard treatment.

Studies have shown that osimertinib can overcome the resistance of acquired T790M mutation, with median PFS of 9.9–12.3 months and ORR of 60–71% (31, 33, 34). A randomized phase III trial, AURA 3, showed that compared with chemotherapy, osimertinib can significantly improve ORR (71% vs. 31%) and PFS (10.1 vs. 4.4 months) in patients with acquired T790M (35). These encouraging results led to the approval of osimertinib as a subsequent treatment for advanced NSCLC patients who developed resistance to prior EGFR-TKIs and acquired a T790M resistance mutation. However, studies have shown that osimertinib also appears to be effective in T790M-negative resistant patients. A study that enrolled 62 T790M-negative patients receiving osimertinib reported a PFS of 2.8 months and an ORR of 21% (31). In a prospective TREM study, 52 EGFR-TKI-resistant patients with T790M-negative status who received osimertinib treatment showed PFS, OS, and ORR of 5.1 months, 13.4 months, and 28%, respectively (22). Furthermore, some retrospective studies have reported that osimertinib had an ORR of 21%–40% and OS of 14–27 months in prior EGFR-TKI-resistant T790M-negative patients (25, 28, 29). This efficacy is similar to the previously reported efficacy of chemotherapy after EGFR-TKI failure. Two studies (AURA3 and IMPRESS) reported PFS of 4.4–5.3 months and ORR of 31.0%–39.5% in patients treated with chemotherapy after resistance to first- or second-generation EGFR-TKIs (35, 36). In our study, the pooled results of osimertinib-treated T790M-negative patients showed similar PFS (3.96 months) and ORR (24.20%) to previous chemotherapy results, indicating that osimertinib may be clinically significant for some patients with a T790M-negative status, although results were not as significant as with T790M-positive patients. However, it is clear that it will be necessary to identify subgroups of these patients that will truly benefit from treatment with osimertinib.

BM progression is a unique disease progression pattern with insufficient response to anti-tumor drugs and poor prognosis because of the active blood–brain barrier (BBB); it accounts for approximately 40% of prior generation EGFR-TKI-resistant

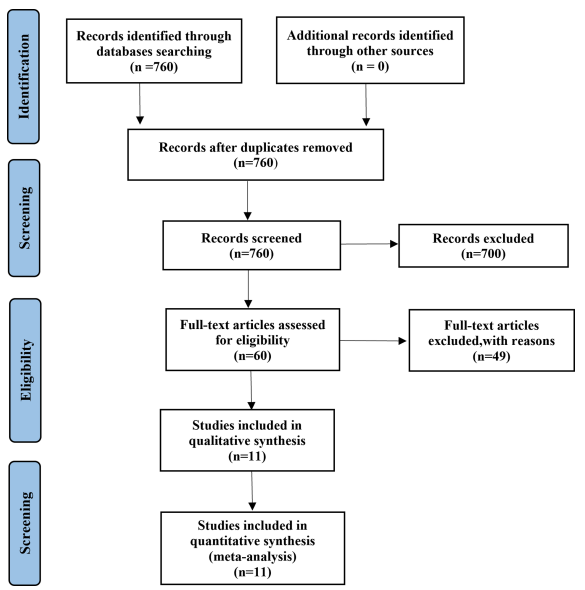

FIGURE 1 | Study selection flow diagram.

metastasis sites (37, 38). In our study, there was no significant OS difference between BM patients with and without T790M, and between those with T790M-positive and T790M-unknown statuses. Furthermore, no significant OS difference was observed in a direct comparison of T790M-positive, T790M-negative, and T790M-unknown patients. These outcomes are generally consistent with the following clinical studies. A retrospective analysis of studies within the AURA series (AURA extension, AURA2, AURA17, and AURA3) exhibited a CNS ORR of 54%–70%, a median CNS PFS of 11.1–11.7 months, and an OS of 18.8 months in T790M-positive patients (33, 34, 39, 40), while some studies also exhibited a CNS PFS of 10.8 months and an OS of 17.2–27 months in T790M-negative patients (24–26). The BLOOM study demonstrated a PFS of 12.3 months and an ORR of 38% in the T790M-unselected population (21). Accordingly, it is worthwhile to discuss whether osimertinib should be used in all patients with progressive BM regardless of T790M status. One of the reasons for the promising efficacy of osimertinib in the CNS may depend on its adequate BBB-penetrating capabilities. The APOLLO and BLOOM studies showed superior BBB penetrations of osimertinib of 31.7% and 16%, respectively (21, 41). However, the BBB penetrations of prior generation EGFR-TKIs were all <6%, with erlotinib at 2.8%–5.1%, gefitinib at 1%–3%, and afatinib at 0.7% (42–45). The insufficient concentration of TKIs in cerebrospinal fluid (CSF), which is less likely to permanently control the dissemination of tumor cells, is crucial in BM after resistance to prior generation EGFR TKIs, apart from the mechanism-induced acquired resistance. Another intriguing circumstance is the mismatching of the T790M mutation detection rate between plasma- or tissue-based genotyping and CSF-based genotyping. A study directly comparing paired plasma and CSF samples in lung adenocarcinoma patients with BM confirmed the lower prevalence of T790M mutation in CSF (3/23) than in plasma (9/23) (46). This result is consistent with other studies reporting a 13%–16% T790M mutation detection rate in CSF, which is significantly lower than the T790M mutation detection rate in plasma of 41%–45% (47, 48). However, one study of 45 EGFR-TKI-treated NSCLC patients with leptomeningeal metastases reported a higher detection rate of the T790M mutation (30.4% vs. 21.7%) and gene copy number variations (CNVs) such as MET (47.8% vs. 0) in CSF than in the plasma, indicating that genetic profiles in CSF may be different from those in plasma, and T790M status in the plasma or primary tumor cannot fully represent the mutation status in CSF (49). In addition, low exposure to first- or second-generation EGFR-TKIs in CSF may also result in "occult" T790M clones within

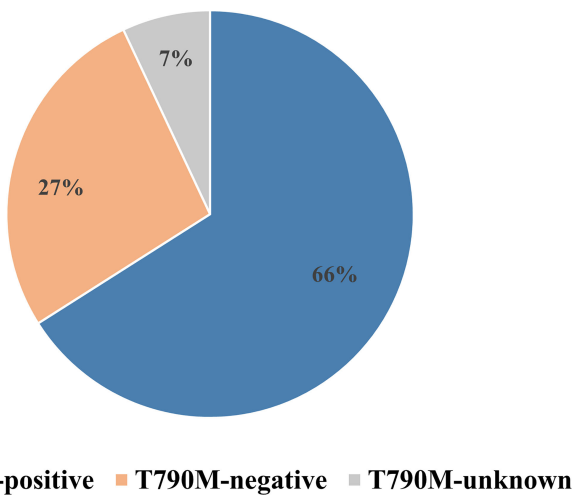

FIGURE 2 | Percentages of T790M-positive, T790M-negative, and T790M-unknown patients in enrolled studies.

TABLE 2 | Pooled results of survival and response rate for the different T790M mutational statuses.

Endpoints	T790M+ vs. T790M–		HR/RR (95% CI)	P value
	No. of studies (patients)	Pooled results (95% CI)		
OS, mos	5 (408) vs. 4 (129)	18.53 (16.48–20.59) vs. 13.90 (11.95–15.85)	0.57 (0.37–0.90)	0.015
PFS, mos	5 (410) vs. 6 (195)	9.14 (8.22–10.06) vs. 3.96 (3.07–4.85)	0.58 (0.36–0.91)	0.017
ORR, %	3 (235) vs. 3 (129)	58.41 (52.82–63.99) vs. 24.20 (16.22–32.17)	2.03 (1.59–2.58)	<0.001
DOR, mos	1 (120) vs. 1 (52)	11.80 (9.85–13.75) vs. 10.70 (5.20–16.20)	NR	<0.001

Abbreviation: 95% CI, 95% confidence interval; DOR, duration of response; HR, hazard ratio; mos, months; NR, not reported; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; RR, relative risk ratio; T790M+, T790M-positive; T790M–, T790M-negative; T790M unk, T790M-unknown.

CSF, i.e., low T790M mutation abundance, which may lead to false-negative test results for the T790M mutation. This may be another reason why some patients with BM progression benefit from osimertinib (50). Recently, a study classifying patients into T790M-positive and T790M-negative cohorts based on detection in CSF showed promising efficacy of osimertinib in the T790M-negative cohort with a median intracranial PFS of 7.0 months (51). Thus, plasma and CSF may be complementary for EGFR-TKI resistance patients with BM progression. However, CSF genotyping-based analyses were not included in this meta-analysis for several reasons. First, these data are from retrospective studies with small sample sizes, leading to various biases, such as low statistical power and inflated effect size estimation. Second, because the absolute amount of tumor-derived cell-free DNA in CSF is very low, the method of detecting mutations in CSF is important to the test results. However, techniques used in CSF detection are under exploration with no definitive conclusion. Therefore, osimertinib may be the better choice for patients with BM progression after prior first- or second-generation EGFR-TKIs, regardless of the T790M status.

Tissue genotyping is currently the standard detection approach due to its sensitivity, but is an invasive procedure that may pose danger or cause treatment delays and is often not feasible. For patients inaccessible to tissue biopsy, liquid biopsy, such as plasma genotyping, may be a non-invasive alternative. In the real world, however, approximately 50% of drug-resistant patients underwent tissue rebiopsy, and 20%–50% patients underwent liquid biopsy (20, 52). Previous studies also showed approximately 70% consistency between liquid biopsy- and tissue rebiopsy-based genetic tests in detecting T790M (18, 19). In our meta-analysis, PFS in plasma T790M-positive and T790M-negative patients was 9.09 vs. 9.84 months. PFS provided by one study in tissue T790M-positive vs. tissue T790M-negative patients was 9.7 vs. 3.4 months. There were dramatic differences observed between tissue and plasma genotyping, indicating that there exist sensitivity differences between these methods. The Cobas EGFR Mutation Test v2 for the analysis of T790M in plasma was approved by the US Food and Drug Administration in 2016 because the detection of L858R point mutation and exon 19 deletions in plasma samples with this test method was highly consistent with that in tissue samples (53). Although plasma genotyping has been widely applied in clinical practice, its sensitivity has not been estimated by well-designed, large-scale prospective randomized trials. In terms of the T790M mutation, Arcila et al. had assessed the credibility of

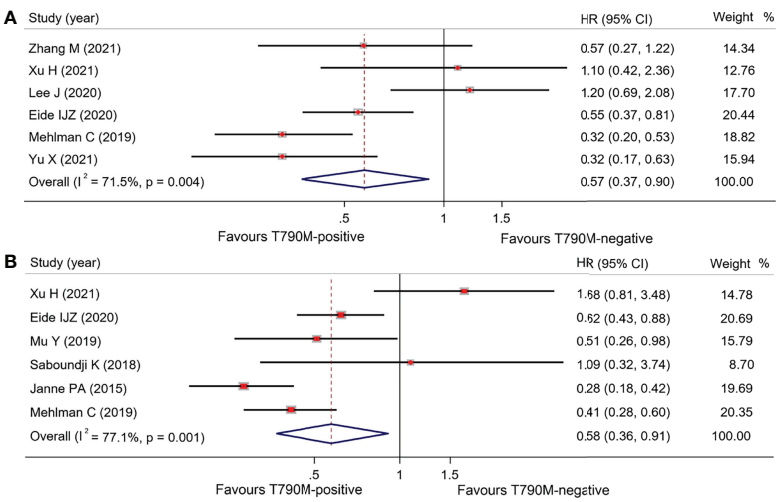

FIGURE 3 | Comparison between T790M-positive and T790M-negative patients of overall group **(A)** and forest plot of OS **(B)**, and forest plot of PFS.

TABLE 3 | Pooled results of survival and response rate the T790M-positive and T790M-negative groups with different genotyping samples.

Genotyping method	Endpoints	T790M+ vs. T790M–		HR/RR (95% CI)	p-value
		No. of studies (patients)	Pooled results (95% CI)		
Plasma	PFS, mos	2 (175) vs. 2 (129)	9.09 (8.16–10.02) vs. 9.84 (8.00–11.69)	1.02 (0.44–2.35)	0.959
	ORR, %	1 (164) vs. 1 (102)	3 (55.50–70.50) vs. 46 (36–56)	1.36 (1.07–1.73)	<0.001
Tissue	PFS, mos	1 (173) vs. 1 (58)	9.70 (7.60–11.80) vs. 3.40 (2.30–4.50)	0.36 (0.26–0.49)	<0.001
	ORR, %	1 (173) vs. 1 (58)	62 (54–70) vs. 26 (14–38)	2.39 (1.52–3.76)	<0.001

95% CI, 95% confidence interval; HR, hazard ratio; mos, months; ORR, objective response rate; PFS, progression-free survival; RR, relative risk ratio; T790M+, T790M-positive; T790M–, T790M-negative; T790M unk, T790M-unknown.

plasma genotyping before the emergence of osimertinib (17). Of 64 patients who were confirmed to harbor the T790M mutation with tissue genotyping, 45 were T790M positive with plasma genotyping, including 11 patients who were positive in the second testing, and the overall sensitivity of plasma genotyping was 70%. In the analysis of AURA extension and AURA studies, the sensitivity was 61% and only 51% in the AURA3 study (33, 34, 39). Furthermore, a cross-comparison study of Cobas, Therascreen, ddPCR, and BEAMing provided sensitivities of 41%, 29%, 71%, and 71%, respectively (53). Plasma genotyping has a relatively high positive predictive value, which can avoid biopsies for most patients, but a large proportion of patients with false-negative T790M mutation may miss the chance of osimertinib treatment. For EGFR T790M-negative patients after prior EGFR-TKI therapy, platinum-doublet chemotherapy is considered the standard treatment with a PFS of 4.5–5.4 months and an ORR of 24–30.9% (54, 55). Data on tissue T790M-negative patients treated by osimertinib after failure of prior generation EGFR-TKI treatment are limited; the only study included in this meta-analysis provided a PFS of 3.4 months (95% CI, 2.3–4.5 months) and an ORR of 26% (95% CI, 14–38%) (18). Therefore, osimertinib appears to have similar efficacy compared to chemotherapy but with more manageable toxicity. As a result, for patients in whom tissue genotyping is ultimately unavailable and are plasma T790M-negative, osimertinib is a moderately recommended subsequent line treatment, and for patients who are tissue T790M-negative, osimertinib may also be a choice given that more than a quarter of patients have a response; at the very least, it has certain advantages over chemotherapy.

There are several limitations to this meta-analysis. First, the number of studies and patients included in this pooled analysis is limited. The major reason is that there are few studies assessing the efficacy of osimertinib in advanced NSCLC patients with T790M-negative or T790M-unknown statuses. Second, the included studies are almost all retrospective, with only one prospective study, so selection bias and public bias are difficult to avoid. Third, we failed to further analyze the different detection methods used in the target population after resistance to prior generation EGFR-TKIs, which may have affected the end results. Therefore, larger-scale clinical studies are needed to confirm the efficacy of osimertinib in advanced

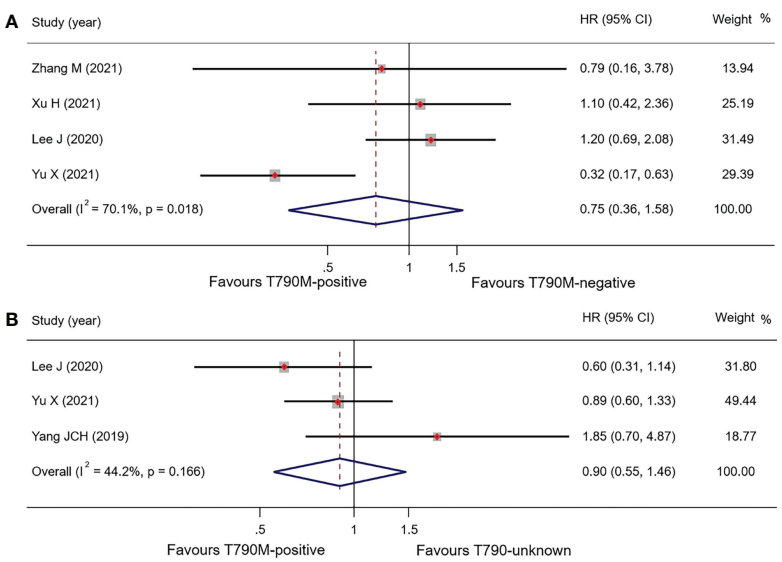

FIGURE 4 | Comparison of BM patients based on T790M mutation status of OS **(A)**, forest plot of comparison of T790M-positive and T790M-negative **(B)**, and forest plot of comparison of T790M-positive and T790M-unknown groups.

TABLE 4 | Pooled results of survival and response rate for the different T790M statuses with brain metastases.

Groups	Endpoints	No. of studies (patients)	Pooled results (95% CI)	HR/RR (95% CI)	p-value
T790M+ vs. T790M–	OS, mos	3 (86) vs. 3 (77)	16.28 (13.62–18.94) vs. 17.50 (14.61–20.39)	0.75 (0.36–1.58)	0.449
	PFS, mos	1 (16) vs. 1 (24)	8.80 (7.30–10.30) vs. 10.80 (7.75–13.85)	NR	<0.001
T790M+ vs. T790M unk	OS, mos	3 (160) vs. 3 (98)	20.78 vs. 22.98	0.90 (0.55–1.47)	0.673
	PFS, mos	1 (20) vs. 1 (21)	8 (3–13) vs. 12.30 (5.95–18.65)	NR	<0.001
	ORR, %	1 (20) vs. 1 (21)	45 (22.50–67.50) vs. 38 (16–60)	1.18 (0.57–2.45)	<0.001
T790M+ vs. T790M– vs. T790M unk	OS, mos	2 (140) vs. 2 (52) vs. 2 (77)	22.59 vs. 21.17 vs. 24.86	NR	NR

Abbreviation: 95% CI, 95% confidence interval; HR, hazard ratio; mos, months; NR, not reported; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; RR, relative risk ratio; T790M+, T790M-positive; T790M–, T790M-negative; T790M unk, T790M-unknown.

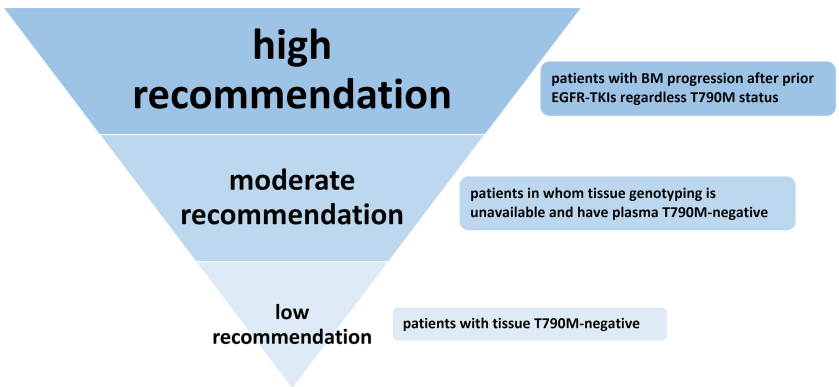

FIGURE 5 | Recommendation level of osimertinib treatment for NSCLC patients with T790M-negative or T790M-unknown status after resistance to first- or second-generation EGFR-TKIs.

NSCLC patients with different T790M statuses following resistance to prior generation EGFR-TKIs.

CONCLUSION

Many studies have shown that when off-target (non-EGFR) pathway resistance mechanisms occur, such as MET/HER2 amplification, BRAF mutation, or RET rearrangement, continuously blocking the EGFR pathway with osimertinib in combination with drugs targeting these off-target activating pathway is a promising treatment strategy regardless of the type of EGFR-TKI treatment previously received. Thus, inhibition of the EGFR pathway is important regardless of the cause of EGFR-TKI resistance. This meta-analysis showed that osimertinib has an encouraging efficacy for plasma T790M-negative patients and progressive BM patients regardless of T790M status after resistance to prior generation EGFR-TKIs. Thus, based on the results of this meta-analysis and given the lack of approved effective targeted therapy, we strongly recommend that patients with progressive BM receive osimertinib treatment, even if the T790M test is negative; we moderately recommend osimertinib as a subsequent treatment for advanced NSCLC patients whose tissue rebiopsy is unavailable (T790M-unknown) and plasma T790M test is negative. Finally, for patients who tested negative for T790M by tissue rebiopsy, we only give a low-level recommendation (**Figure 5**).

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.

AUTHOR CONTRIBUTION

X-FY: writing of the original draft. SJ: data extraction and collection. R-LG: data extraction and collection. LS: software. Z-XW: software. S-LZ: formal analysis. L-TH: table editing. C-BH: conceptualization, methodology, and supervision. J-TM: conceptualization, methodology, manuscript review, and revision. All authors contributed to the article and approved the submitted version.

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Successful sequential tyrosine kinase inhibitors to overcome a rare compound of EGFR exon 18–18 and EGFR amplification: A case report

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Background: New mutational detection techniques like next-generation sequencing have resulted in an increased number of cases with uncommon mutation and compound mutations [3%–14% of all epidermal growth factor receptor (EGFR) mutations]. In rare exon 18 mutations (3%–6%), G719X and E709X represent the majority, but CMut associating these exon 18 points mutations are even rarer, making the understanding of the impact of epidermal growth factor receptor tyrosine kinase inhibitors still limited. Three generations of EGFR tyrosine kinase inhibitors (TKIs) are available to target EGFR mutations, but according to the types of mutations, the sensitivity to TKI is different. Afatinib, osimertinib, and neratinib have showed some effectiveness in single exon 18, but no report has precisely described their efficiency and acquired mechanism of resistance in a CMut of exon 18–18 (G719A and E709A).

Case presentation: We report a case of a 26-year-old woman with bilateral advanced adenocarcinoma of the lung harboring a compound mutation associating G719A and E709A in exon 18, who developed an EGFR amplification as resistance mechanism to osimertinib. She presented a significant clinical and morphological response under sequential TKIs treatment (afatinib, osimertinib, and then neratinib).

Conclusion: A non-small cell lung cancer (NSCLC) with rare compound mutation exon 18–exon 18 (G719A and E709A) and EGFR amplification can be overcome with adapted sequential second- and third-generation TKIs. This

report has potential implications in guiding decisions for the treatment of these rare EGFR mutations.

KEYWORDS

NSCLC, uncommon mutation, exon 18, tyrosine kinase inhibitor (TKI), compound mutations, EGFR

Introduction

Activating epidermal growth factor receptor (EGFR) driver mutations can occur in exons 18–21, and thanks to the development of new techniques of massive sequencing [like next-generation sequencing (NGS)], more than 600 variants of EGFR mutations have been discovered so far. These mutations are classified according to their epidemiological frequencies. On the one side are "common" mutations representing approximately 80% of cases that are constituted by deletions in exon 19 (del 19) and the point mutation L858R in exon 21. On the other side are "rare" or "uncommon" mutations (Umut) with points mutations G719X of exon 18, S768I of exon 20, L861X of exon 21, and insertions/deletions/duplications in exon 20, which represent <20% of the cases. Concerning response to treatment, "common" mutations respond slightly better to EGFR tyrosine kinase inhibitors (TKIs) (1, 2). Exon 18 mutations (detected in 3.6% of all) are mainly represented by the G719X and E709X mutations (3). However, Umut and rare mutations are not fully characterized in their clinical significance and implications, even more when they are associated with each other to form compound mutations (Cmut) (4). Cmut are defined as mutations combining two or more EGFR mutations; they often consist (but not always) of a "common" mutation associated with a "rare" or a "very rare" mutation (2).

To our knowledge, we present here the first case of non-small cell lung cancer (NSCLC) with a rare compound mutation of exon 18–18 (G719A and E709A) treated by a successful sequential use of TKIs (afatinib, osimertinib, and neratinib) and developed an EGFR amplification as mechanism of resistance under osimertinib.

Case report

In January 2016, a 26-year-old Caucasian woman was admitted to the Medical Oncology Department at Avicenne Hospital due to the discovery of a suspicious lung mass in the left lower lobe with bilateral lung lesions on a contrast-enhanced computed tomography (CT) scan performed in the context of low abundance hemoptysis and cervical lymphadenopathy. The patient was healthy in the past, a former light smoker (11 pack-years), and had no disease history reported. Because she had no case of family cancer, germline mutation was not sought. A cervical lymph node biopsy was performed by ultrasound-guided biopsy. The cell morphology was consistent with lung adenocarcinoma, and the tumor cells were positive for thyroid transcription factor-1 (TTF-1). After an extension assessment, the patient was diagnosed with a metastatic lung adenocarcinoma (cT3N3M1a, stage IV A). To clarify the genetic alteration of the tumor, molecular analysis by next-generation sequencing (NGS) of DNA (Tumor Hot Spot MASTR Plus, Multiplicom) was performed on the cervical lymph node biopsy and showed 2 points mutations in EGFR exon 18, namely, p. G719A (C.2156G>T) and p. E709A (C 2126A>C). Immunohistochemistry highlighted a MET hyperexpression (3+) but without MET amplification [fluorescence *in situ* hybridization (FISH), ratio c-MET/chromosome 7 = 2.62]. No other molecular alterations were found.

Afatinib (40 mg daily) was started as first-line treatment based on the UMut. After 2 months, CT showed a partial response (−75%), and she presented a clinical improvement but suffered from a grade 2 (CTCAE v5.0) skin rash, which was relieved by doxycycline. The clinical and morphological response was confirmed for 13 months (Figures 1A, B). In June 2017, faced with an asymptomatic progression on lung lesions and mediastinal lymph node, NGS of circulating DNA (Kit Oncomine Focus Assay) and targeted rebiopsy were done but found no resistance mutations, especially T790M. Several combinations of chemotherapy were prescribed: cisplatin-pemetrexed [progression-free survival (PFS), 15 months] then carboplatin-pemetrexed (PFS, 6 months).

In April 2019, a relapse of thoracic and spleen lesions occurred. Hence, after a molecular board discussion, she received osimertinib (80 mg daily), which allowed an improvement for 16 months until reassessment CT revealed a pulmonary progression. The best morphological response was a stable disease (Figures 1C, D). Osimertinib's plasma concentration monitoring was within standards (231 ng/ml). No mechanism of resistance (T790M and C797S) was found on liquid biopsy, but NGS (Panel Oncomine Comprehensive Assay v3) of new biopsy on lung lesions revealed the same CMut (p.G719A and p.E709A) and a new EGFR amplification (nine copies). Immunohistochemistry highlighted an intermediate ErbB2 hyperexpression (2+) without MET hyperexpression.

From September 2020 to July 2021, she received carboplatin–pemetrexed–bevacizumab (PFS, 5 months), atezolizumab (PFS, 2 months), and gemcitabine (PFS, 3 months).

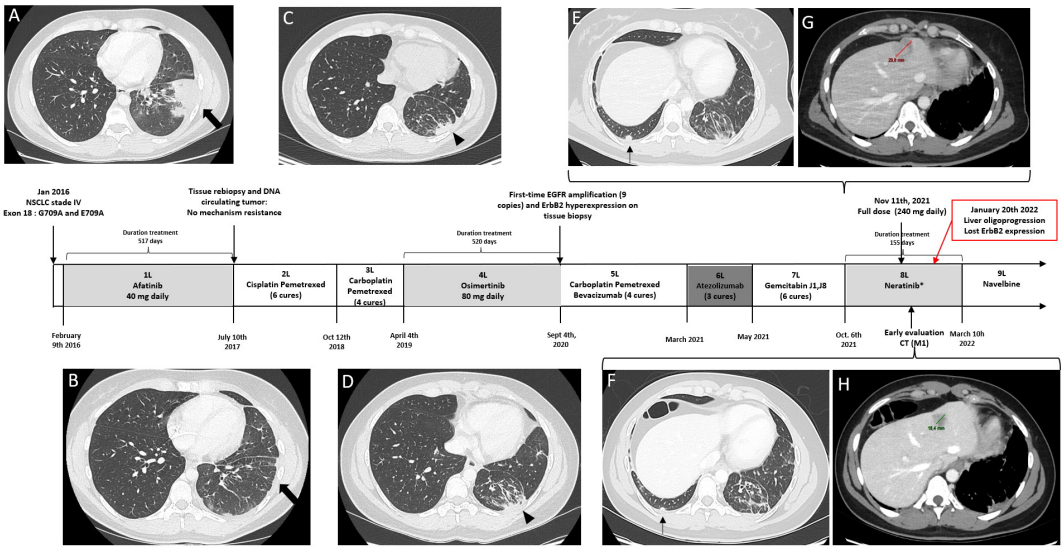

FIGURE 1
Timeline summary with corresponding CT of the different therapeutic lines between February 2016 and November 2021. Baseline chest CT showing **(A)** a lung mass in the left lower lobe. Best response under afatinib with a partial response (**B**, thick arrow). Chest CT before osimertinib's beginning **(C)** and follow-up CT chest with a stable disease as best response (**D**, arrowhead). Baseline chest CT **(E)** and contrast-enhanced liver **(G)** CT before neratinib. At 1 month, early assessment showed a partial response of all lung lesions (**F**, thin arrow) and liver lesions (**H**, a target lesion, 29.8 vs. 18.4 mm, in the longest axis). Red arrow and case: the liver's oligoprogression at second CT evaluation under neratinib and a new liver biopsy was done. Immunohistochemistry showed the loss of ErbB2 hyperexpression. CT, computed tomography.

On 6 October 2021, the patient received neratinib on a compassionate-based access. The TKI was started at 40 mg daily, with a gradual increase of 40 mg/week for a final dose of 240 mg daily; full dose was obtained on 11 November. Early assessment by CT at 4 weeks showed a partial response (−38%) with a decrease in all pulmonary (Figures 1E, F) and liver lesions (Figures 1G, H). She experienced a clinical improvement but suffered from a grade 1 diarrhea. The response was maintained until 20 January 2022, when a dissociated hepatic progression on pre-existing lesions is objectified on the second CT evaluation. At this time, a liver biopsy was performed, followed by a radiofrequency ablation of these lesions while continuing neratinib. On 10 March, a new CT reassessment shows new hepatic and thoracic lesions, corresponding to a clear progression. Molecular analysis on the liver biopsy by NGS (Kit Oncomine Focus Assay) of DNA and RNA found the same CMut of exon 18 and an EGFR amplification without other targetable molecular alteration. Immunohistochemistry found a PD-L1 rate of 50% but no longer found HER2 overexpression. Considering this information, a switch to weekly intravenous navelbine was started.

Discussion

The literature showed that the response to TKIs depends on mutations that form the compound. For instance, in tumors with a CMut including exon 19 deletion or L858R point mutation, Hata et al. showed that they had the same response rate to EGFR-TKI as those with the same mutation alone (5). Therefore, in CMut with common mutations, it is recommended to start osimertinib as a first-line treatment.

For tumors with uncommon CMut, considering the composition of CMut seems also effective. Indeed, for first-generation EGFR-TKI, Chiu et al. demonstrated that patients harboring uncommon CMut (without classical mutations and at least one exon 18 mutation) had a longer PFS than patients with a single exon 18 mutation G719X (11.9 months vs. 6.5 months) (6). This trend is confirmed by Passaro et al. who showed that patients under first- or second-generation TKI with CMut (with an exon 18 mutation) had superior efficacy than patients with a single exon 18 in regard to median overall survival (OS) [hazard ratio (HR), 0.62; 95% CI (0.39–1.00)] (7). Therefore, the question is to know which EGFR-TKI is the most efficient in a CMut exon 18–18.

A *post-hoc* analysis of prospectively collected data from LUX trials showed clinical activity of afatinib in advanced NSCLC harboring UMut (G719X, L861Q, and S768I) (8). Indeed, Yang et al. reported a 71% overall response rate (ORR) and 11 months PFS in this UM. Even better, they found an increase in ORR of 77.8% and PFS of 13.8 months in the G719X subgroup. Hence, afatinib obtained approval by the US Food and Drug Administration (US-FDA) for patients with metastatic NSCLC harboring UMut and by extension, CMut with UMut.

Recently, a multicenter phase II trial (KCSG-LU15-09) demonstrated that osimertinib also has an activity in patients harboring UMut. In this study, osimertinib was given as a front line in 22 cases (61%), and mutations were predominantly G719X (n=19) and L861Q (n=9). Within G719X mutations, four were CMut (two G719 X + L861Q and two G719X + S768I), but none has a CMut exon 18–18, ORR was 53% [95% CI (28%–77%)], and mPFS was 8.2 months [95% CI (6.2–10.2)] (9).

Based on these results, we can say that tumors with a CMut have a preserved sensitivity to afatinib (but lower than common mutation) and that osimertinib is also a good choice in the context of a CMut, even without T790M. Although cross trial comparisons should be performed with caution, it seems more prudent to start with afatinib than osimertinib in view of better results in LUX trials than in KCSG-LU15-09. However, it is important to note that in the *post-hoc* analysis from LUX trials, no information was given concerning the patient's brain status, whereas KCSG-LU15-09 reported 25% patients with brain metastases who could potentially explain this difference. In our case, the patient was free from any cerebral lesions since the beginning, which supports the choice of afatinib before osimertinib.

Moreover, Kobayashi et al. have investigated *in vitro* the sensitivities to three generations of EGFR-TKIs in retrovirally transfected cells that harbor exon 18 (including G719X and E709X) and del 19 mutations. They found that IC_{90} of first- and third-generation TKIs in exon 18 mutations were much higher than those in del 19 (by >11–50-fold), whereas IC_{90} of afatinib were only three- to seven-fold greater than del 19 (3). Therefore, *in vitro* and *in vivo*, second-generation TKI seems more efficient than first or third generations in exon 18 mutation and, by extension, in CMut with exon 18 mutation.

Neratinib is an irreversible pan ErbB (EGFR/HER2/HER4) oral inhibitor, first studied by Sequiest et al. in 2010, who have already demonstrated neratinib efficiency in some patients harboring G719X mutation (10). At this time, it had been more studied for its properties to overcome T790M mechanism resistance than for its high sensitivity to exon 18 mutation. In 2021, the preliminary result of SUMMIT (n=11) confirmed the neratinib efficiency in exon 18 and demonstrated an ORR of 36% [95% CI (11–69)] and a PFS of 6.9 months. Moreover, among them, one patient had the same CMut exon 18–18 as in our case report (G719A and E709A) and presented a partial response lasting for 16 weeks (11). In view of these encouraging results from the SUMMIT trial and the presence of ErbB2 overexpression under osimertinib, neratinib seemed to be the best therapeutic alternative to target at the same time the CMut exon 18–18 and the ErbB2 hyperexpression.

There are limitations to data about sequential treatments for uncommon exon 18 mutation. Indeed, in the KCSG-LU 15-09 trial (9), one of the exclusion criteria was previous treatment with EGFR-TKI, and in the SUMMIT trial (11), 91% (10/11) patients have prior EGFR-TKI, but they have no clue regarding these therapeutic sequence and resistance mechanism prior to EGFR-TKI.

The strength of our case is the precise description of a workable therapeutic sequence of TKIs that allowed prolonged response, causing a dramatically improved OS of an exon 18 mutation and EGFR amplification. Indeed, EGFR amplification is a usual acquired EGFR-dependent mechanism after osimertinib in first (~10%) or second (6–10%) line for patients with common mutations but not yet described in the setting for CMut exon 18–18 (12, 13).

Even more, this case is interesting because she also had a moderate ErbB2 hyperexpression found at progression on osimertinib, which is known to potentially lead to a decreased sensitivity to osimertinib (14). Therefore, the osimertinib resistance mechanism is potentially heterogeneous with association of several resistance mechanisms usually found in common mutation. This hypothesis is reinforced by the fact that the liver's oligoprogression under neratinib was accompanied by the disappearance of ErbB2 hyperexpression, which had probably contributed to an initial and brief sensitivity to the pan ErbB, neratinib.

Concerning alternatives after navelbine, antibody–drug conjugate (patritumab–deruxtecan or HER3-DXd) and bi-specific antibodies (amivantamab) seem to be particularly interesting, especially since these molecules are currently tested in situations of osimertinib failure (15, 16). These antibody drugs are even more attractive, as they have been tested in situations where the resistance mechanisms were not clearly defined. Indeed, Jänne et al. showed recently in a phase II trial that patritumab–deruxtecan was efficient in EGFR-mutated NSCLC heavily pretreated, regardless of the brain status, the resistance mechanism identified, and the level of HER3 expression. They had received, in median, four lines [1–9] of treatment before, including osimertinib (89%), a platinum doublet (80%), and immunotherapy (35%). The objective response rate was 39% [95% CI (26.0–52.4)], the control rate was 72% [95% CI (58.5–83)], and the median progression-free survival was 8.2 months [95% CI (4.4–8.3)] (15). In this case and given the limited state of knowledge on the mechanism of resistance and the sensitivity of TKIs in rare CMut, theses antibody therapies have a place of choice.

Furthermore, it should not be forgotten that a retreatment effect linked to prolonged periods of "TKI holidays" is also possible in our clinical case. Indeed, the effect of retreatment is well described in common mutations (17). It is based on the fact that after one or more lines of TKI, the resistance mechanisms are often heterogeneous and multiple in the same patient (20%–50% of patients according to studies that analyzed resistance mechanisms by circulating tumor DNA after a third-generation TKI) (18, 19). Indeed, these patients will present different subpopulations of cancer cells with heterogeneous mutations, which can be potentially insensitive to EGFR-TKI. The prescription of chemotherapy following EGFR-TKI would make it possible to target all subpopulations and thus reduce the proportion of resistant clones and then to restore sensitivity

to TKI by promoting the re-emergence of clones with a targetable mutation (20). This principle was well described in the study by Ichihara et al. who retrospectively studied the rechallenge of osimertinib after chemotherapy treatment in 15 patients who relapsed after osimertinib. During osimertinib rechallenge, the authors found an ORR of 33%, a disease control rate (DCR) of 73%, and a PFS with 4.1 months, which showed a resensitization to osimertinib. Thus, one of a possible confounding factor in our case is the prescription of chemotherapy between each line of TKI, whose periods could go up to 9 months. Nevertheless, the effect of retreatment is not yet known in UMut and particularly in rare CMut.

As a conclusion, this case emphasizes the potential benefit of sequential TKI therapy of second followed by third generation despite the absence of T790M mutation in patients with UMut. Resistance to third-generation EGFR-TKIs may involve EGFR amplification and probably ErbB2 hyperexpression in CMut exon 18–18 and can be treated by a pan ErbB inhibitor. Reports of these rare compound mutations, mechanisms resistances, and their response to TKIs are necessary to improve knowledge of this UMut. However, more research is needed before neratinib can be recommended as a new standard in CMut exon 18–18.

Data availability statement

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

Author contributions

Conceptualization: PW and BD. Methodology: BD. Investigation: PW, BD, EF, and AM. Ressources: PW. Supervision: BD. Validation: PW, EF, AM, KC, AB, LM, and BD. Visualization: PW and BD. Writing – original draft: PW and BD. Writing – review & editing: PW, EF, KC, LM, and BD. All authors contributed to the article and approved the submitted version.

Conflict of interest

BD: Payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events: Pfizer, Roche, Chiesi. Payment for expert testimony: AstraZeneca, BMS.

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Publisher's note

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Inconsistent clinical outcomes following afatinib treatment in NSCLC patients harboring uncommon epidermal growth factor receptor mutation

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Background: Uncommon epidermal growth factor receptor (EGFR) mutations consist of a heterogeneous population of molecular alterations, and the available clinical data on the outcomes of patients with non-small-cell lung cancer (NSCLC) harboring uncommon EGFR mutations following afatinib treatment are limited. The purpose of this pooled analysis was to investigate the clinicopathological features of patients with uncommon EGFR mutations (um-EGFRms) along with their treatment response and survival outcomes following afatinib treatment.

Methods: We performed a literature search in the NCBI PubMed database to identify relevant articles and conducted this pooled analysis based on 70 studies. The relationships between patient clinical characteristics, EGFR mutation type and the response to afatinib treatment were analyzed using univariate chi-square analysis, and survival analysis was performed using the Kaplan–Meier method.

Results: Data from a total of 99 patients were included in the pooled analysis. The objective response rate (ORR) to treatment with afatinib was53.5%, with a median progression-free survival (mPFS) of 9.0 months. For patients administered first-line afatinib treatment, the ORR and median PFS were 73.5% and 15.6 months, respectively, which were both superior to those of patients treated with second- or later-line treatments (ORR:37.0%, $p < 0.001$; mPFS: 6.0months, $p = 0.001$). Moreover, patients with a single um-EGFRm were more likely to have a favorable response and prognosis benefit after treatment with afatinib than patients with multiple one (ORR: 63.3% vs 38.5%, p=0.017; mPFS: 15.6 months vs 6.0 months,p=0.010). Moreover, single um-EGFRm were independent predictive factors for better treatment response and superior PFS. Subgroup analysis indicated that patients harboring major um-

EGFRms (i.e., L861Q, G719X, and S768I) exhibited the best treatment responses and prognoses (ORR: 74.1%, mPFS: 15.6 months), by contrast, patients harboring multiple um-EGFRms comprising 19del/L858R had the worst treatment responses and prognoses (ORR: 23.5%, mPFS: 5.6months).

Conclusions: Patients with um-EGFRms exhibit favorable but inconsistent responses and survival outcomes following afatinib treatment, which closely related to the mutation pattern and cooccurring partner mutant genes. Administering afatinib for the treatment of patients with um-EGFRm might be considered an effective treatment option in some circumstances, but this recommendation requires further clinical studies for verification.

KEYWORDS

afatinib, uncommon, EGFR, efficacy, prognosis, NSCLC

Introduction

Epidermal growth factor receptor (EGFR) mutations play an important role in the pathogenesis of non-small-cell lung cancer (NSCLC) and are one of the main oncogenic drivers of NSCLC. The frequency of EGFR mutations in NSCLC patients in the Caucasian population is 10%-20%, while it is as high as 30%-60% in the Asian population (1–3). The most prevalent EGFR mutation is exon 19 deletion (19del), followed by point mutation L858R in exon 21 (3). Both are considered to be common and sensitive mutations of EGFR, accounting for 80-90% of mutations in the EGFR gene (3–5). A number of clinical studies have confirmed that, compared with traditional chemotherapy, treatment with EGFR-tyrosine kinase inhibitors (EGFR-TKIs) results in an objective response rate (ORR) as high as 70%-80%, a median progression-free survival (mPFS) of 9.6 months to 18.9 months, and an overall survival (OS) of 21.6 months to 34.1 months (6–12). Nowadays, EGFR-TKIs have become the first-line standard treatment for advanced NSCLC patients with EGFR-sensitive mutations. Additionally, other types of EGFR mutations, such as insertions in exon 20 (20 ins), G719X in exon 18, S768I in exon 20, and L861Q in exon 21 were also found, which are called uncommon EGFR mutation (um-EGFRm), accounting for approximately 10% to 15% of EGFR mutations (13–16). Since patients with um-EGFRms are relatively insensitive to treatment with EGFR-TKIs, which may have a negative impact on research results, most clinical trials investigating the efficacy of EGFR-TKIs do not include patients with this mutation type (13–16). Due to small sample size and high heterogeneity, the efficacy of EGFR-TKIs for patients with um-EGFRms is still unclear. With the rapid development of genetic testing technology, the detection rate of um-EGFRms will continue to increase, and it is of great significance to better understand the sensitivity, efficacy and prognosis of these patients to various TKIs.

It was reported that afatinib, an irreversible ErbB family blocker, is more effective than first-generation TKIs in treating patients with um-EGFRms (14, 17–25). The Food and Drug Administration (FDA) has approved afatinib for the treatment of metastatic NSCLC patients with major um-EGFRms (G719X, S768I, and L861Q). However, unlike common EGFR mutations, which only include two types, um-EGFRms are a class of highly heterogeneous mutations. Due to the low frequency of um-EGFRms and the uncertain efficacy of afatinib, the number of patients receiving afatinib in clinical practice was relatively small (13, 14). Thus, we conducted this pooled analysis to explore the clinical characteristics of patients with um-EGFRms, as well as the efficacy and prognosis following treatment of afatinib, so as to provide a reference for clinicians who formulate treatment plans for patients with rare EGFR mutations.

Methods

Search strategy

We performed a literature search in the NCBI PubMed database to identify all the relevant articles without language restriction (the last search update was June 15, 2021). The following search strategy were used: ((afatinib[title/abstract]) and ((EGFR [title/abstract]) or epidermal growth factor receptor[title/abstract])) and ((NSCLC [title/abstract]) or non-small cell lung cancer[title/abstract]). We also manually checked the reference lists of all related articles to add to the research.

Study eligibility

Two authors independently screened the titles and abstracts of the studies from the search results, and a second screening of the full-text articles was performed. If these two authors failed to reach a consensus, a third investigator was consulted to resolve any disagreements and to reach a consensus on all items. Articles were included if they met the following inclusion criteria: 1) studies focusing on patients with non-small-cell lung cancer; 2) studies in which all patients harbored non-ex20ins, uncommon mutations in EGFR (without restrictions in the method and the biological source for mutation test); 3) patients received afatinib in any treatment line; 4) studies indicating treatment response to afatinib; and 5) studies that reported the PFS of patients.

Study objective

The following data of patients were collected: age, gender, ethnicity, smoking history, tumor stage, mutation type, response to afatinib (objective response (OR) was defined as CR+PR), and PFS. Tumor response was defined as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD) based on Response Evaluation Criteria in Solid Tumors. Objective response (OR) was defined as CR+PR. The primary objective of this study is the clinical outcome of patients applying afatinib treatment, which includes objective response rate (ORR) and progression free survival.

Exploratory analysis

Due to the relatively high incidence of L861Q, G719X, and S768I, they are referred to as major uncommon mutations. Considering that uncommon EGFR-mutant NSCLC is a genetically heterogeneous disease, and the FDA has approved the use of afatinib for the treatment of patients with um-EGFRms of L861Q, G719X, and S768I. We have great interested in the outcomes of afatinib in patients with different types of um-EGFRm. Therefore, we conducted subgroup analyses of afatinib efficacy and survival in patients with different um-EGFRm patterns: Group A for major um-EGFRms (i.e., G719X, S768I, and L861Q), Group B for other single um-EGFRms, Group C for multiple EGFR mutations that contains 19del/L858R, and Group D for multiple EGFR mutations that without 19del/L858R.

Statistical analysis

Fisher's exact or chi-squared tests were used to assess the associations between clinical parameters and afatinib efficacy. The Kaplan–Meier method and the log-rank test were used to analyze the association of clinical parameters with PFS, and the associated 95% CIs were calculated. Multivariate analysis was performed using logistic regression models and Cox proportional hazards models to assess the simultaneous effects of prognostic factors on efficacy and survival. The analyses were performed with SPSS 22.0 program (SPSS Inc, Chicago, IL, USA), a two-sided p-value less than 0.05 was considered statistical significance.

Results

Search results

The flow chart of the study selection process is shown in Figure 1. A total of 679 potentially relevant articles were identified from the PubMed database. Two investigators individually screened the titles/abstracts and full texts and then extracted data separately. Finally, 70 articles were included in the pooled analysis (Supplement Table 1).

Patient characteristics

Data from a total of 99 patients were included in the pooled analysis, with a median age of 58 years and a range of 34 to 84 years. The sex distribution was basically balanced (53 males, 53.5%; 46 females,46.5%), and most were Asian patients (Asian 66, 66.7%; non-Asian 33, 33.3%). Nearly one-third of patients had a history of smoking (60, 60.6%), and most patients had stage IV disease (94, 94.9%). In terms of the mutation type, two-thirds of patients had a single um-EGFRm. The baseline characteristics of the patients are detailed in Table 1. In these 99 patients, there were a total of 50 kinds of um-EGFRms. The top six EGFR mutation types were G719X, 18 del, 19 ins, L861Q, 19del/G724S, and S768I, with 14 cases (14.1%), 8 cases (8.1%), 7 cases (7.1%), 7 cases (7.1%), 6 cases (6.1%), and 6 cases (6.1%), respectively (Figure 2; Supplement Table 2).

Clinical outcomes

After treatment with afatinib, of the 99 patients included, none of the patient had a complete response (0.0%), 53 patients had a partial response (53.5%), and 33 patients had stable disease (33.3&); the other 13 patients experienced disease progression (13.1%) (Table 1). Overall, the objective response rate to the treatment with afatinib was53.5%. In univariate analysis, we found that patients receiving first-line afatinib had an ORR of73.3%, which was significantly better than that of patients receiving second- or later-line therapy ($p<0.001$). In addition, we found that there was a significant correlation between smoking and treatment efficacy and that patients without a history of

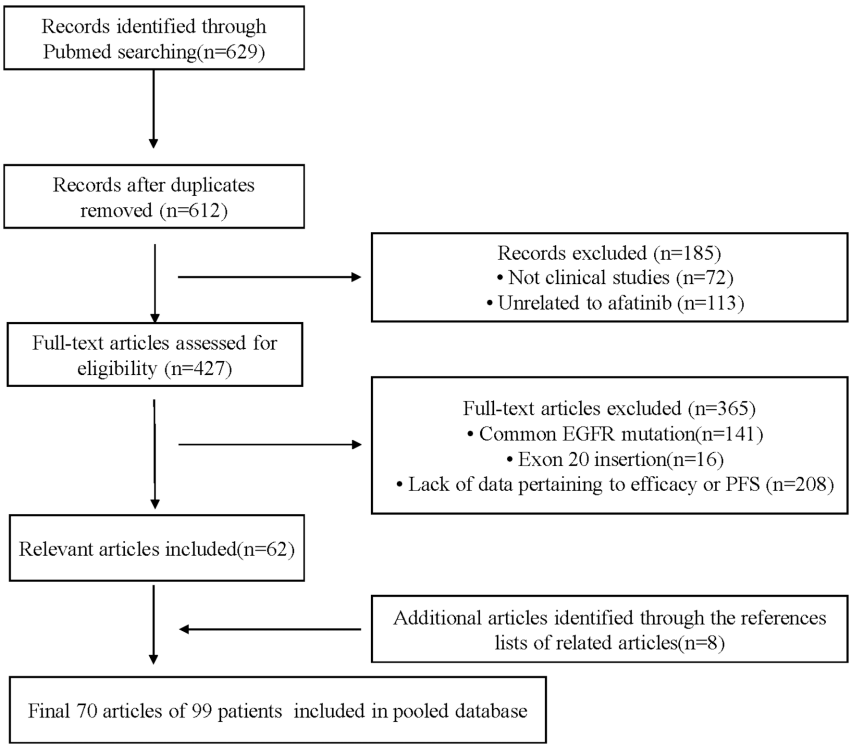

FIGURE 1
Flow chart of study process.

smoking showed a significantly superior tumor response than those who smoked (ORR: 65.0% vs 35.9%, HR: 3.316, 95% CI: 1.428-7.700, p=0.005). Moreover, there is a trend favoring the patients with a single um-EGFRm, the difference in treatment efficacy between patients with single and multiple um-EGFRms was statistically significant (ORR: 63.3% vs 38.5%, HR: 0.362, 95% CI: 0.158-0.831, p=0.017). Other factors (e.g., age, sex, ethnicity, stage) did not show any correlation with efficacy (Figure 3). Further multivariate analysis suggested that not smoking (p=0.020), single um-EGFRm (p=0.040), and first-line treatment (p=0.004) were independent predictive factors for better treatment response (Table 2).

In the overall population, the median progression-free survival time was 9.0 months (Table 1). The mPFS in patients receiving first-line treatment was 15.6 months, which was significantly better than that in patients who received second- or later-line treatment, which was 6.0 months (HR2.346, 95% CI: 1.429-3.849, p=0.001) (Figures 4, 5B). In addition, for patients with a treatment response of OR (p<0.001) (Figure 5C), as well as no smoking history(p=0.012), the PFS was also longer. The Kaplan–Meier curves showed a trend that patients with a single um-EGFRm had longer PFS than patients with multiple um-EGFRms (Figure 5A), the difference was statistically significant (p=0.008). Subsequently, the results of the Cox proportional hazards model showed that the administration of first-line treatment, the objective response to treatment, and with single um-EGFRm were independent prognostic factors for longer PFS (Table 2).

Subgroup analysis

Tumor response and PFS of each individual patient as well as overall tumor response rate and median PFS for each group were shown in Figure 6. The baseline characteristics of the patients in the four subgroups are shown in the Supplement Table 3. Subgroup analysis showed that patients in group A had the best efficacy and prognosis, with an ORR of 74.1% and an mPFS of 15.6 months. In contrast, the treatment efficacy was poorer in patients in Groups C, with ORRs of 23.5%, and corresponding mPFS times of 5.6 months, respectively. While, the efficacy and prognosis of groups B and D were similar, with ORR of 54.5% and 50.0%, and corresponding mPFS were 7.0 months in both groups (Figure 7). When comparing the ORR and mPFS of patients in Group A to those of patients in Group C, there was a statistically significant difference (ORR, HR: 9.286, 95% CI: 2.260-38.150, p=0.002; mPFS, HR: 0.204, 95% CI: 0.094-0.442, p<0.001) (Figure 7). Kaplan–Meier curves also showed that the

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TABLE 1 Baseline characteristics.

Characteristics	No. (n=99)	percentage
Age		
<60	54	54.5%
≥60	45	45.5%
Gender		
Male	53	53.5%
Female	46	46.5%
Ethnicity		
Asian	66	66.7%
Non-Asian	33	33.3%
Smoking		
Yes	39	39.4%
No	60	60.6%
Stage		
I-III	5	5.1%
IV	94	94.9%
EGFR test		
DNA Sanger sequencing	17	17.2%
NGS	35	35.3%
PCR	10	10.1%
ARMS	6	6.1%
NA	30	30.3%
Mutation number		
Single	60	60.6%
Multiple	39	39.4%
Afatinib lines		
1 Line	45	45.5%
≥2 Line	54	54.5%
Response to TKI		
CR	0	0.0%
PR	53	53.5%
SD	33	33.3%
PD	13	13.1%
PFS		
median	9.0 months	–

NGS, Next-generation sequencing; PCR, polymerase chain reaction; ARMS, amplification refractory mutation system; NA, not available; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; PFS, progression-free survival.

PFS was longest in group A and shortest in groups C, while moderate in groups B and C (Figure 8).

Discussion

In this study, the clinicopathological characteristics of 99 NSCLC patients with um-EGFRm were investigated, and their correlation with the efficacy and prognosis following afatinib were analyzed. In generally, the ORR of patients with um-EGFRm was53.5%, and the median PFS was 9.0 month. For patients administered first-line afatinib treatment, the ORR and median PFS were 73.3% and 15.6 months, respectively, which were both superior to those of patients treated with second- and later-line treatments. Moreover, single um-EGFRm and first-line treatment was an independent predictor of favorable treatment response and longer PFS. Subgroup analysis indicated that patients harboring major um-EGFRm had a favorable response to afatinib treatment and prognoses benefit; in contrast, patients harboring um-EGFRm that comprising 19del/L858R had a poorer response to treatment and unfavorable prognoses.

EGFR-mutant NSCLC is a genetically heterogeneous disease that includes more than 200 different mutant subtypes (13, 15). Uncommon and common EGFR mutations have been demonstrated to have similar clinicopathological characteristics (15), but patients with um-EGFRms are less sensitive to first-generation EGFR-TKI therapy (15, 26). Patients with NSCLC harboring um-EGFRm had a poorer response, lower ORR and shorter PFS than those of patients with 19del/L858R after receiving first-generation EGFR-TKIs (13, 15, 19). Regarding afatinib treatment, A *post hoc* analysis of the LUX-Lung 2, 3 and 6 clinical trials revealed that patients with um-EGFRms other than T790M and ex20ins who received first-line afatinib had an ORR of 71.0% and a median PFS of 10.7 months (95% CI: 5.6-14.7) (14). Our results were consistent with those of this study; in the present study, a total of 50 patients were treated with first-line afatinib, and their ORR and median PFS were 72.0% and 12.0 months, respectively. Another study evaluated the clinical efficacy of afatinib in 315 patients with NSCLC carrying um-EGFRms in randomized clinical trials or real-world cases, and the results showed that patients treated with afatinib who harbored major um-EGFRms and harbored multiple um-EGFRms had an ORR of 60.0% and 77.1%, respectively, with a corresponding median time to treatment failure (TTF) of 10.8 months and 14.7 months, respectively (17). These findings suggest that afatinib has favorable activity in patients with um-EGFRms. The results of several real-world observational investigations are consistent with these clinical trial data and demonstrate that afatinib is more efficacious than first-generation EGFR-TKIs in patients with um-EGFRms (18–21). For example, a retrospective study showed that patients with um-EGFRms treated with afatinib had an ORR and disease control rate (DCR) of 75% and 100%, respectively, which were significantly higher than those of patients treated with gefitinib or erlotinib, who had an ORR and DCR of 40% and 80%, respectively. Additionally, afatinib treatment was associated with longer PFS (17.1 months vs. 5.5 months) (18). In another retrospective study of 125 patients with um-EGFRm, compared to those treated with gefitinib and erlotinib, patients treated with afatinib demonstrated a higher ORR (afatinib 78.9%, gefitinib 38.9%, erlotinib 20%, p =0.013), as well as longer PFS (afatinib 10.5, gefitinib 3, erlotinib 0.9 months, p= 0.013) (19). Other real-world research has also demonstrated that patients with um-EGFRm treated with afatinib have more favorable prognoses than those of patients receiving gefitinib or erlotinib (20, 21). Moreover, a recent phase II clinical study (KCSG-LU15-09) of small sample of NSCLC patients harboring um-EGFRm

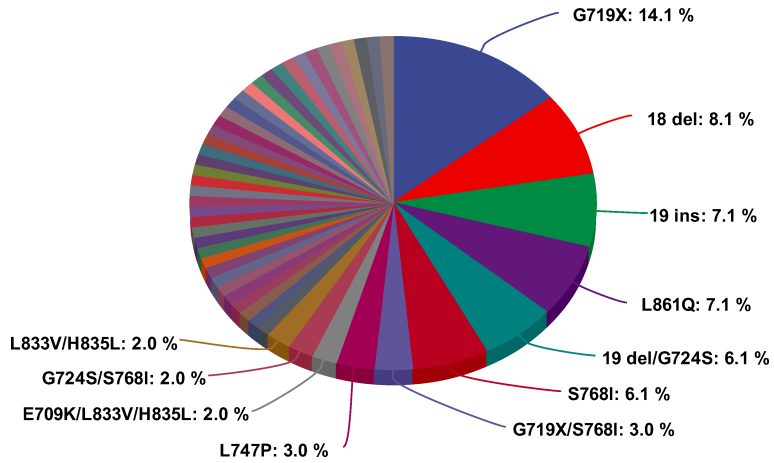

FIGURE 2
Composition of un-common EGFR mutations (n=99).

indicated that osimertinib, a third-generation EGFR-TKI, exhibited clinical activity in patients with um-EGFRms. A total of 36 patients were treated with osimertinib (22 as first-line, 11 as second-line, and 3 as third-line), resulting in an ORR of 50.0% (95% CI: 33%-67%) and a median PFS of 8.2 months (95% CI: 5.9-10.5 months) (27). Presently, the available clinical data on osimertinib treatment for patients with um-EGFRms are limited; only a few cases or case series have reported the efficacy of osimertinib in patients with certain types of um-EGFRms (28–30). In view of the above findings, we can deduce that the second-generation EGFR-TKI, afatinib, has higher clinical activity in patients with um-EGFRms than first- and third-generation EGFR-KTIs, and this is also supported by the results of preclinical studies that um-EGFRms have higher affinity and sensitivity to afatinib (22–25).

Data from clinical trials and real-world research reveal that patients with um-EGFRms exhibit inconsistent responses and survival outcomes following afatinib treatment, which are closely related to the mutation pattern and the cooccurring partner mutant genes (13, 15). Therefore, we performed subgroup analysis to investigate the differences in the treatment efficacy of afatinib among patients with different types of um-EGFRms and the prognoses of these patients to determine which, if any, potential subgroups of patients are more likely to benefit from afatinib treatment. Ex20ins is the third most common EGFR mutation, accounting for approximately 10-12% of all EGFR mutations (31, 32). However, due to steric hindrance at the drug binding pocket, most of the EGFR proteins harboring these mutations are relatively insensitive to EGFR-TKIs, including afatinib (32, 33). In the *post-hoc* analysis of the LUX-lung trials, 23 patients with ex20ins who were treated with afatinib had an ORR of 8.7% and a PFS of only 2.7 months, representing the lowest efficacy of afatinib treatment over other types of um-EGFRms (14). Another study reported a slightly higher efficacy of afatinib in patients with ex20ins, with an ORR of 23.4% and a median TTF of 4.2 months (17). A Spanish multicenter retrospective study also showed that the treatment efficacy of afatinib was significantly lower in patients carrying ex20ins than in patients with other types of mutations, with an ORR of 13.0% and a median OS of 10.7 month (34). Therefore, platinum-based

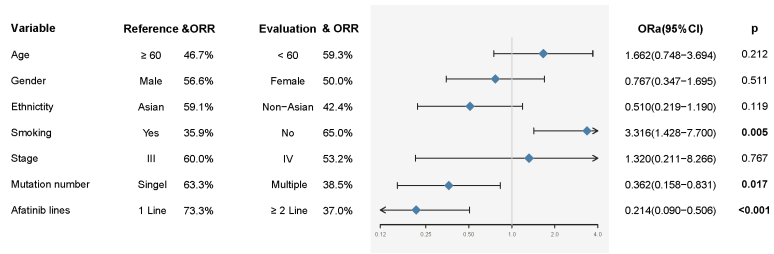

FIGURE 3
Univariate analysis for treatment response. ORR, Objective response rate; ORa, Odds ratio.

TABLE 2 Multivariate analysis for efficacy and PFS.

Variable	Multivariate analysis for efficacy			Multivariate analysis for PFS		
	ORa	95%CI	p	HR	95%CI	p
Smoking						
No	1.00			1.00		
Yes	0.333	0.132-0.838	**0.020**	1.543	0.932-2.555	0.092
Mutation number						
Single	1.00			1.00		
Multiple	0.379	0.151-0.956	**0.040**	1.866	1.128-3.088	**0.015**
Afatinib lines						
1 Line	1.00			1.00		
≥2 Line	0.262	0.106-0.646	**0.004**	1.800	1.057-3.064	**0.031**
TKI response						
OR	–	–	–	1.00		
Non-OR	–	–	–	2.554	1.525-4.277	**<0.001**

OR, objective response; ORa, odds ratio; HR, hazard ratio. P values < 0.05 are highlighted in bold.

combination chemotherapy, rather than afatinib, might be the preferred treatment option for patients with ex20ins. Of note, the FDA currently has approved amivantamab, an EGFR-MET bispecific antibody, for the treatment of patients with locally advanced or metastatic NSCLC patients harboring an EGFR ex20ins mutation who exhibit disease progression during or after platinum-based chemotherapy. In addition to ex20ins, the other most frequently um-EGFRms include G719X in exon 18 (including G719A, G719C, G719D, and G719S and other variants), S768I in exon 20, and L861Q in exon 21, which are known as major um-EGFRms and have been reported to demonstrate sensitivity to afatinib in preclinical and clinical studies (14, 17, 22–25). In *post hoc* analysis of the LUX-lung trials, patients carrying G719X, S768I and L861Q had ORRs of 78%, 100% and 56% after afatinib treatment, respectively, with corresponding median PFS of 13.8, 14.7 and 8.2 months, respectively, which represents the best demonstrated efficacy of afatinib (14). The results of our study are consistent with this finding: the patients carrying major um-EGFRms had an ORR of 74.1% and a median PFS of 15.6 months. This result is also supported by the results of other clinical trials and real-world clinical data (17, 20, 35). Obviously, afatinib should be considered the preferred treatment option for NSCLC patients carrying major um-EGFRms. Single EGFR mutations other than ex20ins and the major um-EGFRms were classified as other single um-EGFRms in this study, and patients with these mutations showed moderate sensitivity to afatinib, with an ORR of 54.5% and a median PFS of 7.0 months. Afatinib showed activity against several mutation types in this class of mutations, such as E709X in exon 18, L747P in exon 19, L774X, R776X, and Q787Q in exon 20, and H833V and H835L in exon 21 (13, 36). However, due to the high heterogeneity of patients with this category of mutations, including different types of mutations and variants, and the low mutation frequency, the available clinical data on the efficacy of afatinib are limited, and conclusions regarding the overall efficacy of afatinib are not uniform. Thus, further studies are warranted.

In addition to single mutations, two or more different types of EGFR mutations may coexist in tumor cells, which accounts for

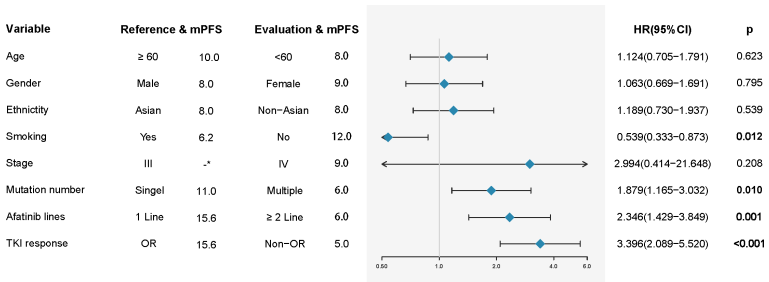

FIGURE 4
Univariate analysis for progression free survival (PFS). NA,s Not available.

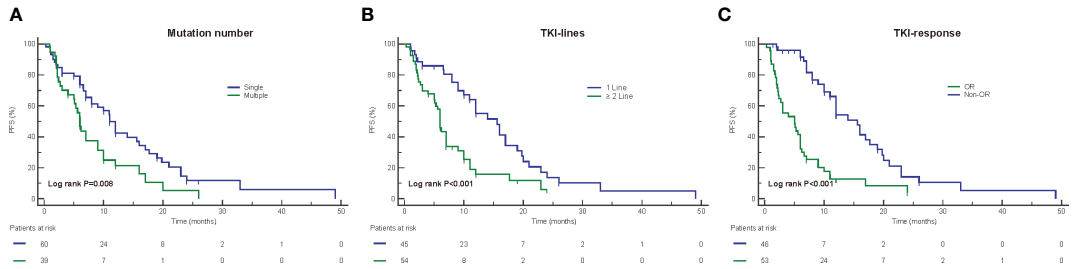

FIGURE 5
Kaplan-Meier curves for progression free survival (PFS) according to mutation numbers **(A)**, TKI-lines **(B)**, and TKI-response **(C)**.

approximately 4-14% of EGFR mutations. Previous studies have indicated that the efficacy of EGFR-TKIs in patients with multiple um-EGFRms may be affected by the sensitivity of the accompanying mutations (13). We found that patients with multiple um-EGFRms containing 19del/L858R had the worst prognoses, with an ORR of only 23.5% and an mPFS of 5.6 months. In contrast, patients with multiple um-EGFRms without 19del/L858R showed a higher sensitivity to afatinib, with an ORR of 50.0% and a median PFS of 7.0 months. This result is similar to the results of a previous retrospective study, in which patients with multiple um-EGFRms who did not harbor 19del/L858R had better PFS than patients with both 19del/L858R (19). This may be due to the presence of major um-EGFRms among patients who harboring multiple um-EGFRms that without 19del/L858R. For example, Yang et al. reported that in patients with multiple um-EGFRms containing a major um-EGFRms, the ORR was 78.3%, and the median duration of response (DoR) was 17.1 months after receiving afatinib (14). This result is consistent with those of preclinical studies concluding that afatinib has broader inhibition than first- and third-generation EGFR-TKIs for patients with multiple EGFR

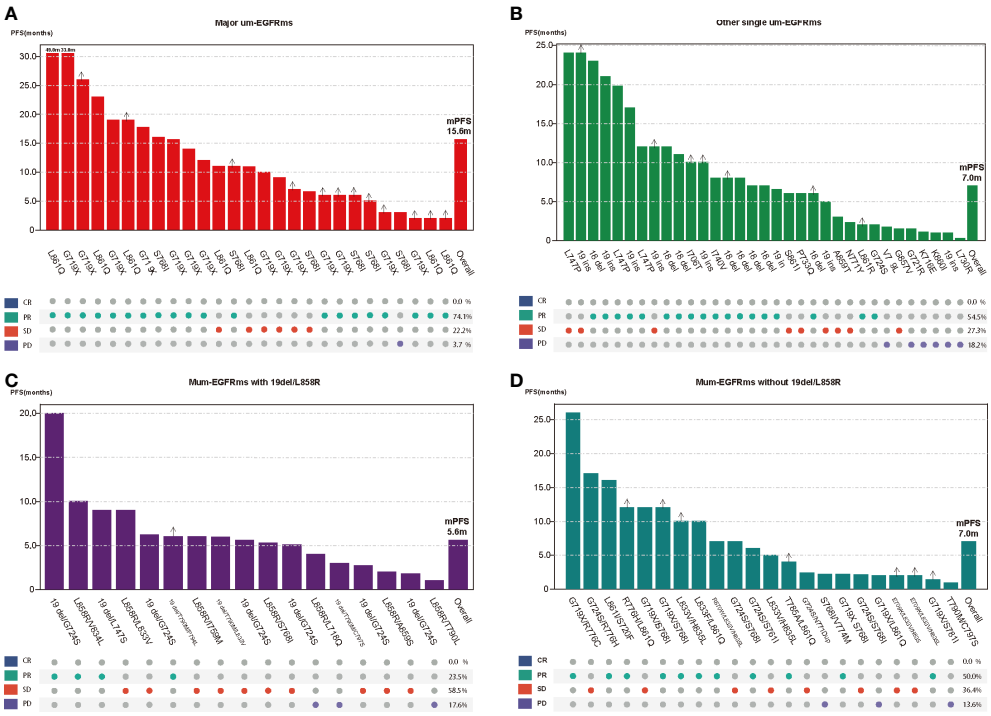

FIGURE 6
Tumor response and progression free survival (PFS) of each individual patient as well as overall tumor response rate and median PFS for each group (**A**: Major ucm-EGFRms; **B**: Other single ucm-EGFRm; **C**: multiple ucm-EGFRms that with 19del/L858; **D**: multiple ucm-EGFRms that without 19del/L858R).

Major um-EGFRms (n=27, 74.1%, 15.6m)	0.483(0.252-0.928) 0.029	0.204(0.094-0.442) <0.001	0.426(0.202-0.894) 0.024
2.381(0.792-7.154) 0.122	**Other single um-EGFRms (n=33, 54.5%, 7.0m)**	0.529(0.274-1.018) 0.057	1.054(0.548-2.029) 1.054
9.286(2.260-38.150) 0.002	3.900(1.049-14.505) 0.042	**Mum-EGFRms with 19del/L858R (n=17, 23.5%, 5.6m)**	1.794(0.859-3.747) 0.120
2.857(0.861-9.483) 0.086	1.200(0.407-3.536) 0.741	0.308(0.076-1.245) 0.098	**Mum-EGFRms without 19del/L858R (n=22, 50.0%, 7.0m)**

FIGURE 7
Odds ratio (ORa) with 95% CI for objective response rate (ORR) (blue)and hazard ratio (HR)with 95% CI for progression free survival (PFS)(green) in subgroup analysis according to mutation patterns (OR and HR was set by column versus row).

mutations, particularly those harboring major um-EGFRms (22–25). In this study, among the 22 patients in Group D, 14 had a major um-EGFRm, while in Group C, only one patient carried a major um-EGFRms. Additionally, among the patients in Group D, three patients also carried the T790M mutation, which is considered a mutation that promotes resistance to afatinib treatment (37, 38). Therefore, administering afatinib for the treatment of patients with multiple um-EGFRm might be considered an effective treatment option in some circumstances. However, given the wide heterogeneity of patients with multiple um-EGFRms and the limited clinical data available, clinicians should make prudent clinical decisions based on a thorough understanding of the sensitivity and resistance of known mutated genes, especially concurrent partner mutations.

There are some unavoidable limitations of this study. Firstly, this is a re-analysis based on published research. This may be affected by, such as, selection bias, publication bias, and other uncontrollable confounding factors. Secondly, due to the variability of the included articles, there were not enough data for comparison of drug toxicity and side effects. Thirdly, the biological source, platform and method for EGFR detection are unclear, which could have an impact on the consistency and rate

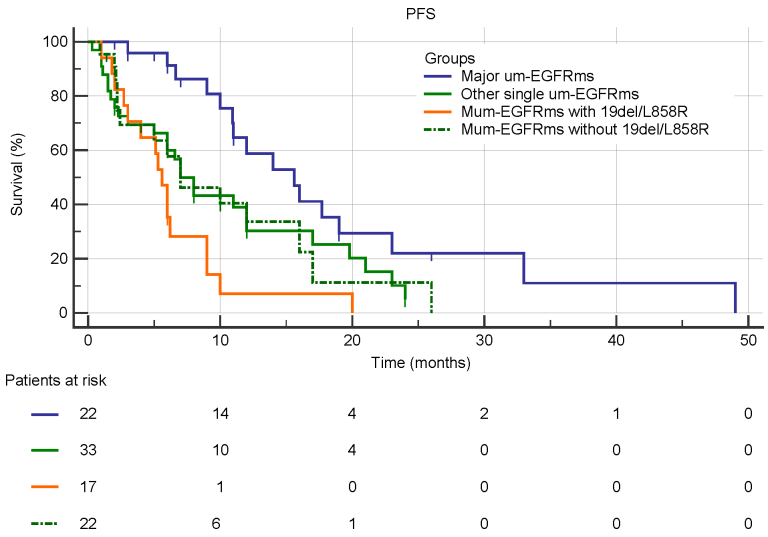

FIGURE 8
Kaplan-Meier curves for PFS in subgroup analysis according to mutation patterns.

of EGFR detection. In addition, due to the heterogeneity of the included literature, we were unable to determine the site of metastasis in each patient and therefore could not investigate the relationship between the sites of metastasis and the type of mutation. Therefore, a further, large-scale, randomized controlled clinical study is needed to validate our conclusions.

Conclusion

In summary, as a special type of EGFR mutation, patients with um-EGFRms exhibit favorable but inconsistent responses and survival outcomes following afatinib treatment. Our findings suggest that NSCLC patients carrying um-EGFRms can be further classified into various mutation subgroups that exhibit different responses and survival outcomes following afatinib treatment, but this conclusion requires further clinical studies for verification.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Yantai Yuhuangding Hospital. The ethics committee waived the requirement of written informed consent for participation.

Author contributions

Conceptualization, SH and ZM; Methodology, CSW and CJW; Software, ZM and CSW; Formal Analysis, CJW and KZ; Resources, CJW; Data Curation, WD; Writing-Original Draft Preparation, CSW and WD; Writing-Review and Editing, SH and CSW; Supervision, SH and CJW. All authors contributed to the article and approved the submitted version.

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This study is a re-analysis of published articles, the included articles are listed in the supplement table and we thank the authors of these articles for their works.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.999606/full#supplementary-material

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Case Report: Long-term remission of malignant pleural and peritoneal effusion in a case of advanced lung adenocarcinoma treated with combined crizotinib and anlotinib therapy

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Malignant pleural and peritoneal effusion are common clinical manifestations in advanced malignant tumors, associated with a poor prognosis. This article presents a case of advanced lung adenocarcinoma with ROS1 rearrangement, characterized by persistent malignant pleural and peritoneal effusion. The patient received combined therapy of Crizotinib and Anlotinib, resulting in a significant reduction and even disappearance of the malignant effusion. Exploratory use of this treatment approach improved the patient's quality of life and holds potential for extending overall survival. However, given the single case report nature, the efficacy of this regimen in treating advanced ROS1-rearranged lung adenocarcinoma should be considered as a supplementary strategy, warranting further validation through multicenter clinical data.

KEYWORDS

malignant pleural effusion, malignant peritoneal effusion, crizotinib, Anlotinib, non-small cell lung cancer

1 Introduction

Non-small cell lung cancer (NSCLC) ranks among the most prevalent and fatal malignancies worldwide, accounting for 80%-90% of cancer cases (1). Malignant pleural and peritoneal effusion can result from local progression or distant metastasis of malignant tumors. Studies have consistently shown that patients with concurrent malignant pleural

and peritoneal effusion have a significantly worse prognosis, with a median survival ranging from 3 to 6 months (2). Moreover, malignant effusion adversely impacts respiratory, circulatory, and digestive functions, diminishing patients' quality of life and compromising the effectiveness of anticancer treatments. Comprehensive management strategies for malignant pleural and peritoneal effusion encompass thoracentesis, closed chest drainage, and pleural catheter placement. Therefore, devising appropriate treatment approaches for effectively managing malignant effusion is pivotal to enhance cancer patients' quality of life and improve treatment outcomes.

In recent years, significant progress has been made in the field of targeted cancer therapy, substantially prolonging the survival of patients with specific genetic mutations. ROS1 gene rearrangements occur in approximately 1-2% of NSCLC patients and represent validated therapeutic driver mutations (3). Crizotinib, a multitargeted tyrosine kinase inhibitor (TKI) targeting MET/ALK/ROS1, has demonstrated notable efficacy in treating NSCLC with ROS1 rearrangements. Anlotinib, a novel multitargeted TKI developed in China, effectively inhibits kinases such as vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor, fibroblast growth factor receptor, and c-Kit, exerting its actions on tumor angiogenesis and growth inhibition (4). Although the combination therapy of Crizotinib and Anlotinib has garnered research attention for small cell lung cancer (SCLC), its clinical application remains limited. Crizotinib targets the anaplastic lymphoma kinase (ALK) gene rearrangement, while Anlotinib is a multitargeted agent that inhibits multiple growth factor receptors and angiogenesis-related pathways. *In vitro* experiments and animal models have suggested a potential synergistic effect of combining Crizotinib and Anlotinib in SCLC treatment. This therapeutic approach holds promise by simultaneously targeting multiple key pathways implicated in tumor growth and angiogenesis, thereby enhancing treatment efficacy.

Following an extensive review of databases such as PubMed, Web of Science, and CNKI, we identified limited literature on the use of Crizotinib and Anlotinib for treating malignant effusion in lung adenocarcinoma in China. Here, we report a case of ROS1-rearranged lung adenocarcinoma with concurrent malignant pleural and peritoneal effusion. The patient achieved complete remission of effusion and long-term relief following maintenance therapy with Crizotinib and Anlotinib.

2 Case report

The patient, a 58-year-old male, was admitted to the Department of Thoracic Surgery at Qingdao Municipal Hospital on June 8, 2017. Upon admission, the patient presented with symptoms of cough, chest tightness, and persistent shortness of breath lasting for over a month. There were no apparent triggering factors in the patient's work environment, and there was no family history of cancer or genetic disorders. The primary symptom reported throughout the course of the illness was respiratory distress. The initial treatment approach involved thoracentesis for pleural effusion drainage, which provided relief for the patient's chest tightness and respiratory symptoms.

A chest enhanced CT scan revealed multiple enlarged lymph nodes in the right pulmonary hilum and mediastinum, multiple nodules in the right pleura, as well as pleural effusion. However, no obvious occupying lesions were seen in both lungs. Further examination of the pleural fluid cytology confirmed the presence of cancer cells. Histological images showed tumor cells can be seen under the microscope, with significant heterogeneity. Some cells are arranged in solid masses or small cords, some are visible as glandular lumen formation, and some are arranged in tubular or adenoid structures. Immunohistochemical results showed the tumor characteristics as follows: Ki-67 (5%+), TTF-1 (+), NapsinA (+), CK7 (+), CR (-), MC (-), Vim (-), EMA (+), CK (+), and CK5/6 (-). Physical examination: no thoracic deformity, bilateral respiratory motion symmetry, turbid sounds on right lung percussion, disappearance of breath sounds in the middle and lower lungs, clear sounds on left lung percussion, no dry and moist rales were heard.

According to the American Joint Committee on Cancer (AJCC) staging system, the patient's tumor size cannot be measured, the tumor metastasis is pleura, and pleural effusion is present, and the lymph node invasion is ipsilateral to the hilar and mediastinum, taking into account the patient's symptoms, imaging findings, pleural fluid characteristics, immunohistochemistry, and staging guidelines, the patient was diagnosed with advanced-stage malignant tumor in the right lung (adenocarcinoma, stage IV A). The patient's treatment journey has spanned over 5 years since June 8, 2017.

The main treatment modality for the patient has been chemotherapy, consisting of multiple cycles. The first chemotherapy cycle was initiated on June 17, 2017, involving pemetrexed 800mg on day 1 and nedaplatin 60mg on day 1. On July 12, 2017, the patient received intrathoracic injections of Endo 45mg and Ganoderma lucidum polysaccharide 4mg. Subsequently, from July to August 2017, the patient underwent 2-3 cycles of chemotherapy with pemetrexed 900mg on day 1 and carboplatin 700mg on day 1. Genetic testing revealed no alterations in EGFR/ALK/ROS1.Between September 2017 and January 2018, the patient underwent 4-7 cycles of intravenous chemotherapy, comprising pemetrexed 900mg on day 1, cisplatin 50mg on day 1, and bevacizumab 600mg. Additionally, intrathoracic injections of cisplatin 80mg and bevacizumab 200mg were administered. After completing 7 cycles of chemotherapy, the patient experienced significant improvement in ascites symptoms and a reduction in dyspnea. On March 6, 2018, the patient received Endo 30mg on days 1-7 and pemetrexed 800mg on day 4, as per the medical instructions. However, the patient did not adhere to the prescribed treatment. Subsequent evaluation revealed an increase in tumor markers, and PET-CT indicated disease progression. Therefore, an additional 2 cycles of chemotherapy were administered using the original regimen: Endo 30mg on days 1-7 and pemetrexed 800mg on day 3. Following the completion of this treatment cycle, the patient was readmitted due to abdominal distension and dyspnea. Subsequent CT scans revealed the presence of malignant pleural and peritoneal effusion (Figure 1; Supplemental Figure).

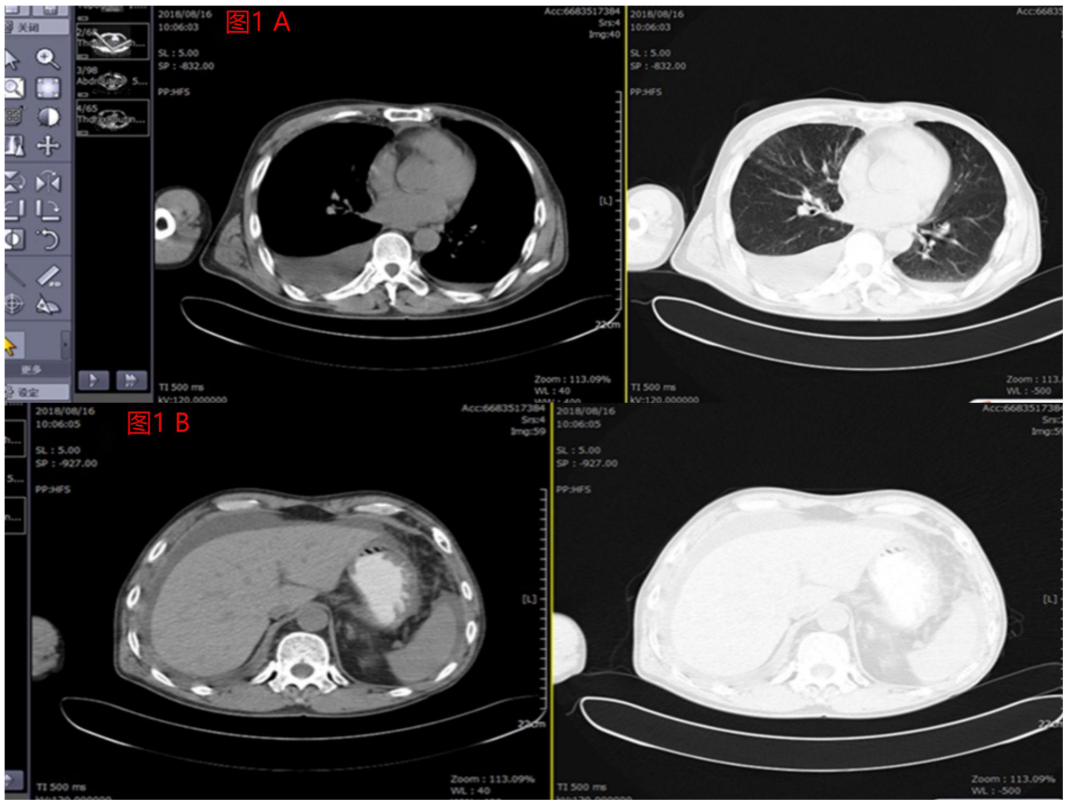

FIGURE 1
(A) Pleural effusion was shown by imaging findings before the patient underwent crizotinib combined with anlotinib treatment in August 2018.
(B) Significantly large perihepatic effusion was shown by imaging findings before the patient underwent crizotinib combined with anlotinib treatment in August 2018.

On August 21, 2018, the patient underwent palliative chemotherapy with cisplatin 40mg on day 1 and 50mg on days 2-3. Due to recurrent pleural effusion and the presence of peritoneal effusion, intraperitoneal infusion of Endo 105mg on day 1 and day 5 was performed on August 24, 2018. The follow-up CT scan showed tumor infiltration of the intestinal tract. Attempted high-throughput sequencing of blood samples revealed a ROS1 rearrangement. Starting from September 5, 2018, the patient initiated targeted therapy with crizotinib 250mg twice daily in combination with anlotinib 10mg once daily. The treatment resulted in significant symptom relief, and the malignant pleural and peritoneal effusion decreased compared to the CT results on August 21, 2018 (Figure 2).

Continuing with the targeted therapy regimen of crizotinib 250mg twice daily and anlotinib 10mg once daily (with a two-week administration of anlotinib followed by a one-week break), the patient's chest and lung signs were evaluated. Subsequent follow-up visits indicated the absence of dyspnea, and imaging examinations of the thoracic and abdominal regions showed no significant malignant infiltrations for over 4 years. The patient developed grade 2 hypertension during the medication period, which was controlled by oral antihypertensive drugs. As of March 2023, the patient's disease has progressed, and palliative treatment has been administered.

3 Diagnosis/follow-up and outcomes

Diagnosis: 1. Pulmonary malignancy stage IV A 2. Malignant pleural effusion 3. Malignant peritoneal effusion 4. Metastatic colonic malignancy

Follow-up and outcomes: The patient is currently undergoing palliative and nutritional support treatment in the Department of Oncology, Qingdao Municipal Hospital for various reasons.

4 Discussion

Lung cancer is a highly prevalent and deadly malignancy in both China and globally, with non-small cell lung cancer (NSCLC) accounting for approximately 80% of cases. About 5% of NSCLC tumors involve rearrangements in the ALK gene located on chromosome 2 (5). In 2007, Soda (6) identified the EML4-ALK fusion gene in NSCLC, where the most common ALK rearrangement involves fusion of the 5' end of the EML4 gene with the 3' end of the ALK gene, resulting in the formation of the fusion oncogene EML4-ALK (7). This fusion protein triggers dimerization of the intracellular kinase domains of ALK, activating downstream oncogenic signaling and leading to disease progression and poor prognosis (8).

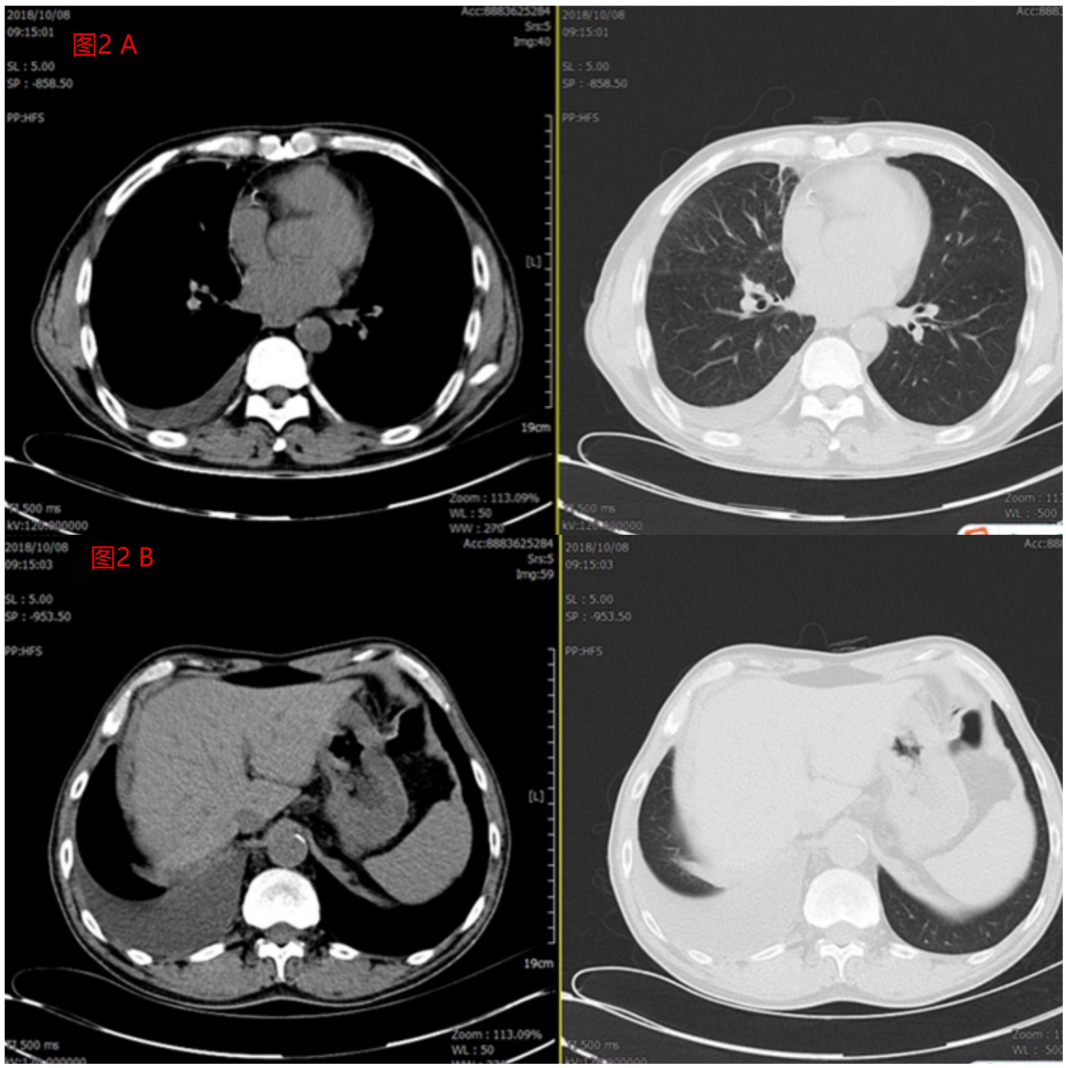

FIGURE 2
(A) The patient's pleural effusion was significantly reduced after 1 month of treatment compared to pre-treatment. **(B)** The patient's peritoneal effusion was significantly reduced after 1 month of treatment compared to pre-treatment.

The formation of malignant pleural and peritoneal effusions is closely associated with vascular endothelial growth factor (VEGF). Tumor proliferation and metastasis are often accompanied by increased vascular density, and VEGF promotes tumor neovascularization by secreting various pro-angiogenic factors, including VEGFA (9). The elevated vascularity of tumor blood vessels contributes to the development of malignant pleural effusion (10). Moreover, VEGF can bind to receptors on mesothelial epithelial cells, increasing their permeability and facilitating fluid reflux, thus disrupting the dynamic equilibrium between fluid production and absorption.

The patient in this case is a middle-aged male with advanced lung adenocarcinoma. Despite undergoing seven cycles of platinum-based chemotherapy, his disease continued to progress, and the control of pleural and peritoneal effusions was suboptimal. Initial genetic testing yielded negative results, but given the worsening condition, a second round of genetic testing revealed a ROS1 rearrangement. The patient received combined treatment with Anlotinib and Crizotinib to control pleural and peritoneal effusions, resulting in a significant reduction in fluid accumulation after one month of treatment. The patient achieved stable long-term relief, with no serious adverse reactions during the course of drug administration.

The combination of Anlotinib and Crizotinib offers several advantages and limitations. As multitarget tyrosine kinase inhibitors, both drugs can simultaneously inhibit multiple key targets such as VEGFR, EGFR, PDGFR, ROS1, and ALK. This multitarget inhibition mechanism may lead to a more comprehensive anti-tumor effect and improved treatment outcomes, particularly in tumors with multiple aberrant signaling pathways. Additionally, combining Anlotinib with Crizotinib may help overcome resistance observed with Crizotinib monotherapy by suppressing chemokine-mediated angiogenesis and enhancing the overall anti-tumor efficacy (11). However, it is important to note that the combined use of Anlotinib and Crizotinib may increase the risk of adverse reactions, as both drugs have their own side effect

profiles including hypertension, hand-foot syndrome, fatigue, and gastrointestinal discomfort. Furthermore, the efficacy of the combination treatment may vary depending on individual tumor characteristics and genetic variations. While preliminary studies have reported favorable results with Anlotinib combined with Crizotinib in lung cancer treatment, larger-scale clinical trials and long-term follow-up data are still lacking to establish the effectiveness and safety of this combination therapy. Therefore, further research is warranted to validate its therapeutic efficacy and determine the optimal treatment strategy.

The patient received multiple cycles of intravenous Endo and Bevacizumab, as well as local injections of Bevacizumab and Endo into the abdominal and pleural cavities, but the outcomes were unsatisfactory. Based on the clinician's experience, the addition of Anlotinib to Crizotinib led to unexpected treatment efficacy for the patient. The combination of Anlotinib and Crizotinib represents a promising approach for ALK-TKI-resistant patients.

In conclusion, Combined treatment with Crizotinib and Anlotinib may demonstrate effectiveness in late-stage ROS1-rearranged lung adenocarcinoma with concurrent malignant pleural and peritoneal effusions, significantly prolonging survival and improving quality of life, as shown in the present case. After an initial negative genetic testing result, targeted therapy should not be permanently abandoned, and a second round of genetic testing should be considered. The clinical practice presented in this case may provide novel insights into the treatment of malignant effusions, although further research is needed to elucidate the treatment mechanism and confirm its efficacy.

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 patient did not have any objection to the treatment plan, fully understood his condition, and expressed happiness and satisfaction with receiving Anrotinib check crizotinib treatment and obtaining long-term remission, and will strictly follow medical advice, actively treat and live optimistically in the future. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

JT and LL are in charge of data acquisition and data analysis. JZ/PL and LZ are in charge of literature review, clinical data collection and validation. JT made a contribution to manuscript editing. HZ and JX are in charge of manuscript review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1191522/full#supplementary-material

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Tumor-associated macrophages mediate resistance of EGFR-TKIs in non-small cell lung cancer: mechanisms and prospects

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Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are the first-line standard treatment for advanced non-small cell lung cancer (NSCLC) with EGFR mutation. However, resistance to EGFR-TKIs is inevitable. Currently, most studies on the mechanism of EGFR-TKIs resistance mainly focus on the spontaneous resistance phenotype of NSCLC cells. Studies have shown that the tumor microenvironment (TME) also mediates EGFR-TKIs resistance in NSCLC. Tumor-associated macrophages (TAMs), one of the central immune cells in the TME of NSCLC, play an essential role in mediating EGFR-TKIs resistance. This study aims to comprehensively review the current mechanisms underlying TAM-mediated resistance to EGFR-TKIs and discuss the potential efficacy of combining EGFR-TKIs with targeted TAMs therapy. Combining EGFR-TKIs with TAMs targeting may improve the prognosis of NSCLC with EGFR mutation to some extent.

KEYWORDS

NSCLC, EGFR-TKIs, resistance, tumor-associated macrophages, exosome

1 Introduction

1.1 Background

The epidermal growth factor receptor (EGFR) is one of the most frequently mutated driver oncogenes in non-small cell lung cancer (NSCLC), and EGFR mutation is found in approximately 50% of the Southeast Asian lung adenocarcinoma population (1). EGFR-tyrosine kinase inhibitors (EGFR-TKIs) such as first-generation EGFR-TKIs gefitinib or erlotinib have shown potent antitumor effects in advanced NSCLC patients with EGFR mutation (2). Osimertinib, a third-generation EGFR-TKI, has been approved as first-line therapy for advanced NSCLC patients with EGFR mutation due to its lower toxicity and stronger antitumor effects (3). However, resistance to EGFR-TKIs is inevitable, and disease progression occurs in most patients. The mechanisms of resistance to EGFR-TKIs are a current research focus in NSCLC. Several resistance mechanisms have been elucidated, including secondary mutations of EGFR, activation of bypass pathways, and histological

transformation (4). The development of fourth-generation EGFR-TKIs targeting the EGFR C797S mutation is underway (5). In recent years, the resistance of EGFR-TKIs mediated by tumor-associated macrophages (TAMs) has received broad attention (Table 1). Previous studies have demonstrated that high infiltration of TAMs is significantly associated with an unfavorable prognosis in NSCLC patients treated with EGFR-TKIs (6–9).

1.2 TAMs in NSCLC

The origin of TAMs in NSCLC is multifaceted, involving both tissue-resident macrophages (TRMs) and monocyte-derived macrophages (MDMs) (10). And TRMs can be classified into lung alveolar macrophages (LAMs) and interstitial macrophages (IMs) based on their anatomical locations. TRMs are present during embryonic development and can self-renew locally, independent of the hematopoietic system (11). They are crucial in coordinating tissue remodeling and maintaining tissue integrity (11). MDMs originate from the hematopoietic system, and many can be observed in inflammatory lesions (12). TAMs from different sources can promote the progression of NSCLC (13). TRMs mainly contribute to tumor generation, while MDMs primarily participate in tumor metastasis (13).

Macrophages can generally be classified into M1 and M2 types based on their polarization status (14, 15). M1-like macrophages secrete pro-inflammatory factors, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-12, and IL-23, to participate in antigen presentation and play a role in immune surveillance (16). And M2-like macrophages secrete inhibitory factors, such as IL-10 and transforming growth factor-β (TGF-β), and have weak antigen-presenting ability (16). TAMs mainly exhibit the M2-like macrophage phenotype (17) and are closely associated with resistance to anti-tumor drugs in various solid tumors, including NSCLC (6, 18–20). Additionally, TAMs exhibit both inter- and intra-tumor heterogeneity. High infiltration of TAMs has been linked to unfavorable prognosis in pancreatic cancer (21), bladder cancer (22), and malignant glioma (23). But in some instances, such as ovarian (24) and colorectal cancers (25), it is associated with a more favorable outcome. In the NSCLC investigation, a high TAMs infiltration level within tumor islets was associated with a favorable prognosis. In contrast, a high level of TAMs infiltration within tumor stroma was linked to unfavorable prognosis (26, 27). The heterogeneity of TAMs in NSCLC may be attributed to tumor hypoxia and the spatial distribution of TAMs within the tumor microenvironment (TME) (28).

TABLE 1 Mechanisms of TAMs mediated resistance to EGFR-TKIs.

Mechanisms	References
Activating bypass pathways	
AKT/mTOR pathway	34
AKT, ERK1/2 and STAT3 pathways	17, 59
LncRNA-MSTRG.292666.16/miR-6836-5p/MAPK8IP3 pathway	35
NF-κB/RELB pathway	36
Suppressing T cells	
NOS and PD-L1 pathways	91
M2-like polarization	
Lipid metabolism pathways	103
STAT3/IL-4 pathway	107
LncRNA SOX2-OT/miR-627-3p/Smads pathway	114
Modulating tumor cell phenotypes	
Stabilizing tumor cell phenotype	115
Promoting the EMT	129, 130

TAM, tumor-associated macrophage; mTOR, mammalian target of rapamycin; RELB, v-rel reticuloendotheliosis viral oncogene homolog B; NOS: nitric oxide synthase; PD-L1, programmed cell death 1 ligand 1; LncRNA SOX2-OT, long non-coding RNA SOX2 overlapping transcript; EMT, epithelial-mesenchymal transition.

1.3 Effects of EGFR-TKIs on TAMs

Jia et al. (29) investigated the impact of EGFR-TKIs on the TME in NSCLC from a dynamic perspective. During early-stage treatment, EGFR-TKIs can increase the infiltration of $CD8^+$T cells and dendritic cells (DC) in TME while inhibiting the infiltration of $Foxp3^+$ regulatory T cells (Tregs) and M2-like polarization of TAMs (29). However, with the continuation of treatment, the immune-activated TME gradually dissipated while the proportion of immunosuppressive cells, myeloid-derived suppressor cells (MDSCs), progressively increased (29). Notably, there was a significant increase in $CD86^+$ macrophage expression driven by EGFR during the initial phase of EGFR-TKIs treatment, which exhibited robust antigen presentation capabilities (29). However, the gradual accumulation of M2-like TAMs, MDSCs, and Tregs during treatment hindered the antitumor immune effects of DC and T cells (29–31).

1.4 Aims

This study aims to comprehensively review the current mechanisms underlying TAM-mediated resistance to EGFR-TKIs and discuss the potential efficacy of combining EGFR-TKIs with targeted TAMs therapy (Figure 1). Combining EGFR-TKIs with TAMs targeting may improve the prognosis of NSCLC with EGFR mutation to some extent.

2 TAMs mediate EGFR-TKIs resistance by activating bypass pathways

2.1 Background

Activation of phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) signaling

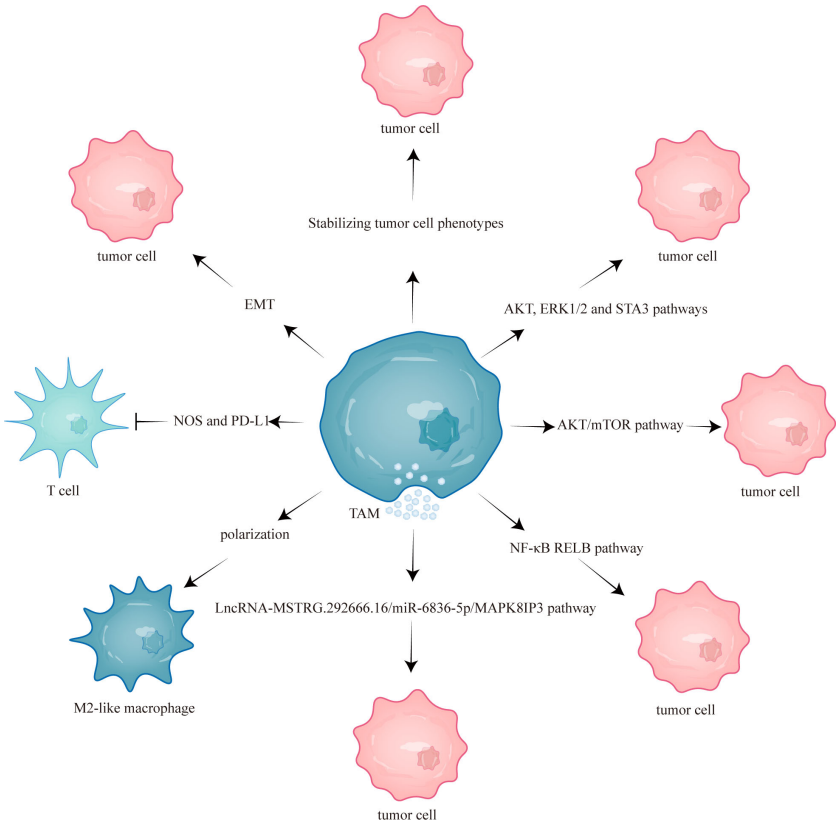

FIGURE 1
TAMs mediated EGFR-TKIs resistance through different mechanisms. TAM, tumor-associated macrophage; mTOR, mammalian target of rapamycin; NOS, nitric oxide synthase; EMT, epithelial-mesenchymal transition; RELB, v-rel reticuloendotheliosis viral oncogene homolog B; PD-L1, programmed cell death one ligand 1.

pathways compensates for the inhibition of EGFR signaling by EGFR-TKIs, promoting resistance of EGFR-TKIs (32). Yuan et al. (33) showed that TAMs can affect the biological behavior of lung adenocarcinoma cells by activating the PI3K/AKT pathway. This suggests that TAM-mediated EGFR-TKIs resistance may be closely related to the activation of bypass pathways. Furthermore, several studies have demonstrated that TAMs contribute to the resistance of EGFR-TKIs by activating bypass pathways, such as AKT/mammalian target of rapamycin (mTOR) pathway (34), AKT pathway (17), extracellular signal-related kinases 1 and 2 (ERK1/2) pathway (17), signal transducer and activator of transcription 3 (STAT3) pathway (17), LncRNA-MSTRG.292666.16/miR-6836-5p/MAPK8IP3 pathway (35) and atypical nuclear factor-κB (NF-κB)/v-rel reticuloendotheliosis viral oncogene homolog B (RELB) pathway (36).

2.2 AKT/mTOR pathway

EGFR-TKIs can increase the content of serum chemokine (C-C motif) ligand 2 (CCL2) (29), which plays an essential role in the process of EGFR-TKI resistance (8). CCL2 in the TME can recruit macrophages (37–39). Xiao et al. (34) showed that gefitinib resistance cell lines increased the release of CCL2 by decreasing the expression of β-catenin protein. Furthermore, tumor cells recruit more M2-like macrophages by releasing CCL2, and these macrophages promote gefitinib resistance by activating the AKT/mTOR pathway (34). As a serine/threonine kinase, mTOR has a catalytic domain similar to PI3K and is considered an atypical protein kinase in the PI3K-related kinase family (40). Through various mechanisms, including activation of growth factor receptor pathway, inhibition of autophagy, and influence on lipid metabolism pathway et al., mTOR could promote tumor development, metastasis, and drug resistance (41, 42). The rapamycin analogs, which inhibit mTOR, have been approved for treating renal cell carcinoma, while several other mTOR inhibitors are currently in development (40).

2.2.1 Prospects

Preclinical studies (43–50) have shown that mTOR inhibitors can improve the resistance of NSCLC to EGFR-TKIs. For example, Wang et al. (51) showed that the combination of ferumoxytol and CpG oligodeoxynucleotide 2395 could effectively suppress EGFR and its downstream AKT/mTOR signaling pathway, thereby enhancing the antitumor activity of macrophages in NSCLC with EGFR mutation. Qu et al. (52) employed a combination of MEK1/2 inhibitor AZD6244 and PI3K/mTOR inhibitor BEZ235 to improve gefitinib resistance in a xenograft model of NSCLC. However, Moran et al. (53) showed that afatinib, in combination with

mTOR inhibitor sirolimus, did not show the expected anti-tumor effect. The toxicity was not tolerable in NSCLC patients with EGFR-TKIs resistance. The intricate resistance mechanism of EGFR-TKIs may account for the limited antitumor efficacy. This implies the necessity of identifying NSCLC patients who are responsive to mTOR inhibitors. Notably, altering the administration mode of mTOR inhibitors to target the TME in NSCLC might alleviate the adverse effects of combination therapy. In conclusion, further exploration is warranted for the combination of mTOR inhibitors and EGFR-TKIs in EGFR-mutated NSCLC based on available evidence (54, 55).

2.3 AKT, ERK1/2 and STAT3 pathways

Exosomes are extracellular vesicles ranging in size from 30 to 150nm, capable of transporting nucleic acids or proteins derived from maternal cells and facilitating intercellular communication (56). Exosomes play a crucial role in the pathogenesis, progression, and metastasis of tumors (57). Yuan et al. (17) investigated the contribution of TAM-derived exosomes to EGFR-TKI resistance and demonstrated that these exosomes could impede the antitumor efficacy of gefitinib. Further protein expression analysis confirmed that TAMs-derived exosomes mediated EGFR-TKIs resistance by activating AKT, ERK1/2, and STAT3 signaling pathways (17). On the other hand, previous studies have shown that epiregulin (EREG), as a ligand for EGFR, can promote the progression of NSCLC (58). EREG-enriched macrophages induce gefitinib and erlotinib resistance by inducing AKT phosphorylation in a human epidermal growth factor receptor 2 (HER-2)-dependent manner (59).

2.3.1 Prospects

The abnormal activation of the AKT pathway is closely related to the resistance of EGFR-TKIs in NSCLC (60, 61). Several studies (62–67) have shown that inhibition of the AKT pathway can improve the resistance of EGFR-TKIs in NSCLC. For example, Lai et al. (68) demonstrated that Polyphyllin I can reverse osimertinib resistance by regulating the PI3K/AKT pathway in NSCLC. Wang et al. (69) showed that combination therapy with gefitinib and miR-30a-5p could overcome acquired resistance to EGFR-TKIs by regulating the PI3K/AKT pathway in NSCLC. However, Clément-Duchêne et al. (70) showed no improvement in progression-free survival (PFS) and overall survival (OS) for EGFR-mutated NSCLC treated with enzastaurin (an oral AKT inhibitor) combined with erlotinib compared to erlotinib alone in a phase II study. This finding contradicts previous preclinical studies and warrants further investigation to identify the subset of NSCLC patients who may benefit from AKT inhibitors. Additionally, TAM-induced AKT phosphorylation is closely associated with HER-2 (59), suggesting that the use of HER-2 inhibitors may improve resistance to EGFR-TKIs in NSCLC (71, 72). Consistent with this hypothesis, Peng et al. (73) have developed a trastuzumab-modified, mannosylated liposome system that effectively targets M2-type TAMs and HER-2 positive NSCLC cells to overcome EGFR-TKIs resistance mediated by the EGFR T790M mutation. Importantly, HER-2 and HER-3 belong to the HER family and have highly similar structures and biological functions (74). Vicencio et al. (75) demonstrated that osimertinib combined with HER-3 antibody therapy could enhance the antitumor effect in NSCLC. Therefore, in addition to directly inhibiting the AKT pathway, combining HER2 or HER3 inhibitors may be a therapeutic strategy for improving the efficacy of EGFR-TKIs.

2.4 LncRNA-MSTRG.292666.16/miR-6836-5p/MAPK8IP3 pathway

Non-coding RNAs (ncRNAs), including circular RNA (circRNA), long ncRNA (lncRNA), and microRNA (miRNA) et al., play an essential role in the initiation and progression of cancer (76). Deng et al. (77) analyzed the serum exosomal-lncRNAs of osimertinib resistant patients and found that the knock of lncRNA MSTRG.292666.16 can improve the osimertinib resistance in NSCLC cells. Furthermore, Wan et al. (35) showed that TAM-derived exosomes promote osimertinib resistance by activating MSTRG.292666.16/miR-6836-5p/1MAPK8IP3 signaling pathway in NSCLC.

2.4.1 Prospects

TAM-derived exosomes play a crucial role in mediating resistance to EGFR-TKIs. Unfortunately, there is a lack of effective methods to target these exosomes. Further investigation is warranted to refrain from the biogenesis of TAM-derived exosomes or impede the binding of exosomes to tumor cells.

2.5 NF-κB/RELB pathway

In pathological conditions like cancer, myeloid cells may transform myeloid-derived suppressor cells (MDSCs), contributing to tumor metastasis and conferring resistance to anti-cancer drugs (78). MDSCs play a crucial role in promoting immunosuppression and inducing the generation of regulatory T cells within the TME (79). Feng et al. (36) suggested that $S100A9^{+}$ MDSC (a subset of monocytic MDSC) derived macrophages induce gefitinib resistance *via* NF-κB/RELB pathway.

2.5.1 Prospects

The oncogenic role of NF-κB has been reported (80). Notably, NF-κB can facilitate the epithelial-mesenchymal transition (EMT) of tumor cells, which may constitute one of the potential mechanisms by which TAMs mediate resistance to EGFR-TKIs (80). Targeting NF-κB has been reported to improve EGFR-TKIs resistance potentially. For example, Yeo et al. (81) improved acquired resistance to EGFR-TKIs by inhibiting NF-κB and activation-induced cytidine deaminase (AICDA) in NSCLC. Liu et al. (82) reported that Liver X receptor ligands could induce apoptosis in EGFR-TKIs resistant cells by inhibiting the AKT-NF-κB pathway in NSCLC. On the other hand, RELB can upregulate the expression of programmed cell death one ligand 1 (PD-L1) and facilitate immune evasion in prostate cancer (83). Previous studies

(84) have shown that PD-L1 expression is also increased in NSCLC patients after developing resistance to EGFR-TKIs, and RELB may up-regulate PD-L1 expression following EGFR-TKIs resistance in NSCLC. Up-regulation of PD-L1 promotes an immunosuppressive TME, which may also be one potential mechanism for EGFR-TKI resistance (84). Therefore, RELB may be a potential target for improving EGFR-TKIs resistance in NSCLC.

3 TAMs mediate EGFR-TKIs resistance by suppressing T cells

3.1 Background

The EGFR signal can reduce chemokine (C-X-C motif) ligand 10 (CXCL10) and CCL5 by reducing interferon regulatory factor-1 (85). EGFR-TKIs can induce an interferon response in NSCLC, and the efficacy of EGFR-TKIs is influenced by immune activation (86). Previous studies (87–89) have shown that macrophages can promote chemotherapy resistance by inhibiting T-cell-mediated responses. Similarly, TAMs mediate T cell inhibition in the TME (90), which causes resistance to EGFR-TKIs related to TAMs (91).

3.2 TAMs inhibit T cells by expressing inducible nitric oxide synthase and PD-L1

Stimulator of interferon genes (STING) regulates the human immune system (92). Lin et al. (91) demonstrated that the enrichment of TAMs impedes T cell activation in NSCLC patients treated with osimertinib. The immunosuppressive TME attenuates the efficacy of EGFR-TKIs in anti-tumor therapy (91). Reprogramming macrophages with STING agonist, MSA-2, can restore T cell activation and reverse osimertinib resistance (91). This implies that the combination of EGFR-TKIs and STING agonists can potentiate the antitumor effects of EGFR-TKIs. In addition, Lin et al. (91) demonstrated that TAMs may mediate T-cell inhibition by up-regulating the expression of inducible nitric oxide (NO) synthase and PD-L1. Studies have shown that NO can promote cisplatin resistance in NSCLC and inhibit T cell proliferation (93, 94). Upregulation of PD-L1 expression in TAMs can increase immunosuppression and tumor aggressiveness in NSCLC (95, 96). These mechanisms provide targets for reactivating T cells in TME. However, Lin et al. (91) did not rule out the possibility that other cells may also be involved in anti-tumor immunity when stimulated by STING agonists, such as dendritic cells and endothelial cells (97, 98).

3.3 Prospects

Further deliberation is warranted on strategies to enhance T cell infiltration in the TME of NSCLC. Immune checkpoint inhibitors (ICIs) can potentially induce M1 polarization of TAMs and reactivate T cells within the TME (99). Theoretically, the combination therapy of ICIs and EGFR-TKIs may enhance the efficacy of EGFR-TKIs in NSCLC. However, the combination of ICIs and EGFR-TKIs has been found to result in intolerable toxicity during clinical trials (100). Strategies to enhance T cell infiltration in the TME of NSCLC, such as reprogramming TAMs or reducing their infiltration, should be developed for clinical implementation.

4 TAMs mediate EGFR-TKIs resistance through M2-like polarization of macrophage

4.1 Lipid metabolism pathways

Lipid metabolism is closely related to TAMs polarization (101). Chen et al. (102) showed that overexpression of sterol regulatory element-binding protein 1 (SREBP1) can mediate osimertinib resistance. Furthermore, Liang et al. (103) analyzed the role of 9 genes related to lipid metabolism in osimertinib resistance. They found that T-cell lymphoma invasion and metastasis 2 (TIAM2) can induce TAMs M2-like polarization mediated osimertinib resistance through PI3K/AKT/mTOR signaling pathway (Figure 2).

4.1.1 Prospects

Targeting lipid metabolic pathways to cause repolarization of TAMs is a feasible approach to improve resistance to EGFR-TKIs. Jin et al. (104) found that simvastatin can mediate TAMs repolarization by targeting cholesterol metabolism. Yin et al. (105) developed a dual-targeting liposomal system for the codelivery of simvastatin/gefitinib to treat NSCLC with brain metastases. Dual-targeting liposomal system with modification of anti-PD-L1 nanobody and transferrin receptor-binding peptide T12 can enter the blood-brain barrier to reverse EGFR T790M mutation-mediated resistance *via* TAMs repolarization (105).

4.2 STAT3/IL-4 pathway

Chen et al. (106) found that T790M-cis-L792F mutation is one of the mechanisms of osimertinib resistance. And Sun et al. (107) found that the expression and secretion of IL-4 increased in T790M-cis-L792F mutant cells, promoting the M2-like polarization of TAMs. Furthermore, Sun et al. (107) demonstrated that TAMs M2-like polarization is one of the downstream mediators of the STAT3/IL-4 signaling pathway, and blocking STAT3 with SH-4-54 and IL-4 with dupilumab can reverse osimertinib resistance to some extent.

4.2.1 Prospects

Targeting STAT3 could be a promising strategy for overcoming resistance to EGFR-TKIs (108). Park et al. (109) showed that the root extract of Scutellaria baicalensis can induce apoptosis in EGFR-TKIs resistant NSCLC by inhibiting STAT3. Shu et al. (110) reversed afatinib resistance in NSCLC by knocking down lncRNA BLACAT1

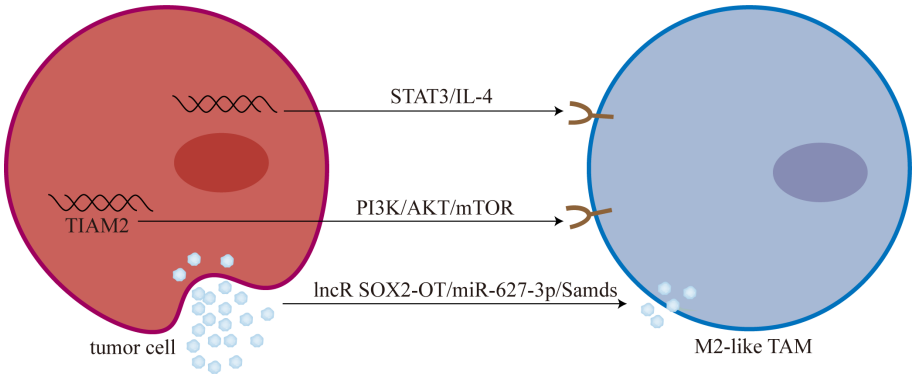

FIGURE 2
Tumor cells promoted M2-like polarization of TAMs. M2-like TAM: M2-like tumor-associated macrophage; mTOR, mammalian target of rapamycin; lncR SOX2-OT, long non-coding RNA SOX2 overlapping transcript; Smads, drosophila mothers against decapentaplegic proteins.

by regulating STAT3 signaling. In addition, it has been previously reported that aberrant activation of STAT3 can promote M2-like polarization of macrophages (111). Lu et al. (111) showed that gefitinib combined with STAT3 inhibitor and anti-CD47 monoclonal antibody could reprogram TAMs and ameliorate acquired resistance to gefitinib in NSCLC. Small molecule inhibitors targeting STAT3 have shown preliminary antitumor effects (112, 113). Further investigation into STAT3 and IL-4 as potential targets is warranted to overcome resistance to EGFR-TKIs.

4.3 LncRNA SOX2-OT/miR-627-3p/Smads pathway

Recently, Zhou et al. (114) found that long non-coding RNA SOX2 overlapping transcript (lncRNA SOX2-OT) is highly expressed in exosomes derived from NSCLC cells. Subsequently, exosomal lncRNA SOX2-OT can promote M2-like polarization of TAMs and promote EGFR-TKIs resistance (114). Mechanistically, lncRNA SOX2-OT promotes M2-like polarization of TAMs by increasing the expression of drosophila mothers against decapentaplegic proteins (Smads) through sponging miR-627-3p (114).

4.3.1 Prospects

lncRNA SOX2-OT/miR-627-3p/Smads axis represents a promising target for reprogramming TAMs. However, there still needs to be more feasible approaches to target this pathway.

5 TAMs mediate EGFR-TKIs resistance by modulating tumor cell phenotypes

5.1 Stabilizing tumor cell phenotypes

Zhao et al. (115) treated NSCLC cells with gefitinib and subsequently co-cultured them with macrophages to mimic the behavior of migrating macrophages. Migrating macrophages contributed to gefitinib resistance by stabilizing tumor cell phenotypes before macrophage polarization. Additionally, Zhao and colleagues (115) postulated that the upregulation of vimentin mediated by TGF-β might also account for the accelerated acquisition of gefitinib resistance in NSCLC cells.

5.1.1 Prospects

Reducing the recruitment of TAMs or depleting the TAMs in the TME of NSCLC may be a potential approach to improve EGFR-TKIs resistance. CCL2-chemokine (C-C motif) receptor 2 (CCR2) signaling and the colony-stimulating factor 1(CSF1)-CSF1 receptor (CSF1-CSF1R) axis are potential therapeutic targets (116, 117). For example, previous studies (118) have shown that CSF1R inhibitors can deplete M2 macrophages in the TME. Sidorov et al. (119) demonstrated that the combination therapy of erlotinib and MLN0128 (an mTOR inhibitor) effectively reduces the infiltration of immunosuppressive chemokines, such as CCL2 and periostin, as well as TAMs in the TME of glioblastoma, leading to a significant improvement in survival outcomes for glioblastoma mice. Schmall et al. (120) demonstrated that inhibiting the recruitment of TAMs and promoting their M1-like polarization through CCR2 inhibition can effectively inhibit lung cancer progression. In addition, targeting surface receptors such as CD52, scavenger receptor-A, folic acid receptor-β, and CD206 represents potential approaches for depleting TAMs (121). Future research endeavors should investigate the clinical applications of these protocols in NSCLC.

5.2 Promoting the EMT

EMT, the process of epithelial-to-mesenchymal transition, plays a crucial role in physiological processes such as wound healing, development, and stem cell behavior (122). However, it is closely associated with tumorigenesis, tumor progression, and drug resistance under pathological conditions (123). Importantly, EMT is one of the mechanisms of acquired resistance to EGFR-TKIs (124). Approaches to overcome EGFR-TKI resistance in NSCLC by reversing EMT are currently under investigation, including the targeting of CD70, cyclin-dependent kinase 7 (CDK7), lipid metabolism pathways, and

fibroblast growth factor receptor 1 (FGFR1) (125–128). Several studies (129, 130) have revealed that TAMs can promote the EMT of tumor cells. Bonde et al. (129) showed that TAMs promote tumor EMT through TGF-β signaling and activation of the β-catenin pathway in NSCLC. And Shen et al. (130) demonstrated that inhibition of TAMs can reverse tumor EMT in NSCLC.

5.2.1 Prospects

Further investigation is warranted to target TAMs to reverse EMT in EGFR-TKI-resistant cells. Reprogramming TAMs to reduce the secretion of pro-EMT signals, such as TGF-β, may represent a promising strategy. Consistent with this hypothesis, Jin et al. (104) showed that targeting lipid metabolism could improve EMT-related drug resistance by reprogramming TAMs in NSCLC.

6 Discussion

Resistance to EGFR-TKIs remains a global challenge, and exploring new methods to enhance the efficacy of EGFR-TKIs is imperative in NSCLC. This review summarizes the multiple mechanisms of TAM-mediated EGFR-TKIs resistance in NSCLC, including activation of bypass pathways, inhibition of T cell activity, M2-like polarization, and regulation of tumor cell phenotypes. Several pertinent issues warrant discussion.

Inhibiting the TAMs-related bypass pathway may be a potential approach to improving resistance to EGFR-TKIs in NSCLC (Table 2). The significance of the mTOR-related pathway in enhancing resistance to EGFR-TKIs warrants reiteration. Previous studies (131) have shown that high expression of mTOR correlates with a diminished therapeutic response to erlotinib in NSCLC. TAMs can induce resistance to EGFR-TKIs by activating the AKT/mTOR signaling pathway directly (34). Additionally, mTOR-related pathways may mediate EMT-related EGFR-TKIs resistance (132). Zhang et al. (133) demonstrated that MTI-31, an inhibitor of mTORC1/2, effectively impedes the progression and EMT of NSCLC while simultaneously enhancing antitumor immunity. Significantly, the PI3K/AKT/mTOR signaling pathway can also facilitate M2-like polarization of TAMs to promote EGFR-TKIs resistance (103). Based on this existing evidence, combination therapy involving mTOR inhibitors and EGFR-TKIs may improve resistance to EGFR-TKIs by blocking multiple resistance signals. However, current clinical trials have demonstrated that the combination therapy does not yield a superior clinical response compared to EGFR-TKIs monotherapy, and its toxicity profile is challenging to manage (53). Further experimentation is warranted to elucidate this phenomenon in the future. On the other hand, targeting STAT3 may represent a promising strategy to enhance the efficacy of EGFR-TKIs by overcoming TAM-mediated resistance. TAMs-derived exosomes can mediate EGFR-TKIs resistance by activating STAT3 signaling pathway (17). Moreover, STAT3 also plays a crucial role in promoting the M2-like polarization of TAMs (111). Combining STAT3 inhibitors with EGFR-TKIs inhibits drug resistance mediated by exosomes derived from TAMs and reprograms TAMs. Previous studies (134–136) have shown the potential of STAT3 inhibitors in combination with EGFR-TKIs for anti-tumor therapy. W2014-S, a novel STAT3 inhibitor, can significantly enhance the anti-tumor effect of EGFR-TKIs in TKI-resistant NSCLC (137). Wang et al. (138) demonstrated that the STAT3 inhibitor BBI608 could potentiate the anti-tumor efficacy of EGFR-TKIs by modulating the ROR1/ABCB1/P53 signaling pathway.

TABLE 2 Strategies for improving resistance to EGFR-TKIs by targeting TAMs.

Strategies	Up/Down	Targets	References
Dictamnine	Down	PI3K/AKT/mTOR and MAPK pathways	43
Torin2	Down	AKT/mTOR pathway	44
Temsirolimus	Down	mTOR	45
BEZ235	Down	PI3K/mTOR pathway	46, 49, 52
Active fraction (HS7) from Taiwanofungus camphoratus	Down	AKT-mTOR, ERK and STAT3 pathways	47
Everolimus	Down	mTOR	45,48, 55
Ku-0063794	Down	mTOR	50
Ferumoxytol and CpG oligodeoxynucleotide 2395	Down	EGFR and AKT/mTOR pathways	51
T0901317 and GW3965	Down	AKT	62
Bufalin	Down	MET/PI3K/AKT Pathway	63
Chloroquine	Down	AKT	64
Norcantharidin	Down	MET/PI3K/AKT Pathway	65
MiR-30a-5p	Down	PI3K/AKT Pathway	66, 69
BMS-708163	Down	PI3K/AKT Pathway	67
Polyphyllin I	Down	PI3K/AKT Pathway	68

(Continued)

TABLE 2 Continued

Strategies	Up/Down	Targets	References
anti-HER-3 antibody	Down	HER-3 and PI3K/AKT Pathway	71
anti-HER-3 antibody	Up	STING	75
HECrossMAb	Down	PI3K/AKT Pathway	72
Gefitinib and Vorinostat	Up	M1-like polarization of TAMs	73
Cosuppression of NF-κB and AICDA	Down	NF-κB and AICDA	81
Liver X receptors agonist	Down	AKT and NF-κB	82
STING agonist MSA-2	Up	M1-like polarization of TAMs	91
Simvastatin	Up	M1-like polarization of TAMs	104, 105
The Root Extract of Scutellaria baicalensis	Down	STAT3	109
Knockdown of lncRNA BLACAT1	Down	STAT3	110
STAT3 inhibitor and an anti-CD47 monoclonal antibody	Up	M1-like polarization of TAMs	111
Simvastatin	Down	EMT	104
MTI-31	Down	EMT	133
W2014-S	Down	STAT3	137
BBI608	Down	STAT3	138

EGFR-TKIs, epidermal growth factor receptor tyrosine kinase inhibitors; TAMs, tumor-associated macrophages; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinases; STAT3, signal transducer and activator of transcription 3; MET, mesenchymal to epithelial transition factor; HER-3, human epidermal growth factor receptor 3; STING: Stimulator of interferon genes; NF-κB, nuclear factor-κB; AICDA, activation-induced cytidine deaminase; EMT, epithelial-mesenchymal transition.

Reprogramming TAMs is a crucial strategy for improving the resistance to EGFR-TKIs in NSCLC. It is worth mentioning that reprogramming TAMs can enhance the efficacy of EGFR-TKIs through a variety of mechanisms, including inhibition of TAM-related drug resistance pathway (34), reactivation of T cells in the TME (91), and reversal of EMT of tumor cells (104). STING (91), lipid metabolic pathways (101), mTOR (34), Smads (114), IL-4 (107), and STAT3 (111) have been reported as targets for reprogramming TAMs in NSCLC. In addition, other strategies for reprogramming TAMs are currently under investigation, which may offer insights into improving resistance to EGFR-TKIs in NSCLC. Parayath et al. (139) reprogrammed TAMs by intraperitoneal injection of Hyaluronic Acid-Based Nanoparticles Encapsulating MicroRNA-125b in NSCLC. Sarode et al. (140) reprogrammed TAMs by targeting the β-catenin/FOSL2/ARID5A signaling pathway in lung cancer. Future research should investigate innovative approaches to reprogramming TAMs in NSCLC with EGFR mutation.

Finally, reducing the number of TAMs in the TME of EGFR-mutant NSCLC, either by inhibiting TAM recruitment or depleting TAMs, may represent a promising strategy to overcome resistance to EGFR-TKIs. The clinical applicability of these methods warrants further investigation (116, 117).

7 Conclusions

TAMs mediate EGFR-TKIs resistance in NSCLC through various mechanisms, including activation of bypass pathways, inhibition of T cell activity, M2-like polarization, and regulation of tumor cell phenotypes. In the future, developing therapeutic regimens that target TAMs, such as interfering with TAM-related pathways, reducing infiltration of TAMs, and reprogramming the macrophage phenotype, could enhance the anti-tumor effect of EGFR-TKIs.

Author contributions

DC, KG, and XY wrote the original draft of the article and drew the illustration. RC, BW, WZ, CF, and MJ contributed to the conceptualization and revised the draft. All authors participated in the revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Sequential treatment in advanced epidermal growth factor receptor-mutated lung adenocarcinoma patients receiving first-line bevacizumab combined with 1st/2nd-generation EGFR-tyrosine kinase inhibitors

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Introduction: The clinical outcomes of sequential treatment of advanced epidermal growth factor receptor (EGFR)-mutated non-small cell lung cancer (NSCLC) patients with first-line bevacizumab combined with 1st/2nd-generation EGFR-TKIs are unclear. Thus, we aimed to analyze the outcomes of these patients.

Methods: Between January 2015 and December 2020, data for 102 advanced EGFR-mutated lung adenocarcinoma patients receiving first-line bevacizumab combined with erlotinib or afatinib followed by treatments at multiple institutions were retrospectively analyzed. All patients with progressive disease (PD) after first-line therapy underwent secondary T790M mutation detection.

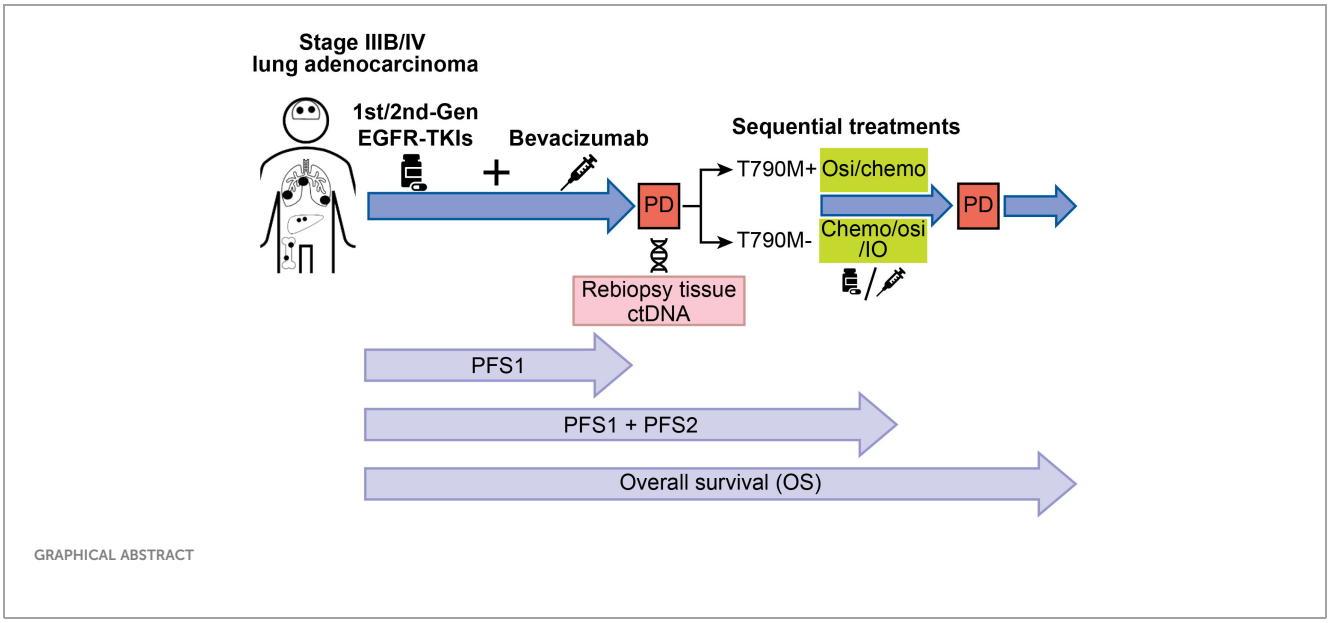
Results: The secondary T790M mutation positive rate of all study patients was 57.9%. First-line erlotinib use and progression-free survival (PFS) after first-line therapy > 12 months were positively associated with the T790M mutation ($P < 0.05$). The response rates (RRs) to second-line treatments were 51.7% and 22.7% for the osimertinib and nonosimertinib groups, respectively ($P = 0.001$).

The median PFS associated with second-line osimertinib and nonosimertinib therapy was 13.7 and 7.1 months, respectively (hazard ratio (HR) = 0.38; 95% confidence interval (CI), 0.23–0.63; P< 0.001). Patients with a secondary T790M mutation receiving second-line osimertinib treatment had a median overall survival (OS) of 54.3 months, and the median OS was 31.9 months for non-T790M-mutated patients receiving second-line nonosimertinib treatments (HR = 0.36; CI: 0.21–0.62, P < 0.001).

Conclusion: The majority of acquired resistance to first-line bevacizumab combined with 1st/2nd-generation EGFR-TKIs is associated with the T790M mutation. Sequential osimertinib treatment in patients with positive secondary T790M mutation is associated with better outcomes among these patients.

KEYWORDS

epidermal growth factor receptor mutation, tyrosine kinase inhibitor, bevacizumab, lung adenocarcinoma, T790M, osimertinib



GRAPHICAL ABSTRACT

1 Introduction

The epidermal growth factor receptor (EGFR) and its downstream signaling pathway play crucial roles in the tumorigenesis of human non-small cell lung cancer (NSCLC) (1, 2). EGFR mutations account for the majority of oncogenic driver mutations in East Asian lung adenocarcinoma patients, and the incidence rate ranges from 40 to 55% (3, 4). The exon 19 deletion (in-frame deletions within exon 19) and L858R (a point mutation at codon 858 within exon 21 by leucine-to-arginine substitution) are the two most frequent (approximately 90%) EGFR mutations in lung adenocarcinoma (1–4). First- and second-generation EGFR tyrosine kinase inhibitors (TKIs), such as erlotinib and afatinib, have been demonstrated to be effective for treating advanced NSCLC harboring exon 19 deletion or L858R EGFR mutations (60-80% objective response rate (RR) and 10-14 months progression-free survival (PFS)) in several prospective clinical trials (5–7). Therefore, erlotinib and afatinib have been used as standard first-line therapies for advanced NSCLC harboring EGFR mutations worldwide.

The vascular endothelial growth factor receptor (VEGFR) signaling pathway has been reported to be involved in tumor growth and progression in various cancer cells (8, 9). Vascular endothelial growth factor (VEGF) is the ligand of VEGFR, and a previous study showed that EGFR-mutated NSCLC cells had increased VEGF protein expression levels compared with wild-type EGFR NSCLC cells (10). Another previous study showed that increased VEGF mRNA expression in plasma and tumor stroma

was associated with resistance to EGFR-TKIs, and combined EGFR-TKIs and VEGF inhibitors had synergistic antitumor effects in an NSCLC mouse model (11). Bevacizumab is a humanized monoclonal antibody targeting VEGF and is used as an angiogenesis agent in anticancer therapies (8, 12). The efficacy of bevacizumab in combination with erlotinib or afatinib for the treatment of untreated advanced EGFR-mutated lung adenocarcinoma has been explored in several previous pivotal clinical trials and clinical studies (13–17). In these previous studies, the combination of erlotinib or afatinib with bevacizumab was demonstrated to have an objective RR of 80% and PFS of 13~24 months (13–17). Therefore, combination therapies have been suggested as a first-line therapeutic option for advanced lung adenocarcinoma patients with EGFR mutations.

The secondary T790M EGFR mutation is the most frequent cause of acquired resistance to first- and second-generation EGFR-TKIs (40%~60%) (18, 19). Osimertinib is a third-generation EGFR-TKI with active targeting of the T790M mutation and was shown to have promising efficacy (71% RR and 10.1 months PFS) in a pivotal clinical trial (AURA3 trial) (20). Therefore, osimertinib has been approved as a therapy for advanced NSCLC patients with secondary T790M mutation with progressive disease (PD) after first- or second-generation EGFR-TKI therapies.

The secondary T790M EGFR mutation appears in advanced lung adenocarcinoma with acquired resistance due to prior bevacizumab treatment combined with erlotinib or afatinib, and osimertinib is administered as a subsequent therapy for T790M-positive patients (15, 16). However, the clinical factors associated with the appearance of a positive T790M mutation in patients receiving first-line combination therapy remain unclear. Thus, we sought to analyze the survival outcomes of patients receiving first-line bevacizumab combined with erlotinib or afatinib followed by sequential systemic therapies (e.g., osimertinib or chemotherapy) after acquired resistance to first-line combination therapy.

2 Materials and methods

2.1 Patients and EGFR mutations

Data from all study patients were retrospectively retrieved from the cancer center database of Linkou, Kaohsiung, Chiayi Chang-Gung Memorial hospitals (CGMHs) and Taipei Tzu Chi Hospital. Between January 2015 and December 2020, 140 advanced lung adenocarcinoma patients with EGFR mutations receiving bevacizumab combined with first- or second-generation EGFR-TKIs as first-line therapy were screened. The inclusion criteria were as follows: (1) patients with primary EGFR mutations without *de novo* T790M; (2) patients with PD after first-line therapy; (3) patients with secondary EGFR T790M mutation tests; and (4) patients receiving subsequent systemic therapies. The exclusion criteria were as follows: (1) no PD after first-line therapy; (2) no tests to detect secondary EGFR T790M mutation; (3) no subsequent systemic therapy administered; and (4) small cell transformation. The summary of study subject screening is summarized in Figure 1.

Amplified refractory mutation system–Scorpion (ARMS/S) assays or next-generation sequencing (NGS) was used to detect primary EGFR mutations and secondary T790M mutations in patients with PD after first-line therapy. The NGS panel used in this study was the same as that described in a previous study (16).

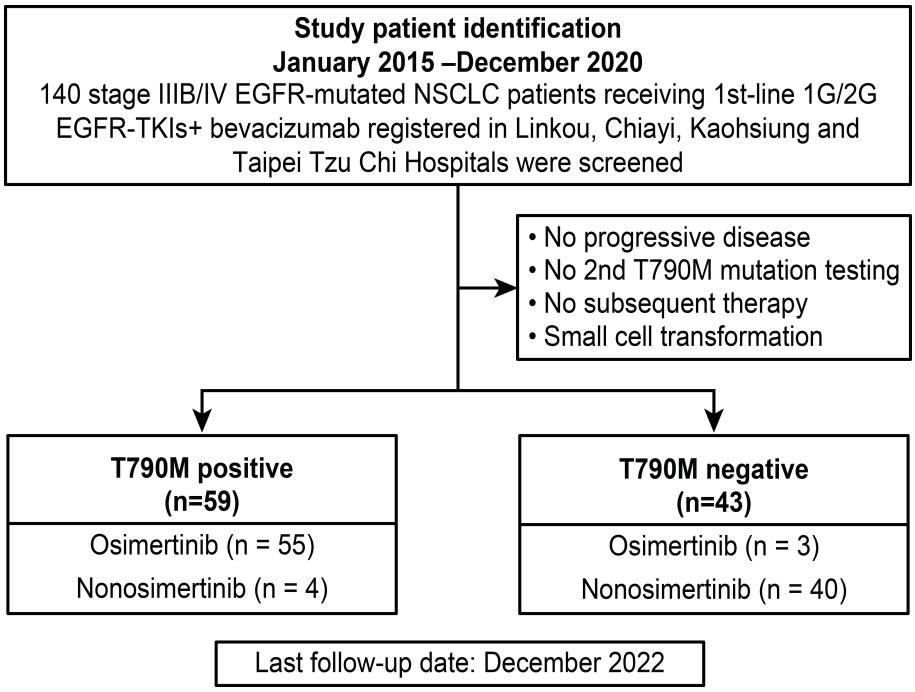


FIGURE 1
Flow chart of the included study patients.

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2.2 Treatment response, survival evaluation, and follow-up

The baseline stages at initial diagnosis of all subjects were determined by computed tomography (CT) with contrast medium enhancement, fluorodeoxyglucose (FDG)-positron emission tomography (PET), and magnetic resonance imaging (MRI) of the brain. All study patients underwent whole-body CT scans every 3 to 4 months to evaluate treatment responses. Additional imaging studies such as sonogram, plain films, MRI and FDG-PET were ordered by physicians based on their need for assistance in disease status assessment.

Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. was used to assess treatment responses. The treatment responses were classified as complete response (CR), partial response (PR), stable disease (SD), and PD. The length of PFS was defined from the treatment start date to the date of first PD images detected or last follow-up. The length of overall survival (OS) was defined from the starting date of first-line therapy to the date of mortality recorded. For patients who survived through the time point of last follow-up (December 31, 2022), the OS was censored at the last recorded clinical visit date.

2.3 Statistical analysis

Categorical variables of study subjects are presented as quantitative variables, and age is presented as the mean ± standard deviation (SD). Cox regression with univariate and multivariate analyses was used to determine the clinical factors associated with the T790M mutation rates. PFS and OS were estimated by Kaplan–Meier survival curves, and two-sided P values were considered statistically significant when they were smaller than 0.05. IBM SPSS Statistics version 22.0 (SPSS Corp., Chicago, IL, USA) was used to perform univariate and multivariate Cox regression analyses. The PFS and OS survival curves were generated by using GraphPad Prism (Version 5.0; GraphPad Software, San Diego, CA, USA).

3 Results

3.1 Baseline patient clinical characteristics and information on sequential treatments

All baseline clinical characteristics of the 102 study patients are shown in Table 1. Ninety-nine (97.1%) patients underwent tissue rebiopsy, and 8 (7.8%) patients had plasma circulating tumor (ct)-DNA liquid biopsy for secondary T790M mutation tests. Five (4.9%) patients had both tissue rebiopsy and ctDNA tests, and 3 (2.9%) patients had ctDNA tests only. Among the 5 (4.9%) patients with both tissue rebiopsy and ctDNA assessment, all the rebiopsy tissues were tested by using NGS, and according to the NGS results, 4 (3.9%) patients were negative for the T790M mutation, and the other 1 (1%)

TABLE 1 Baseline characteristics of all study patients.

Total	N = 102 (%)
Sex	
Male	37 (36.3%)
Female	65 (63.7%)
Age, years (mean ± SD)	57.71 ± 11.02
ECOG PS	
0-1	84 (82.4%)
≧2	18 (17.6%)
Smoking status	
Nonsmoker	25 (24.5%)
Former/current smoker	77 (75.5%)
Histology	
Adenocarcinoma	102 (100%)
Stage	
IIIB	4 (3.9%)
IV	98 (96.1%)
EGFR mutation	
L858R	48 (47.1%)
Exon 19 deletion	52 (51.0%)
Others*	2 (1.9%)
Metastatic sites	
Pleural effusion	31 (30.4%)
Brain	33 (32.4%)
Bone	43 (42.2%)
Liver	18 (17.6%)
Adrenal	6 (5.9%)
First-line EGFR-TKIs + bevacizumab	
Erlotinib	53 (52.0%)
Afatinib	49 (48.0%)
Secondary EGFR-T790M mutation detection methods	
Tissue rebiopsy	99 (97.1%)
Plasma circulating tumor(ct)-DNA	8 (7.8%)
Secondary T790M mutation	
Positive	59 (57.9%)
Negative	43 (42.1%)
Subsequent treatments	
Osimertinib	58 (56.9%)
Chemotherapy	42 (41.1%)
Other EGFR-TKI**	1 (1%)

(Continued)

TABLE 1 Continued

Total	N = 102 (%)
Anti-PD-1/PD-L1 immune checkpoint inhibitors (ICIs)	9 (8.8%)
Chemotherapy + ICI	8 (7.8%)
ICI alone	1 (1%)
Anti-angiogenesis agents	
Bevacizumab	14
Ramucirumab	9

SD, standard deviation; ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; PD-1, programmed death-1; PD-L1, programmed cell death-ligand 1.
* 1 G719X, 1 S768I.
**Afatinib.

was positive. All five (4.9%) patients were negative for the T790M mutation based on ctDNA tests. In the 3 (2.9%) patients who underwent ctDNA testing alone, 2 (1.9%) were positive for the T790M mutation, and 1 (1%) was negative. In the 59 (57.9%) patients positive for the T790M mutation, 55 (53.9%) were administered osimertinib, 3 (2.9%) received platinum-based doublet chemotherapy, and 1 (1%) was switched from first-line erlotinib to afatinib and continued to receive bevacizumab as 2nd-line therapy. In 43 (42.1%) patients negative for the T790M mutation, 3 (2.9%) received osimertinib, 39 received chemotherapy-based therapy, and 1 patient received single pembrolizumab (anti-programmed death-1 (PD-1) inhibitor) as 2nd-line therapy. Twenty-three (22.5%) patients received antiangiogenic agents, including bevacizumab and ramucirumab (anti-VEGFR inhibitor), as second-line therapy. Four (3.9%) patients received second-line osimertinib with continuation of

TABLE 2 Univariate and multivariate analyses of predictors associated with acquired T790M mutation (n=59).

	Number of patients	T790M+ (%)	*P* value	Multivariate analysis	
				Odds ratio (95% CI)	*P* value
Basic data					
Sex			0.584		
Male	20	33.9 (%)			
Female	39	66.1 (%)			
Age (years)			0.466		
≦60	30	50.8 (%)			
> 60	29	49.2 (%)			
ECOG PS			0.219		
0-1	50	84.7 (%)			
≥2	9	15.3 (%)			
Smoking			0.830		
Nonsmoker	45	76.3 (%)			
Current/former	14	23.7 (%)			
EGFR mutation			0.087		
L858R	23	39.0 (%)			
Exon 19 deletion	35	59.3 (%)			
Others	1	1.7 (%)			
Stage			0.999		
IIIB	3	5.1 (%)			
IV	56	94.9(%)			
First-line TKI used					
Afatinib	23	39.0 (%)	0.033	1	
Erlotinib	36	61.0 (%)		2.734 (1.144-6.531)	0.029
PFS (months)			0.013		
≦12	10	16.9 (%)		1	
>12	49	83.1 (%)		2.958 (1.142-7.661)	0.025

ECOG PS, Eastern Cooperative Oncology Group performance status; CI, confidence interval; PFS: progression-free survival; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

bevacizumab, and 6 (5.9%) patients received ramucirumab combined with osimertinib as second-line therapy. Ten (9.8%) patients received bevacizumab combined with chemotherapy, and 4 (3.9%) of the 10 (9.8%) patients received bevacizumab combined with chemotherapy and atezolizumab (anti-programmed cell death-ligand 1 (PD-L1) inhibitor). Three (2.9%) patients received chemotherapy combined with ramucirumab.

3.2 Clinical factors associated with secondary EGFR T790M mutation after first-line bevacizumab combined with 1st-/2nd-generation EGFR-TKIs

The clinical factors associated with secondary T790M mutation after first-line therapy in this study were analyzed by using univariate and multivariate Cox regression (Table 2). In univariate analysis, the primary exon 19 deletion mutation had a trend of a higher secondary T790M mutation-positive rate than the primary L858R mutation, but no statistical significance was achieved. First-line bevacizumab combined with erlotinib had a significantly higher secondary T790M mutation-positive rate than bevacizumab combined with afatinib. In addition, a longer PFS (> 12 months) while on first-line treatment had a significantly higher T790M mutation rate than a shorter PFS (≦12 months). The multivariate analysis showed that first-line erlotinib use (vs. afatinib, odds ratio: 2.734, 95% confidence interval (CI): 1.144–6.531, P = 0.029) and longer PFS while on first-line therapy (vs. ≤12 months PFS, odds ratio: 2.958, 95% CI: 1.142–7.661, P = 0.025) were independent predictive factors associated with secondary T790M mutation. The clinical information comparison between patients treated with first-line afatinib plus bevacizumab and erlotinib plus erlotinib is shown in Supplementary Table S1.

3.3 Analysis of PFS and OS between the two first-line therapy groups

The PFS and OS of the 2 first-line therapies were analyzed by Kaplan–Meier survival curves. There was no significant difference in the median PFS of first-line bevacizumab combined with erlotinib and first-line bevacizumab combined with afatinib (19.6 vs. 18.7 months, hazard ratio (HR) = 1.05; CI: 0.52–1.15, P = 0.201) (Figure 2A). Patients with different primary EGFR mutations (L858R and exon 19 deletion) were divided into 2 groups to analyze the PFS associated with first-line therapies. In L858R-mutated patients, the median PFS was 18.4 and 21.3 months for the bevacizumab combined with erlotinib group and bevacizumab combined with erlotinib group, respectively (HR = 1.05; CI: 0.59–1.87, P = 0.874) (Figure 2B). For patients with primary exon 19 deletion mutations, the median PFS of the first-line bevacizumab combined with erlotinib group was significantly higher than that of the first-line bevacizumab combined with afatinib group (20.7 vs. 13.9 months, HR = 0.53; CI: 0.29–0.94, P = 0.031) (Figure 2C). Regarding OS, no significant difference was noted between the 2 groups of patients receiving first-line bevacizumab combined with erlotinib and first-line bevacizumab combined with afatinib (median OS: 49.4 VS. 42.6 months, HR = 0.841; CI: 0.51–1.38, P = 0.470) (Figure 2D). Patients with baseline brain metastasis were analyzed, and the results are shown in Supplementary Figure S1. The treatment response rate of first-line combination therapy was 84.8%, and median PFS was 14.7months in patients with baseline brain metastasis (Supplementary Figures S1A, B).

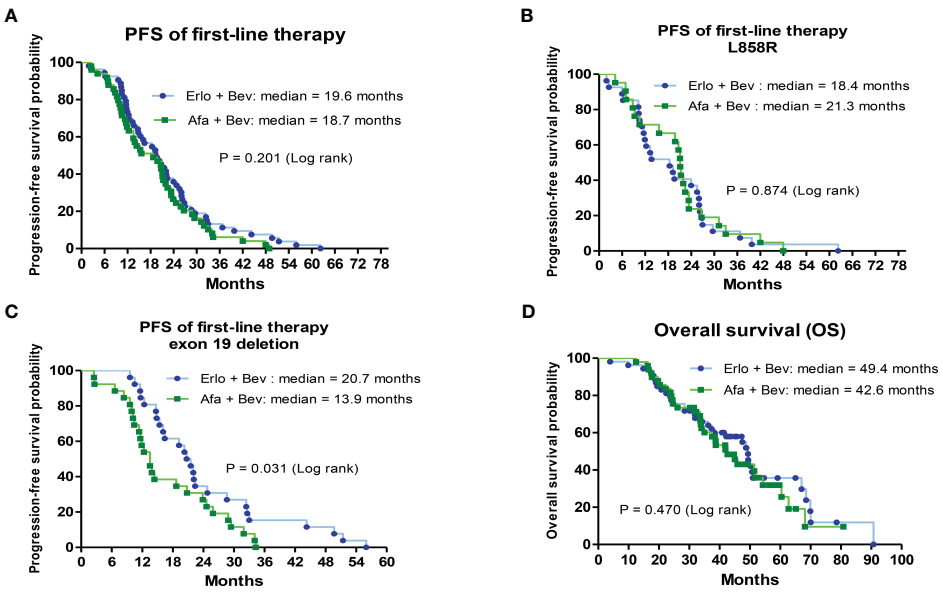

FIGURE 2
Analysis of progression-free survival (PFS) for first-line treatments and overall survival (OS) for first-line treatments by Kaplan–Meier survival curves. **(A)** Comparison of median PFS between bevacizumab combined with erlotinib or afatinib (HR = 1.05; 95% CI, 0.52–1.15; P= 0.201). **(B)** The median PFS between bevacizumab combined with erlotinib or afatinib in primary L858R-mutated patients (HR = 1.05; 95% CI, 0.59–1.87; P= 0.874). **(C)** The median PFS between bevacizumab combined with erlotinib or afatinib in primary exon 19 deletion-mutated patients (HR = 0.53; 95% CI, 0.29–0.94; P= 0). **(D)** The median OS between bevacizumab combined with erlotinib or afatinib (HR = 0.841; 95% CI, 0.51–1.38; P= 0.470).

TABLE 3 Clinical response to 2nd-line therapy.

	Osimertinib N =58 (%)	Nonosimertinib N = 44 (%)	P value
CR	0	0	0.001
PR	30 (51.7%)	10 (22.7%)	
SD	26 (44.8)	24 (54.5%)	
PD	2 (3.5%)	10 (22.7%)	
RR (%)	51.7	22.7	
DCR (%)	96.5	77.3	

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; RR, response rate; DCR, disease control rate.

The median OS of patients with baseline brain metastasis was 34.3 months (Supplementary Figure S1C).

3.4 Treatment outcomes of patients with different T790M mutation statuses and subsequent treatments

Most secondary T790M mutation-positive patients (56 of 59 = 94.9%) who received osimertinib as second-line therapy were divided into osimertinib and nonosimertinib groups for comparison. Second-line osimertinib treatment had a significantly higher objective RR than nonosimertinib therapy (51.7% vs. 22.7%, P = 0.001) (Table 3). All 3 patients who underwent liquid biopsy alone received osimertinib as subsequent treatments, and all patients had SD to osimertinib therapy. The PFS of the 3 patients ranged from 6.37 to 22.17 months. The patient who was T790M negative in liquid biopsy had a 14.83 PFS on osimertinib therapy.

Patients with secondary T790M mutation and second-line therapy had a significantly longer median PFS than those without T790M mutation (15.4 vs. 7.1 months, HR = 0.37; CI: 0.22–0.61, P < 0.001) (Figure 3A). The median PFS of those who received second-line osimertinib therapy was significantly longer than that of those who received nonosimertinib therapy (13.7 vs. 7.1 months, HR = 0.38; CI: 0.23–0.63, P < 0.001) (Figure 3B). The length of PFS of patients who received first-line plus second-line treatments (PFS1 + PFS2) was evaluated. Patients with a secondary T790M mutation had a significantly longer median PFS (1 + 2) than those without a T790M mutation (40.2 vs. 25.3 months, HR = 0.39; CI: 0.24–0.65, P < 0.001) (Figure 3C). Patients with a secondary T790M mutation who received 2nd-line osimertinib had a significantly longer median PFS (1 + 2) than those without a T790M mutation who received nonosimertinib therapy (41.8 vs. 25.9 months, HR = 0.39; CI: 0.23–0.65, P < 0.001) (Figure 3D).

We further analyzed the OS of patients with different secondary EGFR T790M mutation statuses and subsequent treatments. Patients with a secondary T790M mutation had a significantly longer median OS than those without a T790M mutation (54.3 vs. 33.5 months, HR = 0.34; CI: 0.19–0.59, P < 0.001) (Figure 4A). Patients with secondary T790M mutation who received osimertinib as subsequent treatment had a significantly longer median OS than those without T790M mutation who received a nonosimertinib subsequent therapy (54.3 VS. 31.9 months, HR = 0.36; CI: 0.21–0.62, P < 0.001) (Figure 4B).

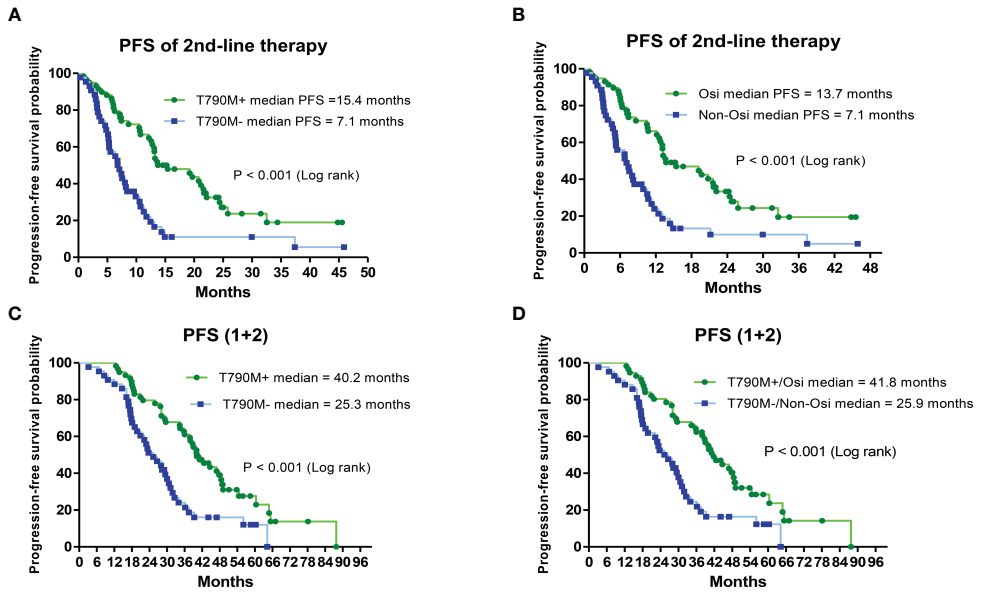

FIGURE 3
Analysis of progression-free survival (PFS) of second-line and first-line plus second-line (PFS1 + 2) therapies by Kaplan–Meier survival curves. **(A)** Comparison of PFS of second-line treatments between T790M mutation-positive and T790M mutation-negative patients (HR = 0.37; 95% CI, 0.22–0.61; P< 0.001). **(B)** Comparison of PFS between second-line osimertinib and nonosimertinib treatments (HR = 0.38; 95% CI, 0.23–0.63; P< 0.001). **(C)** Comparison of PFS (1 + 2) between T790M mutation-positive and T790M mutation-negative patients (HR = 0.39; 95% CI, 0.24–0.65; P< 0.001). **(D)** Comparison of PFS (1 + 2) between second-line osimertinib and nonosimertinib treatments (HR = 0.39; 95% CI, 0.23–0.65; P< 0.001).

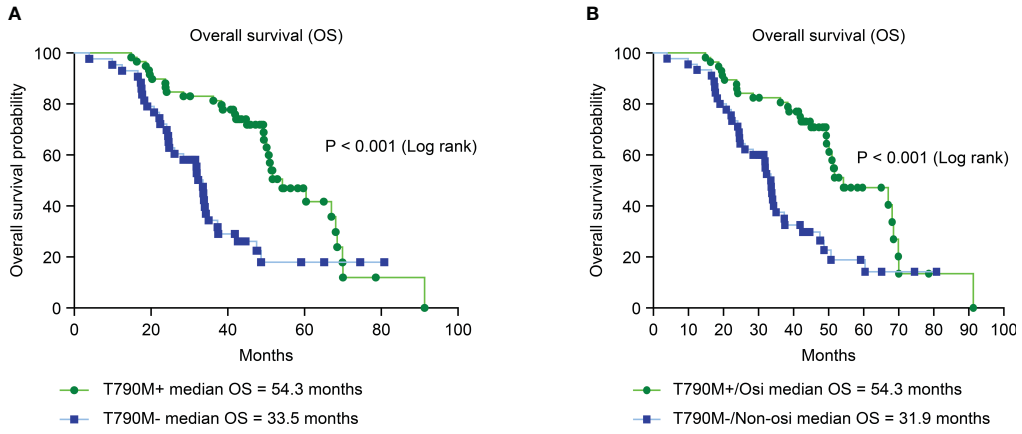

FIGURE 4
Analysis of overall survival (OS) between different T790M mutation statuses and second-line treatments by Kaplan–Meier survival curves. **(A)** Comparison of OS between T790M mutation-positive and T790M mutation-negative patients (HR = 0.34; 95% CI, 0.19–0.59; P< 0.001). **(B)** Comparison of OS between second-line osimertinib in T790M-positive and nonosimertinib in T790M-negative patients (HR = 0.36; 95% CI, 0.21–0.62; P< 0.001).

4 Discussion

The results of this study provide some important clinical information regarding sequential treatments for advanced EGFR-mutated NSCLC patients receiving first-line bevacizumab combined with 1st/2nd-generation EGFR-TKIs. First, the secondary T790M mutation rate after PD in this study was 57.9%. Second, the use of erlotinib in first-line therapy and PFS > 12 months were identified as independent predictive factors associated with higher secondary T790M mutation rates. Third, T790M-mutated patients receiving subsequent osimertinib had a significantly better treatment response and longer PFS than those without the T790M mutation receiving nonosimertinib therapy. In addition, T790M-mutated patients receiving subsequent osimertinib had significantly longer OS than those without the T790M mutation.

The acquired T790M mutation rate in this study was 57.9% and was consistent with that reported in previous studies (18, 21, 22). In contrast to previous studies, all the patients in this study received bevacizumab in addition to 1st/2nd-generation EGFR-TKIs, whereas most patients in previous studies received EGFR-TKI-alone therapies (18, 21, 22). The results of our study indicated that bevacizumab in addition to 1st/2nd-generation EGFR-TKIs does not alter the mechanism of acquired resistance in advanced primary EGFR-mutated NSCLC. Some previous studies showed that prior afatinib therapy was associated with a lower secondary T790M mutation-positive rate when compared with first-generation EGFR-TKIs, and these results were similar to those in our study (23, 24). Some previous studies showed that prior afatinib therapy was not associated with a lower secondary T790M mutation-positive rate and that this rate was even higher than that for first-generation EGFR-TKIs (25, 26). Although there are differences among our study and previous studies, the acquired T790M mutation rates after afatinib therapy in previous studies ranged from 30-50% (22–26). In addition, the small sample sizes in these studies may have led to different statistical significances among these studies. A long PFS of prior EGFR-TKI therapy (> 12 months) was identified as a predictive factor associated with acquired T790M mutation positive rates in previous studies, which was similar to the result in this study (22–26). A previous study showed that prolonging afatinib therapy in EGFR-mutated NSCLC by adding bevacizumab led to a positive acquired T790M mutation conversion, and the results in the same study suggested that prolonging afatinib therapy may induce the clonal selection of acquired T790M-mutated NSCLC cells (27). This clonal selection hypothesis may explain why long PFS of prior 1st/2nd-generation EGFR-TKI therapies is associated with an increased secondary T790M mutation rate. In the analysis of this study, bevacizumab combined with erlotinib had a significantly longer median PFS than bevacizumab combined with afatinib among patients with exon 19 deletion mutations. In addition, more patients in the bevacizumab combined with erlotinib treatment group had a longer PFS (> 12 months) than those in the bevacizumab combined with afatinib group (40 (75.5%) vs. 32 (65.3%)). Our study mainly focused on the rebiopsy results and subsequent therapies, and the study patients were retrospectively selected by selection criteria. Therefore, selection bias may lead to statistical significance in the median PFS between the first-line afatinib and erlotinib combined with bevacizumab groups in patients with exon 19 deletion mutations. Taken together, long treatment PFS is suggested to be the main factor associated with the occurrence of secondary T790M mutation, not afatinib and erlotinib therapies.

In the results of a previous clinical trial (AURA, NCT01802632), osimertinib had a 21% RR and a median PFS of 2.8 months for treating T790M mutation-negative patients with acquired resistance to prior EGFR-TKIs. The results of the AURA trial indicated that osimertinib is less effective in T790M-negative patients than in those with secondary T790M mutations after resistance to prior EGFR-TKI treatments (21). Before osimertinib was approved by the United States Food and Drug Administration (U.S. FDA, November 2015), platinum-based chemotherapy was the suggested subsequent treatment for patients who had PD after 1st/2nd-generation EGFR-TKI therapies (28, 29). Although osimertinib was approved for advanced NSCLC with acquired T790M mutation, chemotherapy

has remained the clinically preferred subsequent treatment for T790M-negative patients with PD after 1st/2nd-generation EGFR-TKI therapies; furthermore, drugs targeting mutations other than T790M are still under investigation in clinical trials (29). Although immunotherapy, such as PD1/PD-L1 immune checkpoint inhibitors (ICIs), has been shown to improve the survival of advanced NSCLC patients without driver mutations (30), the survival benefit of immunotherapy is still very limited for advanced EGFR-mutated NSCLC patients (29, 30).

Osimertinib has been widely used as a late-line therapy for T790M-mutated NSCLC patients based on the results of AURA serial trials (20, 21, 31). In the survival analysis of the NEJ026 trial, patients treated with osimertinib in second-line or later-line therapies had significantly longer OS than those without osimertinib therapy after bevacizumab plus erlotinib or erlotinib alone treatment (32). A previous study also showed that T790M-mutated NSCLC patients receiving subsequent osimertinib therapy had significantly longer OS than those without acquired T790M mutation and subsequent osimertinib therapy. The same study showed that the use of 1st-generation or 2nd-generation EGFR-TKIs in first-line therapies did not affect OS (22). The results of our study are compatible with those shown in 2 previous clinical studies (22, 32). Taken together, these results indicated that the acquired T790M mutation is a key factor associated with OS in advanced EGFR-mutated NSCLC patients receiving 1st-generation or 2nd-generation EGFR-TKIs as first-line therapies.

Osimertinib is suggested as a first-line therapy for advanced EGFR-mutated NSCLC patients because a pivotal clinical trial (FLAURA) showed that osimertinib had a median PFS of 18.9 months and OS of 38.6 months, which were significantly longer than those of comparator therapies (10.2 months of median PFS and 31.8 months of median OS) (33). The median OS associated with first-line osimertinib in the FLAURA trial was 38.6 months (33). In the FLAURA trial (33), patients in the comparator arm received gefitinib or erlotinib alone treatments, whereas all patients in our study received bevacizumab in addition to erlotinib or afatinib.

A previous prospective trial (RELAY) demonstrated that erlotinib combined with ramucirumab had a significantly longer median PFS than erlotinib combined with placebo in untreated advanced EGFR-mutated NSCLC patients (19.4 vs. 12.4 months). Erlotinib combined with ramucirumab has been suggested as a first-line therapy choice for advanced EGFR-mutated NSCLC based on the results of the RELAY trial (34). However, patients with baseline brain metastasis were excluded by the RELAY trial, and the efficacy of ramucirumab combined with erlotinib in EGFR-mutated NSCLC patients with brain metastasis was not clear (34). In the NEJ026 study, 32% of study patients had baseline brain metastasis in the erlotinib combined with bevacizumab and erlotinib alone arms (14). A previous study also reported that bevacizumab in addition to EGFR-TKIs was more effective for brain metastasis control and prevention of the progression of brain metastasis than EGFR-TKI treatment alone in NSCLC with EGFR mutations (35). In addition, some previous studies reported that systemic administration of bevacizumab was effective for the control of NSCLC-related malignant pleural effusion (36). In this study, approximately 30% of patients had baseline brain metastasis and malignant pleural effusion. Regarding the concern regarding metastatic sites and study populations in previous studies, bevacizumab in combination with 1st-/2nd- EGFR-TKIs would be considered as first-line therapy for metastatic EGFR-mutated NSCLC patients.

In the NEJ026 clinical trial, patients who received osimertinib as second-line therapy had a median OS of approximately 50 months, and those who did not receive osimertinib treatments as second-line therapy had a median survival of approximately 40 months (32). In another retrospective clinical study (GioTag study), advanced EGFR-mutated NSCLC patients receiving first-line afatinib followed by osimertinib had a median OS of 37.6 months and 44.8 months in Asian patients (37, 38). A previous study also showed that patients receiving 1st/2nd-generation EGFR-TKIs followed by osimertinib had a median OS over 50 months (22). In this study, patients with acquired T790M mutations receiving subsequent osimertinib had a median OS of 54.3 months, which was compatible with the results of previous studies (22, 32, 37, 38). Together, these results suggest that the OS of advanced EGFR-mutated NSCLC patients who received bevacizumab in combination with 1st/2nd-generation EGFR-TKIs or afatinib alone followed by second-line osimertinib is not inferior to that of patients who received first-line osimertinib therapy. In addition, the median PFS of first-line bevacizumab combined with erlotinib or afatinib in advanced EGFR-mutated NSCLC patients shown by previous studies seems not inferior to first-line osimertinib (13–16). Therefore, bevacizumab combined with erlotinib or afatinib may be a choice of first-line therapy for advanced EGFR-mutated NSCLC patients other than osimertinib.

Rebiopsy, either liquid biopsy or direct tissue biopsy, for secondary T790M detection is recommended as standard care for advanced EGFR-mutated NSCLC with acquired resistance to 1st/2nd-generation EGFR-TKIs (21–26). Most patients in our study underwent tissue rebiopsy or liquid biopsy alone, and only a few (5 = 4.9%) patients underwent both procedures for tests. The second-line osimertinib therapy in this study had a 51.7% RR and a median PFS of 13.7 months, and these results indicated that the T790M mutation testing results were reliable in our study. Some previous studies have suggested that both liquid and tissue rebiopsy be performed for NGS tests because repeated biopsy by liquid or tissue increased the T790M detection rate and may also detect other genomic alterations for optimal subsequent treatment (39–41). In our study, all 5 patients who underwent both liquid and tissue rebiopsies were T790M-negative in liquid biopsy, and one was T790M-positive in tissue rebiopsy. This result indicated that a repeated tissue biopsy converts T790M-negative to T790M-positive results in some patients and is compatible with the findings of previous studies (39–41). In the 3 patients who underwent liquid biopsy alone, 2 had T790M-positive results, and 1 was T790M-negative. Although repeated rebiopsy has been recommended to increase the diagnostic accuracy and T790M positive rate, most patients received only one tissue rebiopsy. The main concerns regarding why patients did not receive repeated biopsies include personal acceptance, procedure-related adverse events, and tumor site procedure-unapproachable tumor sites such as tiny distant metastases (42, 43). Taken together, these findings explain why most patients have a low willingness to undergo repeated tissue rebiopsy in real-world clinical practice.

Small cell lung cancer transformation is a rare (<5%) acquired resistance to previous EGFR-TKI treatment (44). In this study, 2 patients had small cell transformation according to tissue rebiopsy and were excluded from further analysis. For advanced EGFR-mutated NSCLC patients who experienced small cell transformation after previous EGFR-TKI treatments, chemotherapy with platinum-based regimens combined with etoposide is recommended if the patient has acceptable performance status (44). For the 3 patients who underwent liquid biopsies only in this study, all of them were controlled by subsequent osimertinib treatments. Small cell transformation has also been reported as a resistance mechanism to prior osimertinib therapy in a previous study (44). According to the clinical treatment response to osimertinib in the 3 patients who underwent liquid biopsies only, the possibility of small cell transformation was very low, and the 3 patients were still included in this study.

Some limitations of this study should be clarified. First, the study population was East Asian, and whether the secondary T790M mutation rate and outcomes in other ethnic populations are similar to our results is unclear. A recent phase III clinical trial (BEVERLY) investigating the combination of bevacizumab with erlotinib for the treatment of advanced EGFR-mutated NSCLC recruited study patients mainly in European countries (45). In this trial, 24 (49%) patients in the bevacizumab combined with erlotinib arm were reported to receive osimertinib as second-line therapy, but information on the acquired T790M mutation and outcomes was not available (45). Second, the first-line EGFR-TKIs administered in this study were erlotinib and afatinib, and no patients in this study received gefitinib (1st-generation) or dacomitinib (2nd-generation) as first-line treatments. Future studies may be needed to analyze the clinical outcomes of advanced EGFR-mutated NSCLC patients receiving first-line bevacizumab combined with gefitinib or dacomitinib. Finally, the use of multiple genomic alteration detection methods, such as NGS, in NSCLC with acquired resistance to previous bevacizumab combined with erlotinib therapy increases in clinical practice, and resistant genomic alterations other than T790M, such as MET, HER2 or BRAF, can be detected by NGS (29). Targeted therapies for the abovementioned genomic alterations have been developed and explored in clinical trials (29), and patients who receive new targeted therapies may have improved outcomes in the future.

5 Conclusion

Our study clearly demonstrated the clinical perspective regarding sequential treatments with first-line bevacizumab combined with 1st/2nd-generation EGFR-TKIs in advanced lung adenocarcinoma patients harboring EGFR mutations. Secondary T790M mutation detection tests and optimal use of osimertinib may yield favorable survival outcomes.

Data availability statement

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

Ethics statement

This multicenter observational study was approved by the institutional review board (IRB) of the Chang Gung Medical Foundation (no. 202000137B0 and no. 202100379B0) and Taipei Buddhist Tzu Chi General Hospital (no. 09-X-002). Obtaining consent from study subjects was waived by the IRB because of the retrospective nature of this study. All patients in this study received standard cancer care and treatments following the protocol of Chang Gung Medical Foundation and Taipei Buddhist Tzu Chi General Hospital cancer centers. All treatment and evaluation procedures were performed in accordance with the Helsinki Declaration. No identifiable subjective information, such as personal ID or birthday, was presented in this manuscript.

Author contributions

Conception and design: P-CH, C-YH, C-TY. Acquisition of data: P-CH, C-YH, YL, S-HL, L-CC, H-WK, C-CW. Analysis and interpretation of data: P-CH, C-YH, YL, S-HL, L-CC, C-EW, SK, J-SJ, AH, H-WK, C-CW. Writing of the manuscript: P-CH, and C-TY. Review and revision of the manuscript: L-CC, C-EW, SK, H-WK, C-TY. Administrative and funding support: P-CH, C-YH, C-TY. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1249106/full#supplementary-material

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Characterizing the secretome of EGFR mutant lung adenocarcinoma

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Background: Lung cancer is the leading cause of cancer related death worldwide, mainly due to the late stage of disease at the time of diagnosis. Non-invasive biomarkers are needed to supplement existing screening methods to enable earlier detection and increased patient survival. This is critical to *EGFR*-driven lung adenocarcinoma as it commonly occurs in individuals who have never smoked and do not qualify for current screening protocols.

Methods: In this study, we performed mass spectrometry analysis of the secretome of cultured lung cells representing different stages of mutant *EGFR* driven transformation, from normal to fully malignant. Identified secreted proteins specific to the malignant state were validated using orthogonal methods and their clinical activity assessed in lung adenocarcinoma patient cohorts.

Results: We quantified 1020 secreted proteins, which were compared for differential expression between stages of transformation. We validated differentially expressed proteins at the transcriptional level in clinical tumor specimens, association with patient survival, and absolute concentration to yield three biomarker candidates: MDK, GDF15, and SPINT2. These candidates were validated using ELISA and increased levels were associated with poor patient survival specifically in EGFR mutant lung adenocarcinoma patients.

Conclusions: Our study provides insight into changes in secreted proteins during *EGFR* driven lung adenocarcinoma transformation that may play a role in the processes that promote tumor progression. The specific candidates identified can harnessed for biomarker use to identify high risk individuals for early detection screening programs and disease management for this molecular subgroup of lung adenocarcinoma patients.

KEYWORDS

lung cancer, *EGFR*, secretome, transformation, early detection

Background

Lung cancer is the leading cause of cancer mortality in men and women worldwide, contributing to 1.8 million deaths in 2020 alone (1). Lung cancer consists of two subtypes, small cell lung cancer and non-small cell lung cancer (NSCLC), which comprise 15% and 85% of cases, respectively (2). The most common NSCLC subtype is lung adenocarcinoma (LUAD), which comprises ~60% of NSCLC cases (3). LUAD can be classified into oncogenic driver subgroups, where mutations in *KRAS* and *EGFR* are common (4). *KRAS* mutations are associated with smoking; in contrast, *EGFR* mutations are associated with never smokers, especially in women and in East Asia (5, 6). LUAD is thought to arise from a stepwise process of genetic and epigenetic changes, which begins with histologically normal epithelial cells and ends with invasive carcinoma (7, 8). The majority of *in vitro* studies aiming to investigate the genetic alterations required to enable transformation have centered on *KRAS*-driven LUAD, with limited investigation into *EGFR*-driven LUAD (9, 10).

Lung cancer, including LUAD, is typically diagnosed in late or metastatic stages; in these stages, long term patient survival is limited due to less effective treatment methods available such as chemotherapy and radiotherapy (2, 11). Prognosis and clinical stage are directly related, incentivizing earlier lung cancer detection for improvements to patient survival (12). Patients diagnosed at stage I have a five-year survival rate of 68.4%, in contrast with those diagnosed at stage IV, where the five-year survival rate is 5.8% (13, 14). Lose dose computed tomography (LDCT), a radiographic scanning technique used to image the lungs, is the standard for lung cancer screening (15). LDCT was initially demonstrated as an effective annual screening technique for high-risk individuals in the National Lung Screening Trial (NLST), where there was a 20% reduction in lung cancer mortality (16, 17). However, LDCT is limited by non-specificity, over diagnosis of benign pulmonary nodules, and potential harms of repeated radiation (16, 18). Furthermore, LDCT screening is not universally applicable to all populations susceptible to cancer; never smokers were not included in the NSLT study and its effectiveness for this group is unclear (19, 20). One proposed strategy to supplement LDCT for better screening practices is the use of biomarkers of early cancer development (21). Biomarkers would complement LDCT by reducing screening costs through criteria refinement, supporting clinical decision making in unclear situations such as indeterminate pulmonary nodules, and personalized patient screening and treatment planning (21). Blood biomarkers are of particular interest, due to their capability for inexpensive and relatively non-invasive collection (22). However, there are few confirmed protein biomarkers for LUAD and current candidates including NSE, proGRP, CEA, SCCA, and CYRFA 21-1 are non-specific for lung cancer and cannot be used to distinguish histology nor molecular subtype (23).

The secretome consists of proteins transported from a cell into the extracellular space and it is estimated to comprise 15% of all human proteins (24). Secretome proteins include cytokines, growth factors, extracellular matrix-degrading proteinases, and cell motility factors involved in local and systemic signaling (24, 25). A regulated secretome is important in maintaining homeostasis and changes in secretome protein abundance have been implicated in cancer (26). Tumor cells can release proteins that can affect functions such as angiogenesis, immunomodulation, basement membrane degradation, and extracellular matrix modeling (27). Secreted proteins enter bodily fluids such as blood and urine, which enables non-invasive collection and potential biomarker analysis (25, 28). A recent study of 32 types of primary tumors and normal-adjacent tissues found that proteins often found in the secretome are altered at the transcriptional level specifically in cancer, and found common expression decreases of proteins implicated in functions including adhesion and tumor suppression (29). These findings highlight the broad scope changes in the secretome during tumor progression and metastasis; in addition, this study showed the potential of the secretome as a reservoir of biomarker candidates such as matrix metalloprotease (MMP) family members, including MMP9 in breast and lung cancer (29–31). In NSCLC, recent secretome studies have identified proteins affecting erlotinib resistance, biomarkers for cisplatin response, and metastasis (32–34). However, secretome studies often profile immortalized cell lines, where cells are established from patient tumors and have already undergone malignant transformation (35, 36). Studying changes in secreted proteins that occur during the different steps of cancer progression from normal epithelium to invasive and metastatic cancer may therefore generate potential biomarker candidates to aid in early detection, diagnosis and prognosis. This is urgently needed in *EGFR*-driven LUAD, as the transformation process has not been fully elucidated and there are no concrete screening guidelines for the never smoker demographic where mutant *EGFR* LUAD cases are enriched (20, 37).

In this study, we investigated changes in the secretome during malignant transformation and identified potential biomarker candidates by performing proteomic analysis using an *in vitro* model of mutant *EGFR* driven transformation. We generated cell lines modeling the stepwise genetic alterations that occur during transformation, using non-transformed human bronchial epithelial cells (HBEC), and compared these against established LUAD cell lines to profile differences between stages of transformation (38). We initially identified 1020 secretome proteins and progressed through a series of groupwise and individual cell line comparisons to uncover 499 differentially expressed proteins between the untransformed and transformed states. Key selected proteins were validated with gene expression and patient survival data, to determine five biomarker candidates including MDK, GDF15, and SPINT2. This provides the first description of secretome changes during mutant *EGFR*-driven LUAD transformation and provides insight into the biological processes that can be applied for biomarker development.

Abbreviations: BCCA, British Columbia Cancer Agency; DPE, Differential protein expression; DNA, Deoxyribonucleic acid; EGFR, Epidermal growth factor receptor; ELISA, Enzyme-linked immunosorbent assay; GFP, Green fluorescent protein; GSEA, Gene set enrichment analysis; GO, Gene ontology; HBEC, Human bronchial epithelial cells; KRAS, Kirsten rat sarcoma viral oncogene homolog; LFC, Log fold change; LDCT, Lose dose computed tomography; LUAD, Lung adenocarcinoma; MS, Mass spectrometry; NLST, National Lung Screening Trial; NSCLC, Non-small cell lung cancer; PCA, Principal component analysis; RNA, ribonucleic acid.

Methods

Cell culture

All cell lines used were obtained from the American Type Culture Collection (ATCC) or gifted from Dr. Adi Gazdar (UT Southwestern Medical Center). PC-9, H1975 (NCI-H1975), HCC4006, HCC4011, H3255 (NCI-H3255) were cultured in RPMI-1640 (Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific). HBEC (HBEC3-KT) cells were cultured in Keratinocyte serum-free medium (KSFM; Thermo Fisher Scientific), supplemented with accompanying bovine pituitary extract (BPE; Thermo Fisher Scientific), human recombinant epidermal growth factor (EGF; Thermo Fisher Scientific), and 1% Pencillin-Streptomycin. All cell lines were cultured at 37°C, in 5% CO_2.

Expression constructs and cell line generation

Lentiviral vector and overexpression plasmids used to construct overexpression constructs for *EGFR L858R* (Plasmids #82906, #17451) and *GFP* (Plasmid #17445) were obtained from Addgene. Retroviral *TP53* c-terminal fragment (CT) overexpression construct and pCX4 hisD vector control were gifted from Dr. Romel Somwar (Memorial Sloan Kettering Cancer Centre, NY). Lentivirus was produced using HEK 293TD cells (ATCC), psPAX2 (Plasmid #12260; Addgene) and pMD2.G (Plasmid #12259; Addgene). Retrovirus was produced using Phoenix-AMPHO cells (ATCC). HBEC cell lines expressing *GFP, EGFR L858R*, with *TP53* C-terminal (CT) domain dominant negative mutations were generated by lentiviral and retroviral infection, and selected with 5μg/mL blasticidin (Thermo Fisher Scientific), and 2mg/mL L-histidinol (Thermo Fisher Scientific). The HBEC cell line expressing *EGFR L858R* and *TP53* CT was additionally selected in 10μM Nutlin-3a for 6 days (SelleckChem).

Western blot analysis

Protein from cell lysates were obtained by rinsing cells with cold Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific) and lysed in RIPA buffer (VWR) with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Samples were collected on ice, vortexed, and frozen at -80°C before being sonicated and centrifuge-separated at 15,000xg, 4°C for 10 minutes. Protein concentrations were detected using a Pierce BCA protein assay kit (Thermo Fisher Scientific), then samples were heated in 1x diluted NuPAGE LDS sample buffer (Thermo Fisher Scientific) containing 1:10 diluted 2-Mercaptoethanol (MilliporeSigma) at 75°C, for 10 minutes. 20-25μg of samples were run on NuPAGE 4-12% Bis-Tris protein gels (Thermo Fisher Scientific) in NuPAGE MOPS SDS running buffer (Thermo Fisher Scientific) at 200V, for 50 minutes. Samples were transferred from Bis-Tris gel to Immobilon-P PVDF (MilliporeSigma) either at 70V, 4°C, for 2 hours or 30V, 4°C overnight. Membranes were incubated in TBS-T (0.1% Tween-20) (TBS, Bio-Rad; Tween-20, Thermo Fisher Scientific) containing 5% BSA (MilliporeSigma) until primary antibody incubation.

Primary antibodies were prepared following manufacturer's instructions in TBS-T containing 5% BSA or 5% milk (MKP3); specific dilutions are noted. The following primary antibodies were used: p-ERK1/2(Thr202/Tyr204) (p-p44/42 (Thr202/Tyr204); Cell Signaling Technology, 9101); ERK1/2(p44/p42; Cell Signaling Technology, 4695); p-MEK1/2(Ser217/221) (Cell Signaling Technology, 9121); MEK1/2 (Cell Signaling Technology, 9122); p-EGFR(Tyr1068) (Cell Signaling Technology, 2234); EGFR L858R (Cell Signaling Technology, 3197); EGFR (Cell Signaling Technology, 2232); MKP3 (DUSP6) (Santa Cruz Biotechnology, sc-377070, 1:200); p53(Cell Signaling Technology, 2527); p53, to detect TP53 CT (MilliporeSigma, SAB4503011); GFP (Cell Signaling Technology, 2956); β-Actin (Cell Signaling Technology, 12620, 1:2000). Membranes were incubated in primary antibodies at 4°C overnight, then HRP-conjugated secondary antibodies according to manufacturer recommendations (Cell Signaling Technology). Proteins were detected after incubation with ECL, SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) on a ChemiDoc MP imager (Bio-Rad).

Secretome sample collection

Cells were seeded at approximately 80% confluency in 6cm plates in triplicate overnight: 900,000 (HBEC); 1,000,000 (PC-9); 1,000,000 (H1975); 3,500,000 (HCC4006); 7,000,000 (HCC4011); 2,500,000 (H3255). Plates were rinsed twice with DPBS and media was changed to supplement-free KSFM containing 1% Penicillin-Streptomycin (HBEC) or serum-free RPMI-1640 containing 1% Pencillin-Streptomycin (PC-9, H1975, HCC4006, HCC4011, H3255). Plates containing only media were also prepared, and all plates were incubated for 24 hours at 37°C. Conditioned media was collected, centrifuged at 1000 RPM for 5 minutes, at 4°C, and filtered with a 0.45μM filter (Sarstedt) to remove cell debris. The complete 4mL volume of filtered conditioned media was centrifuged in a Vivaspin Turbo 3kDa ultrafiltration unit at 3220xg, at 8°C until media was concentrated to approximately 150-200μL. Concentrated media was buffer exchanged, where samples were centrifuged twice with 4mL 50mM HEPES buffer, pH 7.0, then once with 1mL HEPES at 3220xg, at 8°C to a final volume of 150-300uL. Samples were stored at -80°C until mass spectrometry sample preparation.

Proteomic analysis

Samples were prepared for tandem mass spectrometry (MS/MS) analysis through a protocol of reduction, alkylation, and protein digestion. Samples were reduced by incubating with 16μL

of 200 mM dithiothreitol (DTT, Bio-Rad) for 30 minutes at 55°C, then alkylated by incubating with 32 µL of 400 mM iodoacetamide (IAA, Bio-Rad) for 30 minutes at room temperature. Samples were quenched with an additional 16 µL 200 mM DTT. Trypsin/Lys-C mix was prepared for sample digestion, where 200µL of 200 mM HEPES pH 8.0 was added to 20 µg Trypsin/Lys-C (Promega). Samples were digested by incubating with 16µL Trypsin/Lys-C mix on a ThermoMixer (Eppendorf) at 1000 RPM, overnight at 37°C. One tenth of each sample was pooled and prepared to confirm quality. Peptides were acidified by adding 10% (v/v) trifluoroacetic acid (TFA, Thermo Fisher Scientific) and diluted to a concentration of 1% TFA, then desalted following a Stop And Go Extraction (STAGE) tip protocol (39). Briefly, STAGE tips were packed with 3 punches of C18 resin which was washed (100 uL 0.1% TFA in acetonitrile) and equilibrated (2x100 uL 0.1% TFA in 18 MΩ water) then peptide was loaded. Salts were removed by rinsing (200 uL 0.1% formic acid in HPLC water) then eluted in 100 uL 0.1% formic acid in 60/40 acetonitrile/HPLC water. Desalted peptides were eluted and solvent evaporated by centrifuging samples in a SpeedVac Vacuum Concentrator (Thermo Fisher Scientific) until dry. Peptides were reconstituted in a 0.1% formic acid, 1% DMSO aqueous solution and assessed for quality on a LTQ Orbitrap Velos™ (Thermo Fisher Scientific).

The remaining digested peptides were tandem mass tag (TMT) labeled using a TMT 11-plex kit (Thermo Fisher Scientific) following manufacturer's instructions. Post-labeling, samples were pooled, dried by speed vacuum to evaporate excess solvent, and acidified with TFA as described above. Peptides were desalted following the STAGE tip protocol and excess solvent was reduced by vacuum centrifugation (39). Peptides were constituted in a 0.1% formic acid, 1% DMSO aqueous solution and run on an Orbitrap Eclipse™ mass spectrometer (Thermo Fisher Scientific) set to MS2 mode. MS spectra were searched with Proteome Discoverer suite (v.2.4.0.305, Thermo Fisher Scientific) against Swissprot human reference database (20585 sequences, October 2020). Precursor and fragment ion tolerance were set to 20 ppm and 0.05 Da, respectively. Dynamic modifications included Oxidation (+15.995 Da, M), Acetylation (+42.011 Da, N-Term), and static modification included Carbamidomethyl (+57.021 Da, C) and TMT (+229.163 Da, K, N-Term). Peptide-spectrum matches (PSMs) were validated with Percolator, where only PSMs with false discovery rate (FDR)< 0.01 were retained in the analysis.

PSMs were filtered by removing PSMs with average signal-to-noise (SN) ratio lower than 10 and isolation interference higher than 50% and SN was summarized to the protein level for analysis. Protein level data was $\log_2$-transformed and median normalized, where normalization was performed by taking the median total signal, calculating respective normalization factors for samples and media controls, and then missing values were imputed. Samples were compared against the appropriate media control (HBEC, KSFM; other cell lines, RPMI) on the $\log_2$ scale, and enriched proteins were determined by analyzing the intersection between sample and media. Differences between technical replicates and cell lines were assessed with principal component analysis (PCA). Proteins were filtered prior to statistical analysis, where only proteins seen in 2 or more technical replicates were retained. The average $\log_2$ HBEC GFP;$p53^{wt}$ signal intensity was subtracted from all samples to generate average $\log_2$ fold changes. Sample $\log_2$ fold changes were analyzed with the limma package (40) (version 3.50.0) with the moderated *t*-test in R, and adjusted *P*-values were calculated with the Benjamini-Hochberg procedure, with those<0.05 considered significant (R version 4.0.5).

Protein annotation and gene ontology enrichment analysis

The high-throughput model of DeepLoc 2.0 was used for the prediction of subcellular localization for the identified proteins (41). Functioning as a multi-label predictor, it possesses the capability to anticipate one or more localizations for a given protein. Selected gene lists were analyzed for Gene Ontology (GO) terms using the clusterProfiler package in R for enrichment in biological processes, molecular function, and cellular compartments (42). *P*-values were adjusted using the Benjamini-Hochberg procedure and terms with *adjp*< 0.05 were retained (R version 4.0.5). Reactome enrichment analysis was performed using ShinyGO 0.77 with EnsemblIDs corresponding to the individual proteins where available (43, 44). The 'Curated.Reactome' database was assessed using default settings, consisting of FDR< 0.05, min pathway size n=2, and max pathway size n=2000 and the top 20 pathways were plotted, sorted by fold enrichment.

Microarray analysis

Z-score normalized Affymetrix gene expression data collected from 199 primary lung tumors was retrieved from Memorial Sloan Kettering Cancer Center (45). Statistical analysis was performed with the Wilcoxon rank-sum test (Mann-Whitney *U* test) and adjusted *P*-values were calculated using the Benjamini-Hochberg procedure. Analysis was performed using base R functions (version 4.0.5). Statistically significant microarray probes were mapped to their corresponding gene with the R package hgu133a.db (46) (version 3.2.3). Genes were filtered for optimal 1:1 probe:gene mapping with the R package jetset (47) (version 3.4.0) to yield a final gene list.

Survival analysis

Differentially expressed genes were analyzed for differences in patient survival with NCBI GEO gene expression dataset GSE31210 (48). Probes were mapped to the corresponding genes with the R package hgu133plus2.db (49) (version 3.2.3). Median overall survival was calculated by applying a median split in gene expression and the Logrank test in Graphpad Prism 6.

ELISA

Quantification of GDF-15 in the secretome was performed using the Human GDF-15 Quantikine ELISA Kit (R&D Systems, DGD150) according to the kit instructions. MDK and SPINT2 were quantified using the Human MDK ELISA Kit (Invitrogen, EH319RB) and SPINT2 (HAI-2) Human ELISA Kit (Invitrogen, EH319RB and EHSPINT2) according to kit instructions. Quantification was performed on conditioned media samples collected under secretome collection conditions, in parallel with secretome experiment collection. All samples were run in duplicate. Sample concentrations were determined by subtracting the media background control signal, then interpolating with a standard curve. Differences in concentrations were statistically computed with the unpaired Student's T-test in Graphpad Prism6.

Trypan Blue viability stain

Cells were seeded at approximately 80% confluency in 6-well plates overnight: 350 000 (HBEC); 340,000 (PC-9); 340,000 (H1975); 1,200,000 (HCC4006); 2,400,000 (HCC4011); 850,000 (H3255). Plates were rinsed twice with PBS and media was changed to supplement-free or serum-free conditions and incubated for 24 hours. HBECs were incubated with either supplement-free KSFM and 1% Penicillin-Streptomycin, or KSFM supplemented with BPE (50µg/mL), EGF (5ng/mL), and 1% Penicillin-Streptomycin. NSCLC cell lines were incubated with either serum-free RPMI-1640 and 1% Penicillin-Streptomycin, or RPMI-1640 supplemented with 10% FBS and 1% Penicillin-Streptomycin. Post-incubation, cells were trypsinized with 0.05% Trypsin-EDTA (Thermo Fisher Scientific; HBEC) or 0.25% Trypsin-EDTA (Thermo Fisher Scientific; NSCLC). Trypsinization was neutralized with either trypsin neutralizer (Thermo Fisher Scientific; HBEC) or RPMI-1640 containing 10% FBS (NSCLC), cells mixed in 0.4% Trypan Blue solution (Thermo Fisher Scientific) at a 1:1 ratio, and live cell population determined with a TC20 automated cell counter (Bio-Rad). The average percent live cell population was determined from the average of 3 wells with the unpaired Student's T-test in Graphpad Prism6 (2 counts per well).

Propidium Iodide viability stain

Cells were seeded and treated under supplement-free or serum-free conditions for 24 hours as described in the Trypan Blue viability analysis section. Post-treatment, cells were incubated with 1µg/mL Hoescht 33342 (Thermo Fisher Scientific) for 30 minutes, and 1µg/mL propidium iodide (Thermo Fisher Scientific) for 10 minutes, respectively. Stained cells were imaged with an EVOS FL fluorescence microscope (Thermo Fisher Scientific). Live cell population was determined by quantifying the average live cell population from 2 images per well using ImageJ software, and applying the unpaired Student's T-test in Graphpad Prism6. The formula 100 % − ((*PI stained cell population* ÷ *Hoescht* 3342 *cell population*) × 100) was used to determine the live population.

Results

Mass-spectrometry secretome profiling of mutant EGFR lung cell models

To study potential changes in secreted proteins during malignant mutant *EGFR*-driven LUAD transformation, we generated an *in vitro* model approximating mutant *EGFR* malignant transformation with HBEC stable cell lines and selected *EGFR* mutant NSCLC cell lines (Figure 1A). HBECs, a bronchial epithelial cell line immortalized with non-viral proteins hTERT and CDK4, was selected due to its ability to maintain a non-transformed phenotype post-immortalization *in vitro* and *in vivo* (38). HBEC cell lines stably expressing $EGFR^{L858R}$ or GFP control, with or without dominant negative p53 C-terminal domain alterations ($p53^{CT}$) (GFP;$p53^{wt,}$ GFP; $p53^{CT}$), $EGFR^{L858R}$;$p53^{wt}$, $EGFR^{L858R}$;$p53^{CT}$ represent a profile of commonly mutated genes observed in *EGFR* mutant LUAD (4, 50). To confirm gene expression, HBEC cells were treated with the MDM2 inhibitor Nutlin-3a for 24 hours to assess p53 levels (51). HBEC GFP;$p53^{CT}$ showed minor changes in p53 expression, consistent with a mutant p53 phenotype, comparable to the mutant p53 NSCLC cell line H1975 (52) (Figure 1B). Cell lines expressing $p53^{wt}$ showed increased p53 expression, which is consistent with Nutlin treatment (53). Expression of $EGFR^{L858R}$ was confirmed using mutant specific antibodies (Figure 1B).

For secretome collection and analysis, HBEC and *EGFR* mutant NSCLC cell lines were starved under supplement-free and serum-free conditions for 24 hours, with cell-free media serving as controls (Figure 1C). This was performed to improve the detection of low abundance proteins masked by FBS and minimize non-human contamination, which is common practice in secretome experiments (54). However, starvation conditions can negatively affect cell viability and increase cell cytolysis, potentially contaminating media with intracellular proteins (35, 54). To investigate whether supplement- or serum-free conditions would have an effect in this regard, we assessed cell viability prior to secretome collection (Supplementary Figures 1, 2). Cell lines treated with Alamar Blue or propidium iodide (PI) showed no major changes in cell viability, measured as a live cell population percentage (Supplementary Figures 2B, C).

Upon secretome collection, samples were analyzed by TMT-11 MS/MS. To improve detection of low abundance proteins, conditioned media was concentrated prior to mass spectrometry (Figure 1C). Data was checked for quality and potential sources of variation introduced during sample processing using principle component analysis (PCA), confirming that technical replicates were generally clustered per cell line tumor or tissue origin, separate from media control samples (Supplementary Figure 3). We identified 1020 proteins post-secretome collection. The initial output was filtered to identify proteins in conditioned media by removing the intersection between media control and cell line samples, yielding 852 candidate secreted proteins (see methods). We then assessed this subset for those predicted to contain a signal peptide for secretion and their predicted subcellular localization

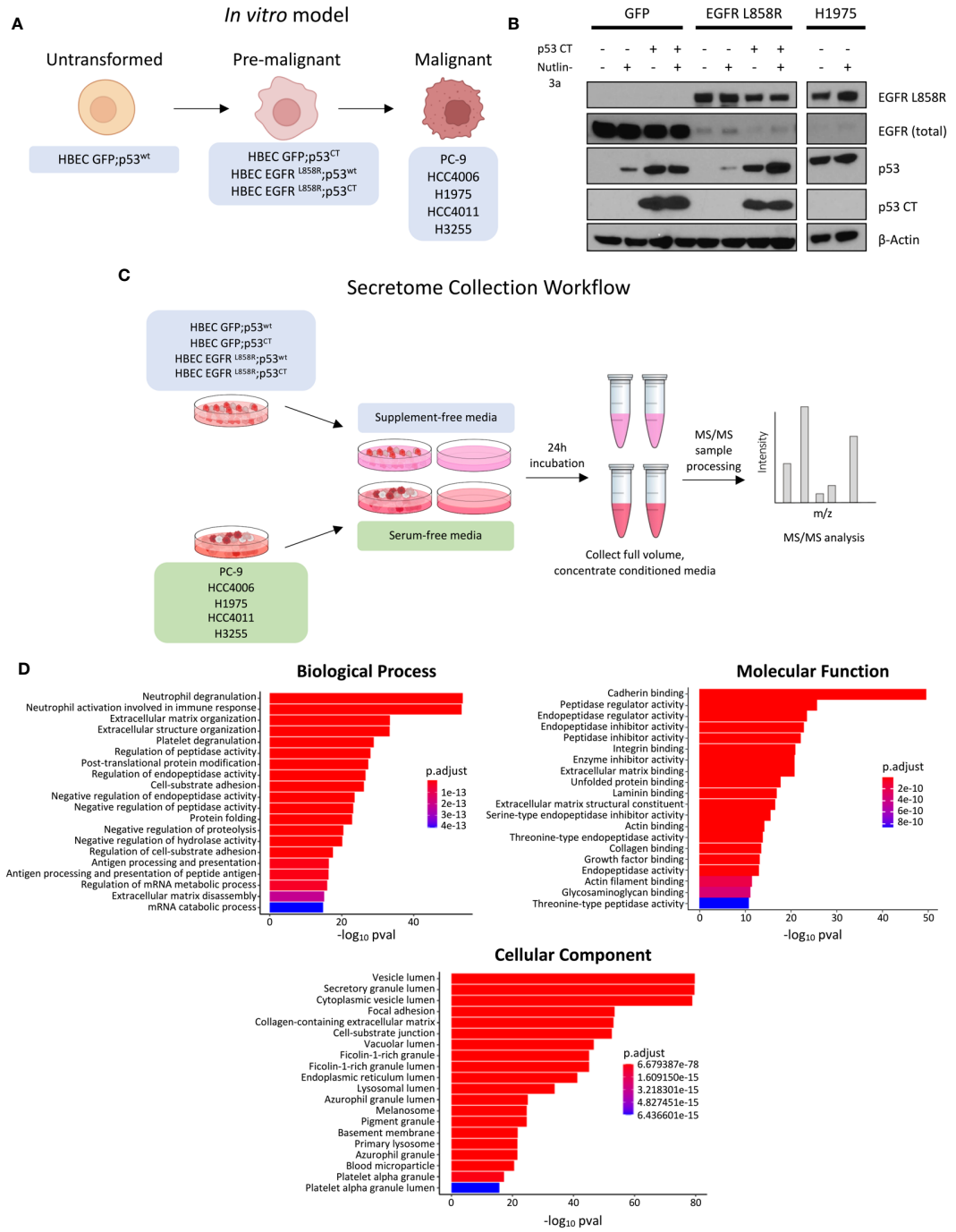

FIGURE 1
Experiment overview, model expression validation, and GO enrichment analysis of identified proteins. **(A)** Schematic overview of model, where each cell line represents a stage of malignant transformation. HBEC GFP;p53wt represents the wild-type, untransformed state, HBEC cell lines expressing mutations in EGFR-driven transformation represent an intermediate pre-malignant stage, and EGFR mutant NSCLC cell lines PC-9, HCC4006, H1975, HCC4011, and H3255 represent the transformed, malignant state. **(B)** Western blot expression validation of HBEC stable cell lines expressing GFP, EGFR L858R, in combination with p53 CT. Cell lines were treated with 10μM Nutlin 3-a, an MDM2 inhibitor, for 24 hours to confirm mutant p53 expression. EGFR L858R basal expression was also confirmed. H1975 was used as a positive control. **(C)** Schematic overview of the secretome collection experiment. **(D)** Bar plots showing the top 20 GO enrichment terms sorted by adjusted p-value (p adj< 0.05, Benjamini-Hochberg adjustment) for biological processes, molecular functions, and cellular components, for proteins identified in 2 or more technical replicates (prior to statistical analysis).

using DeepLoc 2.0 (41, see methods). This predictive model can distinguish among 10 distinct localization and has the ability to forecast the presence of sorting signal peptides that influence the prediction of subcellular localization.

In total, image 359 (42%) of the candidate proteins are predicted to contain a signal peptide and 217 (26%) are predicted to have extracellular localization (Supplementary Table 1). This includes the proteins HSPG2, LAMA5, and AGRN that have previously been

demonstrated to be secreted (33). An additional 123 (14%) of the candidate secreted proteins were predicted to be localized to the cell membrane, including EGFR which is known to undergo shedding into the extracellular space (Supplementary Table 1) (55).

The enriched protein subset was examined for associated GO terms to further assess if proteins were secreted (Figure 1D). Cellular component GO terms were commonly associated with vesicular protein transport; the top enriched terms included "vesicle lumen", "secretory granule lumen", and "cytoplasmic vesicle lumen" (Figure 1D) (56). Other terms were associated with cellular compartments such as the lysosome and endoplasmic reticulum, which could suggest affiliation with either conventional or unconventional secretion (57, 58). Secretion-associated cellular component terms were complemented by biological process terms that are associated with extracellular proteins, with examples including "neutrophil degranulation", "extracellular matrix organization", and "platelet degranulation" (59–61). Of the molecular function GO terms identified, cadherin binding was the most significantly enriched; this may be attributed to cadherin and the associated catenin binding to facilitate cell adhesion (62). Proteolysis terms, such as peptidase and endopeptidase regulation, were also enriched (Figure 1D). These terms are consistent with protease functions, which range from cell proliferation to the immune response (63). To interrogate specific signaling pathways associated with the identified proteins we performed a separate enrichment analysis interrogating the Reactome database (see methods). This revealed the top enriched pathways to include post–translational protein phosphorylation, IGF signaling among others including platelet/neutrophil degranulation and non–integrin membrane–ECM interactions that closely resemble the results from the GO analyses (Supplementary Figure 4).

Identification of differentially expressed secreted proteins corresponding to stages of mutant EGFR mediated lung cell transformation

Differential protein expression (DPE) analysis was performed to investigate differences in secreted proteins between the pre-malignant and malignant stages of *EGFR* mutant LUAD transformation (Figure 1A). This was performed by comparing HBEC cell lines expressing mutant *EGFR* and/or p53 to *EGFR* mutant LUAD cell lines and assessing differences specific to each group (Figure 2A). 91 proteins were found to be differentially expressed, with 64 under-expressed and 27 over-expressed in the malignant vs non-malignant states, respectively (Figure 2A). Hierarchical clustering based on the differentially expressed proteins revealed distinct grouping between HBEC cell lines and *EGFR* mutant NSCLC cell lines (Figure 2B). This suggests that there may be distinct secretome profiles between pre-malignant and malignant stages of lung transformation. We assessed the top five over-expressed and under-expressed proteins in malignant vs non-malignant states for their potential as biomarker candidates and found that two, NPC2 and MDK - both of which are predicted to have a signal peptide and extracellular localization (Supplementary Table 1) – have been found to be over-expressed in mouse LUAD plasma and NSCLC patient serum, respectively, confirming their secretion (64, 65). We queried the 91 differentially expressed proteins for GO terms associated with biological processes, molecular functions, and cellular components (Figure 2C). Four of the top five enriched cellular compartment GO terms were associated with secretory pathways, such as "secretory granule lumen" and "cytoplasmic vesicle lumen", suggesting the presence of secreted proteins or proteins involved in secretion; this includes conventional secretion, but also unconventional secretion mediated by lysosomes, autophagosomes, and multivesicular bodies that become exosomes (58, 66). "Collagen-containing extracellular matrix" was the top enriched cellular component GO term, which could reflect the predominant role of collagen in extracellular matrix formation and integrity, as well as functions such as cell adhesion (67, 68). Similar to the observations made during initial secretome profiling, terms related to proteolysis represented the top 5 molecular function terms (Figures 1B, 2C). This is reflective of the broad functions of proteases which include extracellular matrix assembly and remodeling and aligns with the top GO cellular component terms (69). Likewise, protease-related functions were represented in biological process GO terms; interestingly, immune cell functions were also represented (Figure 1B). The presence of immune cell-related terms suggests changes in immune regulatory programs that occur during transformation. This aligns with previous observations where PD-L1, a key protein in immune homeostasis, was upregulated in *EGFR* mutant NSCLC cell lines and expression associated with clinical LUAD samples (70, 71). Reactome analysis also revealed enrichment in numerous immune related signaling pathways in addition to ATF6 and JAK-STAT signaling (Supplementary Figure 5).

To investigate the differences in secreted proteins during transformation, we analyzed differences in secreted proteins between untransformed and pre-malignant stages (Figure 1A, Supplementary Figure 6). This was done through comparison across the HBEC cell lines expressing different mutant proteins. DPE analysis was performed where HBEC GFP;$p53^{CT}$, HBEC $EGFR^{L858R}$;$p53^{wt}$, and HBEC $EGFR^{L858R}$;$p53^{CT}$ were individually compared to HBEC GFP;$p53^{wt}$ cells. In contrast to the malignant vs non-malignant comparison, only PFKP, FN1, SERPINA3, and SERPINB7 were found to be differentially expressed in the pre-malignant and non-transformed states (Supplementary Figures 6A–C). SERPINA3 and SERPINB7 were also differentially expressed in the malignant vs non-malignant comparison (Figure 2B). This suggests that their expression levels may change during multiple stages of transformation. As few differentially secreted proteins were identified through this analysis, it is possible that expression of cancer genes alone is insufficient to dramatically alter the secretome or that the pre-malignant stage of transformation may not be distinct from the histologically normal, untransformed stage in terms of secreted profiles.

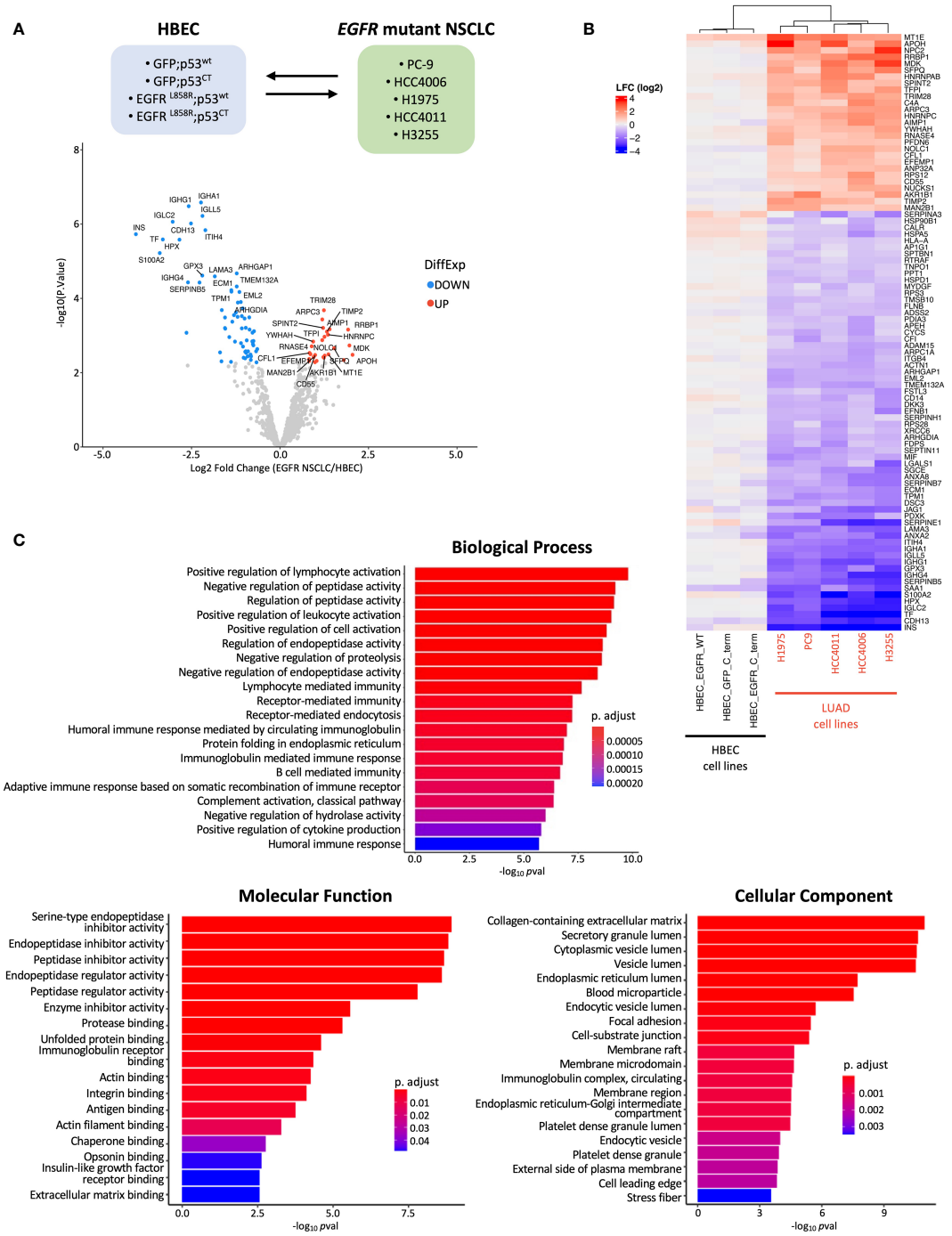

FIGURE 2
Groupwise comparison between HBEC cell lines and EGFR mutant NSCLC cell lines. **(A)** Schematic showing comparison performed during statistical analysis and volcano plot of log_2fold change (LFC) differentially expressed proteins identified from MS/MS analysis. The top 20 significantly over- and under-expressed proteins (p adj< 0.05 and absolute LFC > 0.6) are colored in red or blue, respectively, and labeled. **(B)** Heatmap of differentially expressed proteins identified from group-wise comparison in A., with hierarchical clustering (n=91) (cell line and protein, hierarchical clustering; cell line clustering distance, complete; protein clustering distance, average). **(C)** Bar plots showing the top 20 GO enrichment terms sorted by adjusted p-value (p adj< 0.05, Benjamini-Hochberg adjustment) for biological processes, molecular functions, and cellular components for differentially expressed proteins found during MS/MS analysis.

Identification of secreted biomarker candidates specific to the malignant state

To identify protein candidates for further analysis and validation, we also performed DPE analysis of *EGFR* mutant NSCLC cell lines individually against HBEC GFP;p53^{wt}, defined as the initial, untransformed stage (Figure 1A). This was done to capture cell line-specific differentially expressed proteins not observed in a group-wise comparison. Figure 3A outlines the filtering pipeline to identify potential protein biomarker

candidates using p adj< 0.05 and LFC > 0.6. The number of differentially expressed proteins ranged from 119 to 316 per LUAD cell line, in contrast to 91 from the group-wise comparison (Supplementary Figure 7). Individual comparisons may highlight specific genetic alterations per cell line, as each cell line has varying *EGFR* and *TP53* mutations (72–74). Clustering based on differentially expressed proteins demonstrated that EGFR L858R driver mutation cell lines HCC4011 and H1975 grouped together, while EGFR exon19 deletion cell lines HCC4006 and PC-9 clustered together. The exception was H3255, an EGFR L858R mutant, which may be attributed to other differences in genetic alterations; an example is H1975 possessing CDKN2A and PIK3CA mutations that are not found in H3255 (74, 75). We then profiled the 499 differentially expressed proteins found across all LUAD cells compared to the non-transformed state for enrichment in GO terms associated with biological processes, molecular functions, and cellular compartments (Figure 3C). Similar to the group-wise comparison, GO cellular component terms were associated with the extracellular matrix, such as "collagen-containing extracellular matrix", "laminin complex", and "basement membrane" (60, 76). Also common were secretion associated terms, with "secretory granule lumen", "cytoplasmic lumen", and "vesicle lumen" comprising 3 of the top 5 cellular component terms (Figure 3D) (58, 66). Reactome analysis revealed enrichment in similar signaling pathways as the groupwise comparison, with the noted addition of MET related signaling as one of the most enriched pathways (Supplementary Figure 8).

Proteins identified from the individual cell line comparisons were filtered for further analysis (Figure 3A). Differentially expressed proteins were filtered for overlap in three or more *EGFR* mutant LUAD cell lines, and then for expression in the same direction. This resulted in 130 proteins for further investigation. As there was no relevant *EGFR* mutant LUAD proteomic dataset available, we aimed to assess whether the expression of the secreted proteins are specific to EGFR mutant LUADs using transcriptomic data. Differential gene expression analysis was performed on a cohort of 39 *EGFR* mutant and 154 *EGFR* wild-type tumors (45). This analysis revealed 16 genes differentially expressed between EGFR mutant and wild-type LUAD tumors with corresponding proteins that were differentially secreted in the EGFR mutant LUAD cell lines (Table 1, Figure 3D). NPC2 demonstrated the highest level of expression, consistent with previous findings where NPC2 expression was greater in LUAD compared to other lung tumor types (77, 78). ENO1 and RAC1 also showed high levels of expression in EGFR mutant LUADs (Figure 3D) aligning with previous studies demonstrating that ENO1 expression is greater in LUAD tumor samples relative to non-cancerous tissue, and the RAC1 splice variant RAC1B enhances LUAD tumor formation *in vivo* (79, 80). The analysis of our MS/MS results identified differentially expressed proteins from our secretome experiment with evidence that they may be useful candidate biomarkers in the clinical setting.

Secreted proteins with gene expression levels associated with poor outcome in EGFR mutant LUAD

The 16 protein candidates found to have EGFR specific expression levels in LUAD tumors were subsequently analyzed for survival difference between 125 *EGFR* mutant and 68 wild-type patient tumors in an independent dataset (Table 1) (48). Kaplan-Meier curves were plotted, where overall survival duration between patients with high and low expression of genes was compared based on *EGFR* mutation status (Figure 4). High expression of *ENO1* ($p< 0.001$), *PFKP* ($p< 0.05$), *RAC1* ($p< 0.001$), and *SPINT2* ($p< 0.05$) in patients with *EGFR* mutant tumors was associated with shorter overall survival than *EGFR* mutant tumors with low gene expression (Figures 4A–D). The association with poor survival was not seen in patients with *EGFR* wild-type tumors, suggesting that survival differences could be *EGFR* mutation-specific. High *MDK* expression was also associated with lower overall survival, in *EGFR* mutant and wild-type patient tumors ($p< 0.05$) (Figure 4E). This observation aligns with a previous study where NSCLC patients displayed increased protein expression of serum MDK compared to healthy individual controls, and expression was associated with lower overall survival (65).

Orthogonal validation of secreted proteins

To validate levels of secreted protein expression in the EGFR mutant LUAD cell lines, MDK, GDF15, and SPINT2 were assessed by ELISA assays (Table 1, Figure 5). MDK and SPINT2 were selected due to the observed differences in overall survival while GDF15 levels have been associated with different stages of lung cancer in patients (81). Protein concentration was measured in conditioned media samples collected and concentrated in parallel with the MS/MS samples. As we were interested in comparing between malignant and untransformed, histologically normal states, we compared protein concentration between HBEC GFP;$p53^{wt}$ and the selected LUAD *EGFR* mutant cell lines (Figure 5). The mean MDK concentration in LUAD cell lines ranged from 0.94 - 17.28 ng/mL, and when compared to HBEC GFP;$p53^{wt}$, concentrations were significantly different ($p< 0.01$; Figure 5A). GDF15 mean protein concentration in NSCLC cell lines varied between 5.85 pg/mL - 6.44 ng/mL, compared to the mean HBEC GFP;$p53^{wt}$ concentration of 2.41 pg/mL. This corresponded to an increase of secreted GDF15 concentration in NSCLC cell lines up to 2000x that observed in HBEC GFP;$p53^{wt}$ (PC-9, $p< 0.0001$; HCC4006, $p< 0.001$; H1975, $p< 0.001$; HCC4011, $p< 0.001$; H3255, $p< 0.0001$; Figure 5B). SPINT2 mean protein concentration in NSCLC cell lines ranged between 98.30 - 580.97 pg/mL, relative to 98.58 pg/mL in HBEC GFP;$p53^{wt}$. With the exception of PC-9, protein concentrations were significantly greater than HBEC GFP;53^{wt}, where concentrations were 1.7- 5.9 times greater (HCC4006, $p<$

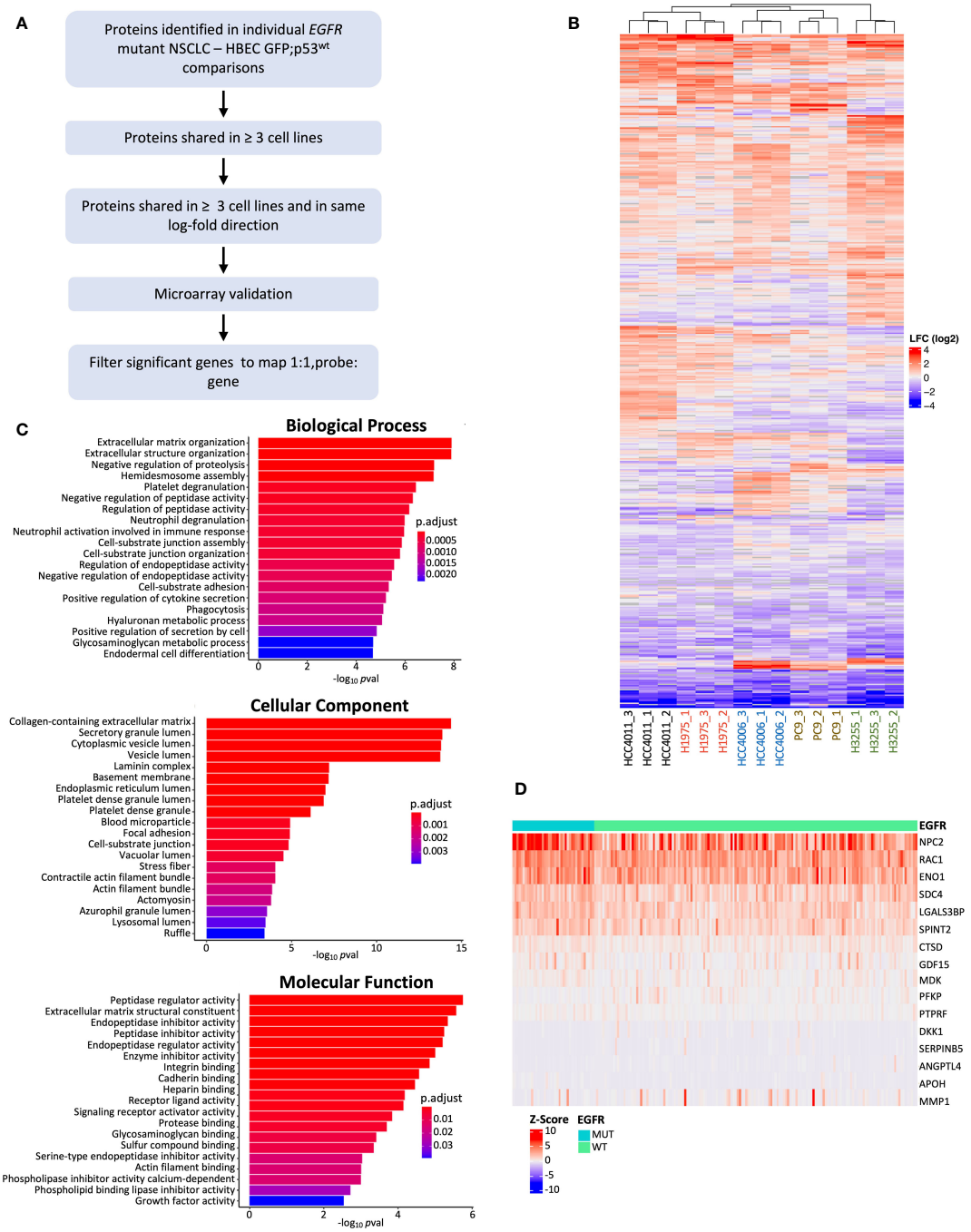

FIGURE 3
Individual EGFR mutant NSCLC cell line comparison against HBEC GFP;p53^{wt} and filtering pipeline. **(A)** Filtering pipeline used to identify protein candidates for biomarker validation. Differentially expressed proteins from cell line comparisons with HBEC GFP;p53^{wt} (p adj< 0.05, absolute LFC > 0.6) were determined, and filtered for microarray gene expression validation if found in 3 or more *EGFR* mutant cell line comparisons and LFC expression was in the same direction (LFC values were all positive or negative). Filtered differentially expressed proteins were analyzed for differential gene expression with Z-score normalized Affymetrix gene expression data from 199 primary LUAD tumors (45), and differentially expressed genes that were found to be significant (p adj< 0.05, Benjamini-Hochberg adjustment) were further filtered for optimal 1:1 probe to gene mapping for additional stringency (47). **(B)** Heatmap of differentially expressed genes found from individual comparison between *EGFR* mutant NSCLC cell lines and HBEC GFP;p53^{wt} (cell line and protein, hierarchical clustering; cell line clustering distance, complete; protein clustering distance, average). LFC ranges from high (red) to low (blue). **(C)** Bar plots showing the top 20 GO enrichment terms sorted by adjusted p-value (p adj< 0.05, Benjamini-Hochberg adjustment) for biological processes, molecular functions, and cellular components for differentially expressed proteins found during individual cell line comparisons between *EGFR* mutant NSCLC cell lines and HBEC GFP;p53^{wt}. **(D)** Heatmap of differentially expressed genes post-microarray analysis for further validation (p adj< 0.05). Samples are grouped by EGFR status (mutant, wild type) (gene clustering method, Euclidean; gene clustering distance, complete).

TABLE 1 Protein candidates identified from microarray gene expression analysis, listed by gene symbol, sorted by adjusted p value (p adj< 0.05; Benjamini-Hochberg adjustment).

Accession	Gene	Peptides	Unique peptides	Quantified peptides	adj.P.Val
Q08380	LGALS3BP	23	23	20	9.5519E-05
P61916	NPC2	10	10	10	9.5519E-05
O43291	SPINT2	2	2	1	0.001714715
P06733	ENO1	30	28	27	0.003759408
P21741	MDK	12	12	11	0.003759408
P63000	RAC1	3	2	1	0.005590461
Q01813	PFKP	2	2	1	0.007683377
P31431	SDC4	7	7	5	0.012165019
Q99988	GDF15	6	6	4	0.020488018
P02749	APOH	2	2	2	0.02368962
P36952	SERPINB5	14	14	8	0.027201045
P10586	PTPRF	19	19	15	0.031704624
P07339	CTSD	20	20	20	0.034872449
O94907	DKK1	7	7	7	0.034872449
P03956	MMP1	2	2	2	0.035149978
Q9BY76	ANGPTL4	6	6	4	0.03699355

0.001; H1975, $p< 0.0001$; HCC4011, $p< 0.01$; H3255, $p< 0.01$; Figure 5C). Together, these assays confirm the MS results, validating the increased secretion in EGFR mutant LUAD.

Discussion

Earlier detection of LUAD is key to long-term patient survival, where LDCT screening could benefit from the inclusion of biomarkers to complement screening (12). This is especially important given that LDCT screening for never smokers, which have increased incidence of *EGFR* mutant LUAD, does not have concrete guidelines (20, 37). Compared to ever smokers, one study found that the rate of diagnosis with LDCT screening for a never smoker cohort was 0.45%, which was lower than the NLST ever smoker rate of 1.0% (18, 82). More recently, a LDCT screening study for primarily non-smoking Asian women, the demographic commonly associated with *EGFR* mutant LUAD, found an increase in cancer incidence in early-stage cancers (stages 0-I) yet no change in late-stage incidence (stages II-IV) (83). These findings suggested that additional cases identified by LDCT were attributed to over-diagnosis, and that LDCT would have limited use for populations affected by *EGFR* mutant LUAD, furthering the necessity of biomarker-based detection to inform clinical decisions (21, 83). Currently, NSCLC biomarker candidates are limited and are neither specific for lung cancer nor histology (23). The cancer secretome is a valuable resource to uncover prognostic and diagnostic biomarker candidates (25, 84).

To date, secretome studies focusing on NSCLC have primarily analyzed established cancer cell lines, identifying changes in secreted proteins that provide further insight into processes such as tumor growth and metastasis (33, 85). However, cancer cell lines may not capture changes that occur during earlier stages of malignant transformation, resulting in missing potential biomarkers for earlier detection. Using an *in vitro* model representative of malignant transformation, this study analyzed the secretome of *EGFR*-driven LUAD malignant transformation *in vitro*. Our study began with generating a model and serum-free culture conditions compatible with mass spectrometry analysis to profile the secretome. Due to the lack of a transformation phenotype *in vitro* and *in vivo*, the HBEC cell line was selected to serve as an untransformed basal state to compare secretome changes during different transformation stages (38). By introducing common genetic alterations found in *EGFR* and *p53* into HBECs, we could also profile secretome changes during an intermediate, pre-malignant stage of transformation (50, 86, 87). An additional benefit to the HBEC cell line was its ability to be cultured in non-serum conditions; serum proteins often obscure low abundance proteins and may introduce non-human contamination (33, 54).

Together with selected *EGFR* mutant NSCLC cell lines representing the transformed state, we generated a model that represents key genetic alterations occurring during *EGFR*-driven malignant transformation (Figures 1A, B) (9, 50). We initially identified 1020 proteins, where 852 proteins from secretome conditioned media were enriched. HSPG2, LAMA5, and AGRN were identified which is consistent with a previous NSCLC secretome study, suggesting that secreted proteins could be detected with our approach (33). We further validated the presence of secreted proteins by performing GO analyses, where

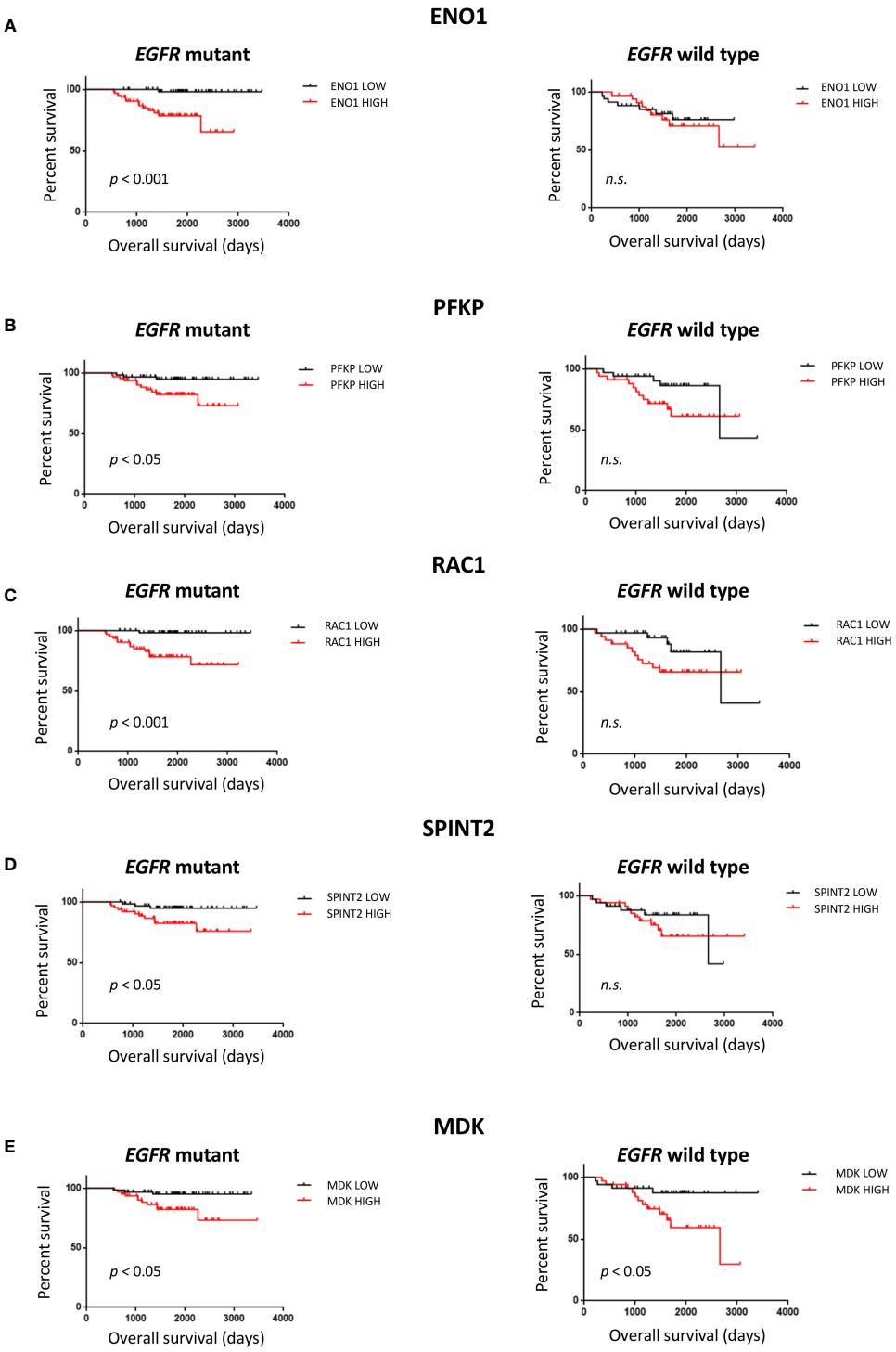

FIGURE 4
Survival analysis of protein candidates identified from filtering pipeline. Overall patient survival was analyzed (log rank test, median split) for *EGFR* mutant tumors, n=125 and *EGFR* wild type tumors, n=68 from the NCBI GEO GSE 31219 dataset. **(A)** ENO1. **(B)** PFKP **(C)** RAC1 **(D)** SPINT2 **(E)** MDK. *n.s.* non-significant.

cellular component terms referenced secretory pathways or locations associated with secretion (Figure 1D) (58, 66). We identified 91 differentially expressed proteins in *EGFR* mutant NSCLC cell lines relative to HBEC cell lines (Figures 2A, B) with secretome profiles of the transformed states including changes in immune functions. This aligns with previous NSCLC studies where EGFR mutations were associated with immune changes such as increased expression of PD-1 and PD-L1, and decreased CD8+ T cell infiltration (Figure 2C) (88, 89). We were unable to identify notable differences when comparing the basal HBEC GFP;$p53^{wt}$ cell

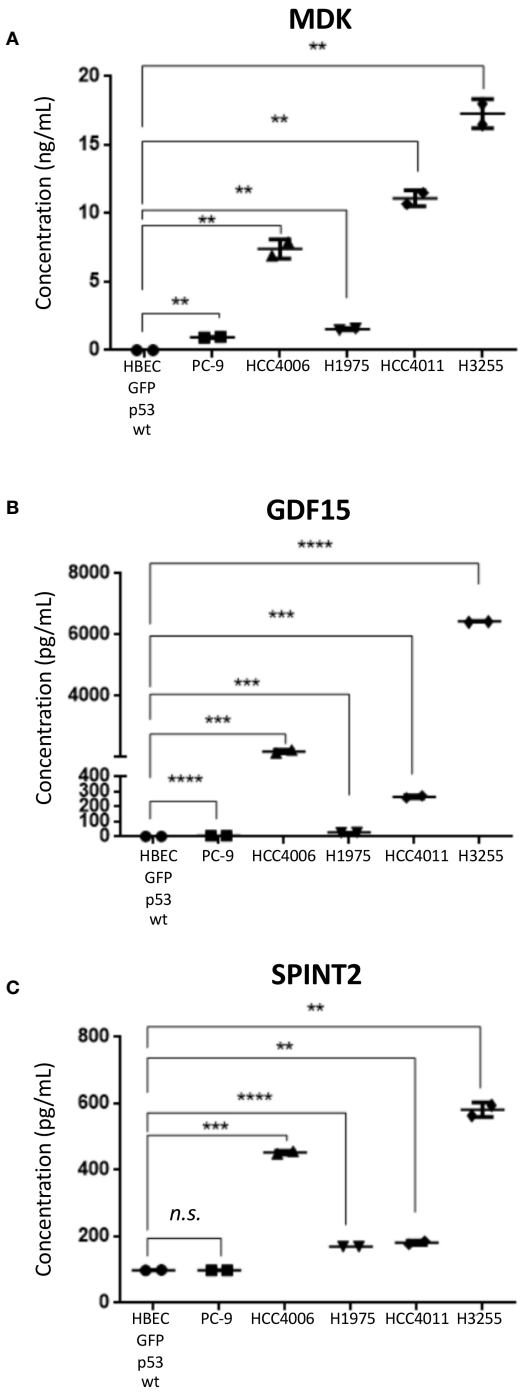

FIGURE 5
ELISA analysis of selected protein candidates in secretome conditioned media. Mean values ± SD are shown, experiment performed in technical duplicate (Student's unpaired T-test, two tailed). **(A)** MDK **(B)** GDF15 **(C)** SPINT2. ** $p< 0.01$, *** $p< 0.001$, **** $p< 0.0001$, *n.s.* non-significant.

line to HBEC cell lines expressing *EGFR* and *p53* alterations (Supplementary Figure 5) suggesting the introduction of additional genetic alterations may be needed to establish a more advanced pre-malignant state (9).

To broaden the pool of potential candidates and identify the most notable differences between untransformed and transformed states, we also compared *EGFR* mutant NSCLC cell lines individually to the basal HBEC cell line (Figure 3). 499 proteins across all comparisons were identified, with 130 proteins differentially expressed (Figures 3A, B). The secretome profile of the LUAD cell lines had a broad scope of biological functions (Figure 3C) which may be due to additional genetic changes specific to each NSCLC cell line, beyond EGFR and p53, such as p16 that can affect the secretome profile (90, 91). Using transcriptome data, we found genes for 16 of these proteins which were expressed specifically in EGFR mutant LUAD, suggesting they may be biomarker candidates for this molecular subtype of lung cancer (Figure 3D) (65, 78). A subset of these were also associated with patient survival in EGFR mutant LUAD, and we validated MDK, GDF15, and SPINT2 by ELISA, confirming their secretion and association with the malignant state.

MDK is a growth factor that binds to heparin and is involved in promoting cell growth and survival *in vitro* and tumor growth *in vivo* in a model of LUAD (92). GDF15 is a member of the transforming growth factor-β superfamily and varying biological effects have been observed with expression changes (93). In one study, GDF15 overexpression suppressed cell proliferation *in vitro* and tumor formation *in vivo*, while in another study overexpression promoted tumor growth *in vivo*, and proliferation *in vitro* when stimulated with C5a (94, 95). SPINT2 is a serine protease inhibitor where decreased expression facilitated STYK1-mediated tumor progression (96). With the exception of the PC-9 cell line when measuring SPINT2 concentration, *EGFR* mutant NSCLC cell lines had significantly higher concentrations of the selected proteins than HBEC GFP;$p53^{wt}$ (Figure 5). This suggests that there may be changes in MDK, GDF15, and SPINT2 expression during *EGFR*-driven malignant transformation that could be indicative of progression and studied for biomarker use (81, 97, 98).

While our study sought to identify changes in the secretome during LUAD transformation *in vitro*, there are limitations that should be considered for future studies. Firstly, the HBEC cell lines which represented the pre-malignant model stages were not validated for transformation capacity anchorage-independent growth *in vitro* or growth *in vivo* (99). As a result, this hampered the accuracy of the model when compared to clinical stepwise transformation and thus the accuracy of secretome changes occurring during the pre-malignant stage (50). Secondly, there may be additional genetic alterations that occur during EGFR-driven transformation. A previous study modeling transformation in HBECs found that *EGFR* and *TP53* mutations were unable to promote transformation *in vivo*, while another identified that alterations in APC, RB1, and RBM10 promoted tumor growth *in vivo* (9, 100). Thirdly, limited incubation time under serum-free conditions can restrict the scope of secretome profiling, as secretome protein abundance has been observed to increase over time, despite minimizing cell death (35, 101, 102).

Conclusions

In summary, we have profiled the secretome of non-transformed and EGFR mutant transformed lung cells and

identified 3 protein candidates that were validated for differential expression in EGFR mutant patients. These proteins show promise as candidates for lung cancer biomarker applications, although further mechanistic and validation studies are needed. The data and findings shown provide an insight into secretome changes under a variety of conditions and will serve as a valuable resource to support future studies in LUAD biomarker discovery and molecular changes occurring during EGFR-driven malignant transformation.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: [ProteomeXchange/PXD045328].

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

JL: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. FJ: Investigation, Methodology, Writing – review & editing. JJ: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. TS: Investigation, Methodology, Writing – review & editing. RS: Investigation, Methodology, Writing – review & editing. DL: Investigation, Methodology, Writing – review & editing. DF: Investigation, Methodology, Writing – review & editing. SS: Investigation, Methodology, Project administration, Writing – review & editing. GL: Investigation, Methodology, Writing – review & editing. GM: Project administration, Supervision, Writing – review & editing. WL: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1286821/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Cell viability analysis of HBEC stable cell lines under secretome conditions. HBEC cell lines were seeded and incubated overnight, then media was changed to secretome media conditions (KSFM, 1% PenStrep) or standard culture conditions (KSFM, supplemented with BPE, EGF, and 1% PenStrep) for 24 hours, then cell viability was assessed with Trypan Blue and Propidium Iodide (PI) staining. **(A)** Quantification of HBEC live cell population, as stained with Trypan Blue. **(B)** Quantification of HBEC live cell population, as stained with PI. **(C)** Representative images of DAPI and PI channels used to quantify PI staining. Experiment was performed in biological triplicate. *n.s.* non-significant.

SUPPLEMENTARY FIGURE 2
Cell viability analysis of EGFR mutant NSCLC cell lines under secretome conditions. Cell lines were seeded and incubated overnight, then media was changed to secretome media conditions (RPMI, 1% PenStrep) or standard culture conditions (RPMI, supplemented with 10% FBS, and 1% PenStrep) for 24 hours, then cell viability was assessed with Trypan Blue and Propidium Iodide (PI) staining. **(A)** Quantification of live cell population, as stained with Trypan Blue. **(B)** Quantification of live cell population, as stained with PI. **(C)** Representative images of DAPI and PI channels used to quantify PI staining. Experiment was performed in biological triplicate. ** $p < 0.01$, *n.s.* non-significant.

SUPPLEMENTARY FIGURE 3
Secretome experiment PCA. PCA was performed on all proteins identified during MS/MS **(A)** PCA excluding media control samples. **(B)** PCA including media control samples.

SUPPLEMENTARY FIGURE 4
Reactome pathway analysis of candidate secreted proteins from all cell lines. Plot showing the top 20 pathways identified using the Curated.Reactome database (FDR< 0.05). Minimum pathway size was n = 2, maximum pathway size n = 2000.

SUPPLEMENTARY FIGURE 5
Groupwise Reactome analysis of secreted proteins between HBEC cell lines and EGFR mutant NSCLC cell lines. Plot showing the top 20 pathways identified using the Curated.Reactome database (FDR< 0.05). Minimum pathway size was n = 2, maximum pathway size n = 2000.

SUPPLEMENTARY FIGURE 6
Differential protein expression analysis of HBEC cell lines expressing EGFR L858R, with or without expression of p53 c-terminal, relative to HBEC GFP; $p53^{wt}$ with different absolute LFC parameters to identify differentially expressed proteins. The top 20 significantly over- and under-expressed proteins (p adj< 0.05 and absolute LFC > 0.6) are colored in red or blue, respectively, and labeled. **(A)** HBEC GFP;$p53^{CT}$ i) minimum absolute LFC< 0.6 ii) minimum absolute LFC< 0.3 **(B)** HBEC $EGFR^{L858R}$;$p53^{wt}$ i) minimum absolute LFC< 0.6 ii) minimum absolute LFC< 0.3 **(C)** HBEC $EGFR^{L858R}$; $p53^{CT}$ i) minimum absolute LFC< 0.6 ii) minimum absolute LFC< 0.3 **(D)** Venn diagram describing overlap in differentially expressed proteins among HBEC stable cell lines. "abs" = absolute.

SUPPLEMENTARY FIGURE 7
Differential protein expression analysis of EGFR mutant NSCLC cell lines, relative to HBEC GFP;$p53^{wt}$. The top 20 significantly over- and under-expressed proteins (*p* adj< 0.05 and absolute LFC > 0.6) are colored in red or blue, respectively, and labeled. **(A)** PC-9. **(B)** HCC4006. **(C)** H1975 **(D)** HCC4011 **(E)** H3255.

SUPPLEMENTARY FIGURE 8
Reactome pathway enrichment of proteins detected in individual EGFR mutant NSCLC cell lines vs HBEC GFP;$p53^{wt}$. Plot showing the top 20 pathways identified using the Curated.Reactome database (FDR< 0.05). Minimum pathway size was n = 2, maximum pathway size n = 2000.

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The great need to overcome osimertinib resistance in advanced non-small cell lung cancer: from combination strategies to fourth-generation tyrosine kinase inhibitors

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1 Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide, surpassing the mortality of breast, colorectal and prostate cancers combined. The 5-year overall survival is around 18% including all stages of non-small cell lung cancer (NSCLC). It is likely that screening, targeted therapy, and immunotherapy will improve this poor prognosis in the coming years (1).

Currently, the choice of the treatment for patients with advanced NSCLC is based on the integrated evaluation of some parameters: histology (squamous versus non-squamous); presence of driver molecular alterations (sensitizing mutations of EGFR and/or BRAF, and rearrangements of ALK and/or ROS1 and/or NTRK); PD-L1 expression level; patient clinical characteristics such as age, performance status (PS) and comorbidities. The presence or absence of those driver molecular alterations allows distinguishing oncogene-addicted disease from non-oncogene-addicted disease, which presents different therapeutic approaches (2).

Many driver molecular alterations have been known in NSCLC till now. Some of these can be already targeted by means of specific drugs, while others could be targetable. These include: KRAS gene mutations (20-30%), EGFR (10-15% of Caucasian patients and up to 40% of Asian patients), BRAF (2-4%), ALK rearrangements (3-7%), ROS1 (1-2%), RET (1-2%), NTRK (0.5-1%), HER2 gene mutations (1-2%) and MET gene amplifications or mutations (2-4%) (3).

EGFR tyrosine kinase inhibitors (TKIs) represent the recommended first-line treatment in patients with advanced NSCLC and common mutations, i.e. exon 19 deletions (Ex19dels) and exon 21 point mutation (L858R). Many randomized phase III trials showed, in patients with advanced NSCLC and common EGFR mutations, the superiority of EGFR TKIs, i.e. gefitinib, erlotinib, afatinib, in the first line of treatment compared to standard platinum-based chemotherapy, in terms of both RR and PFS (4). The majority of patients treated with these drugs, around a half, develop the resistance point mutation T790M. To overcome this resistance mechanism, osimertinib was developed as second-line treatment. Subsequently its superiority in terms of overall survival (OS) over first- and second-generation TKIs was demonstrated also in the first-line setting (5, 6). As a consequence, osimertinib had been the best option for the treatment of advanced NSCLC patients with activating EGFR mutations until now (2). However, the recent results of FLAURA-2 trial and MARIPOSA trial showed that upfront combination strategies delayed resistance occurrence. Indeed, osimertinib plus platinum-pemetrexed and amivantamab plus lazertinib, respectively, achieved longer PFS compared to single agent osimertinib (7, 8).

2 Osimertinib resistance

Osimertinib has largely improved the survival of advanced NSCLC patients with EGFR mutation both in the first- or second-line setting, but the emergence of acquired resistance remains a great need.

The resistance mechanisms to osimertinib may be different depending on whether this drug is administered in the first or in the subsequent lines. In fact, osimertinib was initially developed exclusively for patients who had developed a T790M mutation during treatment with first or second generation TKIs. In this setting, the most frequent on-target resistance mechanism (approximately 14%) is the point mutation of EGFR exon 20, C797S. Instead, MET amplification occurred in 19% of patients, in 7% in conjunction with the C797S mutation. Furthermore, HER2 and PIK3CA amplifications, RET and NTRK rearrangements, and BRAF V600E mutation were each observed in 3–5% of cases (9, 10).

As regards the use of osimertinib as first-line treatment, some literature data reported an intrinsic resistance. The relative mechanisms include the HER2 and MET amplifications, as observed in *in vitro* studies, but also the combination of the KRAS G12D mutation and PTEN loss, and CDCP1 or AXL RNA overexpression, as reported in some NSCLC patients (11–13).

The main information about the acquired resistance to first-line osimertinib derives from the phase III trial FLAURA, while some other literature data are from case reports or small case series. Cell-free DNA from blood samples of patients included in FLAURA trial was analyzed via NGS (14). The authors did not find emergent T790M mutation. The most common resistance mechanism was MET amplification (15%), followed by less frequent EGFR amplification (9%), as well as C797S mutation (7%). S768I mutation or other combined EGFR mutations, such as exon 19 deletion + G724S (exon 18), L718Q (exon 18) + EGFR exon 20 insertion (exon 18 + 20), L718Q + C797S or L718Q + L797S (exon 18 + 20), are very rare (<1%). These findings suggest that secondary EGFR mutations are not the main mechanism of resistance to osimertinib.

Many EGFR-independent mechanisms were observed when resistance to first- or second-line osimertinib developed. These mechanisms are more evident with osimertinib than on-target mutations possibly because of its stronger EGFR inhibition compared to first or second-generation TKIs (15). Moreover, during the treatment with osimertinib EGFR-independent resistance mechanisms tend to occur earlier than EGFR-dependent ones, maybe because of pre-existent subclones rapidly arising under the selective pressure of treatment (16). This kind of mechanisms includes MET amplifications, HER2 amplifications, PI3KCA, BRAF and RAS mutations, ALK or RET rearrangements, cell cycle gene alterations (17). The ongoing ELIOS trial (NCT03239340) is designed to investigate prospectively the mechanisms of acquired resistance to first-line osimertinib, given that paired tissue biopsies (pre-treatment and at progression) are collected. Moreover, HER3 expression was found in around 80% of NSCLC and in more than 85% of those harboring EGFR activating mutations. It emerged as a further mechanism of resistance to EGFR TKIs and favors metastatic progression and worse prognosis (18).

Finally, phenotypic changes were also observed as resistance mechanisms in up to 15% of patients treated with first- or later-line osimertinib. These include the epithelial-mesenchymal transition and the transformation of adenocarcinoma to small-cell carcinoma, this latter associated with RB1 and TP53 mutations (19).

Patients experiencing progression during first-line osimertinib should receive chemotherapy regimens used in non-squamous NSCLC. The combination of carboplatin, paclitaxel, bevacizumab and atezolizumab could represent a treatment option for them (20). Anti-PD-1/PD-L1 drugs as monotherapy have not proven to be a successful option in this subgroup of patients (21). However, three phase III trials studied chemoimmunotherapy after osimertinib resistance. CheckMate-722 confirmed the lack of benefit in these patients (22). KEYNOTE-789 and ORIENT-31 are still ongoing.

To prolong the benefit obtainable with osimertinib, similarly to first-generation TKIs, this drug could be continued "beyond progression" in selected cases with some conditions, such as exclusively radiological progression of mild entity, slow tumor kinetics, absence of clinical symptoms, and in case of oligoprogressive disease local therapies can be associated (23).

Here, we discuss the evolution of treatment strategies to achieve the best outcomes for patients progressing during first-line osimertinib. The majority of the studies addressing this aim are still ongoing.

3 Combination strategies

Many trials were designed to combine osimertinib with other new targeted agents directed against the emerging resistance mechanisms. These drugs can be classified in specific inhibitors, antibody-drug conjugates, and bispecific antibodies (24) (Table 1).

Given that MET alterations are the most frequent resistance mechanism to osimertinib, three MET inhibitors were developed and combined with osimertinib, i.e. savolitinib, tepotinib and capmatinib. However, other specific inhibitors are available against MEK (selumetinib), AXL (DS-1205c), ERK (ERAS-007), mTOR (sapanisertib), PI3Kα/δ (TQ-B3525), JAK1 (itacitinib), CDK4/6 (abemaciclib), AURKA (alisertib).

Antibody-drug conjugates (ADC) are composed of a link between a specific monoclonal antibody directed against a tumor molecule and a cytotoxic payload. After the bond of the monoclonal antibody with its own target, the drug is internalized and the linker is degenerated releasing the cytotoxic payload, with consequent antitumor effect. Some of these drugs have been already available in trials for NSCLC patients and specifically target MET (telisotuzumab vedotin), HER2 (trastuzumab deruxtecan), HER3 (patritumab deruxtecan), TROP2 (datopotamab deruxtecan and SKB264). All these antibody-drug conjugates are studied after osimertinib resistance. However, only patritumab deruxtecan and telisotuzumab vedotin are combined with osimertinib. Interestingly, the efficacy results from phase I and phase II trials with patritumab deruxtecan were achieved across a broad range of pretreatment HER3 expression in the tumor membrane and across the various EGFR-TKI resistance mechanisms (25, 26).

Finally, the category of bispecific antibodies in this setting is represented only by amivantamab until now. This is a fully humanized antibody directed against mutated EGFR and mutated or amplified MET. It exerts its function both via the inhibition of the ligand binding and antibody-dependent cellular cytotoxicity (27). This drug is combined with lazertinib, a potent brain-penetrant third-generation EGFR TKI, and investigated in three clinical trials (CHRYSALIS, CHRYSALIS-2, MARIPOSA-2) for patients progressing during or after osimertinib.

ORCHARD is a biomarker-directed Phase II platform trial. This study is evaluating the optimal treatment strategy depending on the underlying resistance mechanism to first-line osimertinib. For this reason, treatment assignment is based on a molecular tumor characterization from a tissue biopsy performed at progression during osimertinib. Each patient will be assigned to treatment with the combination of osimertinib and a specific targeted drug for the resistance mechanism detected, e.g.

TABLE 1 Drugs in clinical development for patients progressing during or after osimertinib.

Target	Category	Name	3rd gen EGFR TKI combined	Trial phase advancement
EGFR-directed	Allosteric inhibitors	BLU-701	Yes	I/II
		BLU-945	Yes	I/II
		BBT-176	No	I/II
		JIN-A02	No	I/II
Other-target-directed	MET inhibitors	Savolitinib	Yes	III
		Tepotinib	Yes	II
		Capmatinib	Yes	III
	MEK inhibitors	Selumetinib	Yes	Ib
	ERK inhibitors	ERAS-007	Yes	I/II
	PI3K inhibitors	TQ-B3525	Yes	I/II
	mTOR inhibitors	Sapanisertib	Yes	I
	CDK4/6 inhibitors	Abemaciclib	Yes	II
	AXL inhibitors	DS-1205c	Yes	I
	JAK1 inhibitors	Itacitinib	Yes	I/II
	AURKA inhibitors	Alisertib	Yes	I
	EGFR-MET bispecific antibodies	Amivantamab	Yes	III
	Antibody-drug conjugate	Trastuzumab deruxtecan	No	Ib
		Patritumab deruxtecan	Yes	III
		Datopotomab deruxtecan	No	III
		SKB264	Yes	III
		Telisotuzumab vedotin	Yes	III

osimertinib + savolitinib for MET amplification, osimertinib + necitumumab for EGFR amplification, and so on. If a resistance mechanism is not found, the patient will be assigned to platinum-based chemotherapy with durvalumab. Those patients with resistance mechanisms not targetable will be treated according to local practice. This trial also includes an adaptive design to allow the addition of new emerging drugs (28). Recently, an interim analysis of safety and efficacy showed that osimertinib + necitumumab in patients with a secondary gene alteration in EGFR, i.e. amplification, L718 or G724 mutation, exon 20 insertion, met futility criterion so that recruitment for this treatment was closed (29).

4 Fourth-generation EGFR-TKIs

The therapeutic strategies for the acquired resistance to osimertinib we discussed before are suitable for EGFR-independent resistance mechanisms, which are more frequent for patients treated with first-line osimertinib. However, some of these patients develop on-target EGFR mutations, such as C797S point mutation in exon 20, mainly in *cis* with the activating mutation. To target this alteration a specific drug does not yet exist. Brigatinib, an ALK inhibitor showed activity against cells with this mutation, but it needed the combination with cetuximab to be effective *in vivo* (30). In a retrospective analysis including 15 patients who developed resistance to osimertinib because of C797S mutation, brigatinib plus cetuximab achieved the 10% objective response rate and 60% disease control rate (31).

Among the various EGFR inhibitors under development, there are new TKIs, which are "fourth generation" or allosteric EGFR inhibitors (Table 1). It means that these drugs bind to the receptor outside the ATP pocket of the kinase domain. This bond selectively alters EGFR conformation and bypasses the resistance mechanisms mediated by new mutations in the ATP-binding domain, mainly C797S (32). Among these drugs, a limited activity was obtained with EAI-045, the first one identified in this class, when used as a single agent, but it has to be combined with cetuximab to be effective in cell lines with triple EGFR mutations (L858R/T790M/C797S). Similarly, JBJ-04-125-02 achieved increased cell death and more effective inhibition of cell proliferation when combined with osimertinib, compared with single agent alone (33). CH7233163, a further fourth generation EGFR TKI, exhibited even more potent antitumor activity against the EGFR triple mutation (ex19del/T790M/C797S) (34). Some other allosteric EGFR inhibitors were included in the design of phase I/II clinical trials, i.e. BLU-701 (HARMONY trial), BLU-945 (SYMPHONY trial), BBT-176, JIN-A02. The first two of these are studied both as single agent and in combination with osimertinib in patients who progressed during or after a previous EGFR TKI.

5 Discussion

From the scenario of these new therapeutic strategies emerges the possibility of avoiding or delaying the chemotherapy option for patients who develop resistance to first-line osimertinib. We still do not have sufficient efficacy data available to allow considering some therapeutic strategies over others. However, based on the molecular

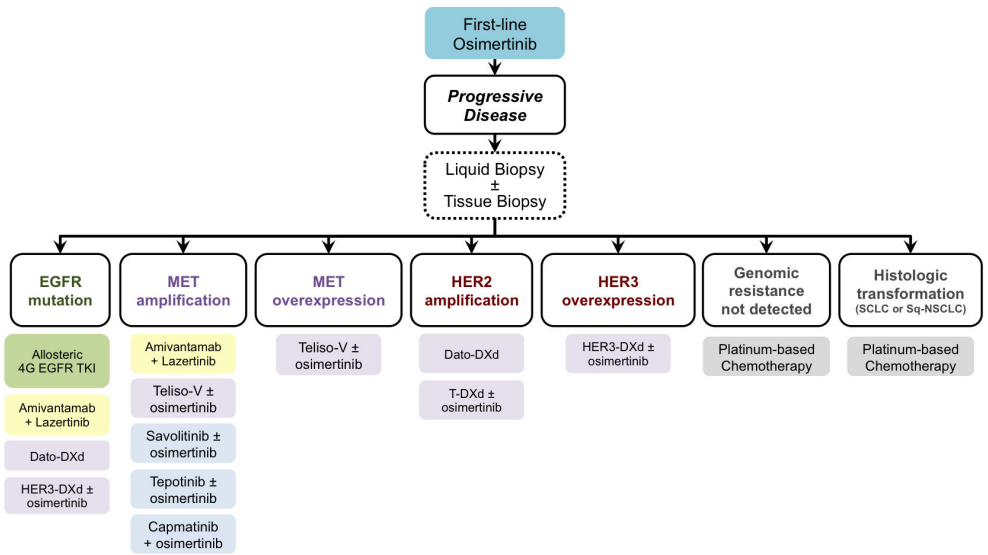

FIGURE 1
Future scenario for treating patients who developed resistance during or after osimertinib according to post-progression molecular characterization. EGFR, Epidermal Growth Factor Receptor; MET, MET Proto-Oncogene, Receptor Tyrosine Kinase; HER2, Human Epidermal Growth Factor Receptor 2; HER3, Human Epidermal Growth Factor Receptor 3; 4G, fourth generation; Dato-DXd, Datopotamab Deruxtecan; HER3-DXd, Patritumab Deruxtecan; Teliso-V, Telisotuzumab Vedotin; T-DXd, Trastuzumab Deruxtecan; SCLC, small cell lung cancer; Sq-NSCLC, squamous non-small cell lung cancer.

targets at which these new drugs are directed, when patients treated with osimertinib become refractory to this drug, it is necessary to evaluate which resistance mechanism has developed. The search for these resistance mechanisms involves the need to carry out tissue biopsies, possibly associated with a blood sample for the detection of gene alterations in cell-free DNA. This combined, liquid and tissue, evaluation would allow defining the prevailing resistance mechanism, on the basis of which the most appropriate molecular treatment can be indicated. In principle, the therapeutic choice could be oriented towards combination strategies of osimertinib plus a specific inhibitor or antibody-drug conjugate or bispecific antibody in the case of EGFR-independent resistance mechanisms (e.g. MET overexpression or MET amplification, as highlighted in the biomarker analysis of CHRYSALIS-2 trial) (35). Instead, the use of allosteric EGFR TKIs would be more suitable in case of the prevalence of new EGFR mutations, especially at the ATP-binding site. A relevant issue that could be resolved by the results of these ongoing clinical trials concerns the usefulness of combining these new drugs with the continuation of osimertinib versus its suspension. We summarized a possible future scenario in Figure 1 to attribute a next-generation therapeutic option to each of the more frequent mechanisms of resistance to first-line osimertinib. On the basis of the findings that are emerging from many clinical trials, we suppose that in the next future oncologists will be able to address each patient, who experience resistance to upfront osimertinib, toward a different treatment strategy according to the molecular alterations highlighted via circulating cell-free DNA and/or DNA from tumor tissue biopsy. However, the results of upfront combination strategies, such as those investigated in FLAURA-2 and MARIPOSA trial, will further change the scenario, and we think that a different spectrum of resistance mechanisms could emerge. To face with the complexity of gene alterations that can lead to the resistance to first-line treatment, i.e. osimertinib single agent or combination strategies, liquid biopsy will be mandatory and, if not informative, it can be completed with tissue biopsy. However, we believe that the design of platform trials could be the best option to manage the complexity of resistance occurrence.

Author contributions

GB: Conceptualization, Data curation, Formal Analysis, Methodology, Writing – original draft, Writing – review & editing. AB: Data curation, Investigation, Software, Visualization, Writing – original draft, Writing – review & editing. LCa: Data curation, Formal Analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. LCr: Conceptualization, Data curation, Investigation, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

LCa has served as consultant or advisor to Bristol-Myers Squibb, Merck Sharp and Dohme, and has received compensated educational activities from Bristol Myers Squibb, Astrazeneca, and Sanofi.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

ADC	Antibody-drug conjugate
ALK	anaplastic lymphoma kinase
ATP	Adenosine Triphosphate
AXL	AXL receptor tyrosine kinase
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CDCP1	CUB-domain-containing protein 1
CDK4/6	Cyclin-dependent kinase 4 and 6
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Signal-Regulated Kinase
HER2	Human Epidermal Growth Factor Receptor 2
HER3	Human Epidermal Growth Factor Receptor 3
JAK1	Janus kinase 1
KRAS	Kristen Rat Sarcoma viral oncogene
MEK	Mitogen-activated protein kinase kinase
MET	MET Proto-Oncogene, Receptor Tyrosine Kinase
mTOR	Mammalian target of rapamycin
NGS	next-generation sequencing
NSCLC	Non-Small Cell Lung Cancer
NTRK	Neurotrophic tyrosine receptor kinase
OS	Overall survival
PD-1	Programmed death-1
PD-L1	programmed death-ligand 1
PFS	Progression-free survival
PI3Kα/δ	Phosphoinositide 3-kinases α/δ
PI3KCA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PTEN	Phosphatase and TENsin homolog deleted on chromosome 10
RB1	retinoblastoma susceptibility gene 1
RET	rearranged during transfection
ROS1	ROS Proto-Oncogene 1
RR	Response rate
TKI	Tyrosine-Kinase Inhibitor
TP53	Tumor protein P53
TROP2	trophoblast antigen 2

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Addition of bevacizumab to EGFR tyrosine kinase inhibitors in advanced NSCLC: an updated systematic review and meta-analysis

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Background: The synergistic effects of antiangiogenic inhibitor bevacizumab and epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKI) therapy were encouraging in patients with EGFR-mutant advanced NSCLC, though some controversy remains. The specific subgroup of patients who might benefit most from the EGFR-TKI and bevacizumab combination therapy is yet to be determined.

Methods: Randomized clinical trials (RCTs) that had compared the clinical efficacy of EGFR-TKI and bevacizumab combination therapy with EGFR-TKI monotherapy in treating EGFR-mutant advanced NSCLC patients published before 23 December 2022 were searched in the Cochrane, PubMed and Embase. We performed a meta-analysis for the overall survival (OS), progression-free survival (PFS), objective response rate (ORR), and treatment-related adverse events with a grade equal or more than 3 (grade≥3 TRAEs). Subgroup analyses of PFS and OS stratified by clinical characteristics and treatment were conducted.

Results: We included 10 RCTs involving 1520 patients. Compared with EGFR-TKI monotherapy, addition of bevacizumab to EGFR-TKI resulted in a significantly higher PFS (hazard ratio (HR) = 0.74, 95% confidence interval (95% CI): 0.62–0.87)) and ORR (risk ratio (RR) = 1.07, 95% CI: 1.01–1.13). However, no significant difference in OS (HR = 0.96, 95% CI: 0.83–1.12) was noticed. Patients with EGFR-mutant advanced NSCLC receiving combination therapy showed PFS improvement regardless of gender (male or female), Eastern Cooperative Oncology Group performance status (0 or 1), baseline central nervous system (CNS) metastasis (presence or absence) and EGFR mutation type (19del or 21L858R). Subgroup analyses showed that, with the treatment of bevacizumab and EGFR-TKI, patients who ever smoked achieved significantly better OS and PFS benefits (HR = 0.68, 95% CI: 0.48–0.95; HR = 0.59, 95% CI: 0.46–0.74, respectively), and those aged <75 years and the Asian population had significantly prolonged PFS (HR = 0.69, 95% CI: 0.52–0.91; HR = 0.71, 95% CI: 0.58–0.87; respectively). The superiority of EGFR-TKI and bevacizumab combination therapy against EGFR-TKI monotherapy in improving PFS was more significant in the erlotinib regimen subgroup. The risk of grade≥3 TRAEs was remarkably higher in the combination therapy group (HR = 1.73, 95% CI: 1.39–2.16).

Conclusion: Addition of bevacizumab to EGFR-TKI therapy provided significantly better PFS and ORR for EGFR-mutant advanced NSCLC patients, though with higher risk of grade≥3 TRAEs. Patients who ever smoked, aged <75 years, and Asian population might benefit more from the combination regimen.

Systematic Review Registration: This systematic review and meta-analysis was registered in the PROSPERO database (CRD42023401926)

KEYWORDS

EGFR, NSCLC, bevacizumab, EGFR-TKI, combination therapy

Introduction

Lung cancer is one of the most common leading causes of death worldwide (Miller and Hanna, 2021). Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) account for nearly 85% and 15% of all lung cancers, respectively (Molina et al., 2008; Wang et al., 2021). Epidermal growth factor receptor (EGFR), a transmembrane receptor tyrosine kinase in the ERBB family, plays fundamental role in cell proliferation and survival (Jorissen et al., 2003). The overall EGFR mutation frequency was about 50% in Asia-Pacific patients and 15%–

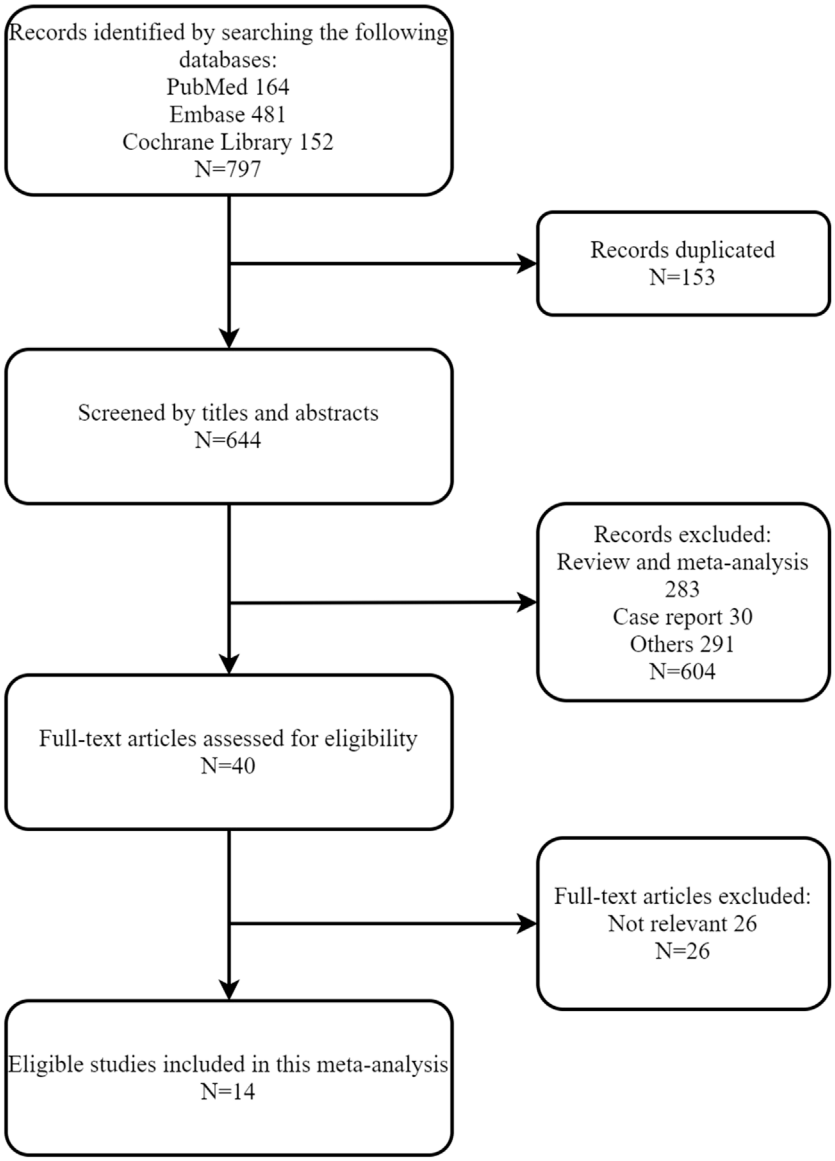

FIGURE 1
Flowchart of study selection.

TABLE 1 Baseline characteristics of included studies in the meta-analysis.

Study/year	Design	Histology/ Stage	Treatment		Treatment line	Median follow-up	Randomization	Outcomes
AfaBev-CS (2022)(26) jRCTs061180006	Phase II	Non-squamous NSCLC	Afatinib (30 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Afatinib once daily (40 mg)	First-line	31.3 months	1:1	PFS, ORR, AEs
ARTEMIS-CTONG1509 (2021)(25) NCT02759614	Phase III	NSCLC/Stage IIIB-IV, recurrence	Erlotinib (150 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Erlotinib once daily (150 mg)	First-line	NA	1:1	PFS, OS, ORR, AEs
BEVERLY (2022)(31) NCT02633189	Phase III	NSCLC/Stage IIIB, IV	Erlotinib (150 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Erlotinib once daily (150 mg)	First-line	36.3 months	1:1	PFS, OS, ORR, AEs
BOOSTER (2021)(18) NCT03133546	Phase II	Non-squamous NSCLC/Stage IIIB-IV	Osimertinib (80 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Osimertinib once daily (80 mg)	Second-line	33.8 months	1:1	PFS, OS, ORR, AEs
JO25567 (2014)(20) JapicCTI-111390	Phase II	Non-squamous NSCLC/Stage IIIB-IV, recurrence	Erlotinib (150 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Erlotinib once daily (150 mg)	First-line	20.4 months for PFS	1:1	PFS, ORR
JO25567 (2018)(24) JapicCTI-111390	Phase II	Non-squamous NSCLC/Stage IIIB-IV, recurrence	Erlotinib (150 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Erlotinib once daily (150 mg)	First-line	34.7 months for OS	1:1	OS
JO25567 (2018)(21) JapicCTI-111390	Phase II	Non-squamous NSCLC/Stage IIIB-IV, recurrence	Erlotinib (150 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Erlotinib once daily (150 mg)	First-line	27 months for monotherapy; 25.9 months for combination therapy	1:1	AEs
NEJ026 (2019)(22) UMIN000017069	Phase III	Non-squamous NSCLC/Stage IIIB–IV	Erlotinib (150 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Erlotinib once daily (150 mg)	First-line	12.4 months for PFS	1:1	PFS, ORR, AEs
NEJ026 (2022)(27) UMIN000017069	Phase III	Non-squamous NSCLC/Stage IIIB–IV	Erlotinib (150 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Erlotinib once daily (150 mg)	First-line	39.2 months for OS	1:1	OS
Stinchcombe et al. (2019)(19) NCT01532089	Phase II	Non-squamous NSCLC	Erlotinib (150 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Erlotinib once daily (150 mg)	First-line	33 months	1:1	PFS, OS, ORR

(Continued on following page)

TABLE 1 (*Continued*) Baseline characteristics of included studies in the meta-analysis.

Study/year	Design	Histology/ Stage	Treatment		Treatment line	Median follow-up	Randomization	Outcomes
WJOG8715L (2021)(23) UMIN000023761	Phase II	NSCLC/Stage IIIB-IV, recurrence	Osimertinib (80 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Osimertinib once daily (80 mg)	Not First-line	16.2 months for monotherapy; 16.0 months for combination therapy	1:1	PFS, OS, ORR, AEs
WJOG9717L (2022)(28) UMIN000030206	Phase II	Non-squamous NSCLC/Stage IIIB-IV, recurrence	Osimertinib (80 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Osimertinib once daily (80 mg)	First-line	19.8 months	1:1	ORR, AEs
WJOG9717L (2022)(30) UMIN000030206	Phase II	Non-squamous NSCLC/Stage IIIB-IV, recurrence	Osimertinib (80 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Osimertinib once daily (80 mg)	First-line	36 months	1:1	Updated PFS, OS
Youngjoo Lee et al. (2022)(29) NCT03126799	Phase II	Stage IIIB/IV NSCLC	Erlotinib (150 mg) once daily + bevacizumab (15 mg/kg) every 21 days	Erlotinib once daily (150 mg)	First-line	38.9 months	1:1	PFS, OS, ORR, AEs

20% in western NSCLC patients, with higher frequency in women compared with men, as well as in non-smokers compared with ever-smokers (Midha et al., 2015). Moreover, exon 19 (19del) deletion and L858R point mutation are most prevalent (Lee, 2017). The mutation and overexpression of EGFR was the pharmaceutical basis for the development and employment of EGFR-tyrosine kinase inhibitors (EGFR-TKI), and it has been widely adopted in front-line treatment for NSCLC patients with EGFR mutation (Lau et al., 2019; Li et al., 2019; Ito et al., 2020). Nevertheless, most patients inevitably develop resistance to these TKIs within 9–13 months (Lee, 2017), which has been found to be associated with increased vascular endothelial growth factor (VEGF) levels (Hung et al., 2016). It is reported that inhibition of angiogenesis could effectively enhance the anti-tumor activity of EGFR-TKI by targeting both the EGFR and VEGF pathways (Zhang et al., 2020; Watanabe et al., 2021). Therefore, addition of antiangiogenic agents might be able to prevent EGFR-TKI resistance and exert synergistic anti-tumor effects.

Bevacizumab is a kind of recombinant, anti-VEGF monoclonal antibody, which targets vascular endothelial growth factor-A (Goyal et al., 2022). The addition of bevacizumab to chemotherapy or immune check point inhibitors in the treatment of advanced NSCLC was demonstrated to be favorable (Systematic review and meta, 2013; Socinski et al., 2021; Sugawara et al., 2021), whereas its role in EGFR-TKI combination therapy remains controversy. The combination of erlotinib and bevacizumab was shown to be encouraging and has been accepted as an alternative choice of front-line therapy (Hsu et al., 2018). However, in the trials that mostly included non-Asian patients, no superiority of the combination regimen was found in terms of the anti-tumor effect, as compared with EGFR-TKI alone (Stinchcombe et al., 2019; Soo et al., 2021).

As more clinical trials had reported the outcome of combination therapy involving bevacizumab and different EGFR-TKIs, the present study aimed to clarify the clinical value of bevacizumab and EGFR-TKI combination therapy in EGFR-mutant advanced NSCLC patients, and further explore its role in predefined subgroups, in an attempt to provide evidence for selection of NSCLC individuals who might benefit most by adding bevacizumab to EGFR-TKI.

Methods

Search strategy

This systematic review and meta-analysis was registered in the International prospective register of systematic reviews (PROSPERO) database (CRD42023401926). We conducted a thorough search to identify relevant RCTs that had compared the clinical efficacy of combination EGFR-TKI and bevacizumab therapy with EGFR-TKI monotherapy in the treatment of advanced NSCLC using the following databases: PUBMED, EMBASE, and Cochrane. The last retrieval was performed on 23 December 2022. The keywords used were as follows: all terms related to "NSCLC," "bevacizumab," "erlotinib," "gefitinib," "icotinib," "afatinib," "Osimertinib," and other EGFR-TKIs, "epidermal growth factor receptor," "EGFR," all terms related to clinical trial. The retrieval strategy for the PubMed database is listed in Supplementary Table S1.

Eligibility criteria

Studies fulfilling all the following criteria were included (Miller and Hanna, 2021) RCTs; (Molina et al., 2008) studies that had

TABLE 2 Baseline characteristics of patients included in the meta-analysis.

Study/year	Treatment	Patients (n, female%)	Age (median, years)	Smoking history (never smoker, smoker, other)	Ethnicity	EGFR mutation (19del, L858R, other)	ECOG score (0, 1, 2)	CNS metastasis
AfaBev-CS (2022)(26) jRCTs061180006	Afatinib + Bev	50	NA	NA	Japanese centers	NA	NA	NA
	Afatinib	50	NA	NA	Japanese centers	NA	NA	NA
ARTEMIS-CTONG1509 (2021)(25) NCT02759614	Erlotinib + Bev	157 (61.8)	57 (33–78)	NA	Asian (100%)	(82, 75, 0)	(25, 132, 0)	44 (28%)
	Erlotinib	154 (62.3)	59 (27–77)	NA	Asian (100%)	(79, 75, 0)	(17, 137, 0)	47 (30.5%)
BEVERLY (2022)(31) NCT02633189	Erlotinib + Bev	80 (65%)	65.9 (57.9–71.8)	(46, 34, 0)	Italian centers	(44, 34, 2)	(52, 26, 2)	None
	Erlotinib	80 (62.5%)	67.7 (60.7–73.6)	(37, 43, 0)	Italian centers	(44, 32, 4)	(47, 29, 4)	None
BOOSTER (2021)(18) NCT03133546	Osimertinib + Bev	78 (60.3%)	68 (34–85)	(44, 34, 0)	Asian (41%)	(58, 20, 0)	(22, 51, 5)	13 (16.7%)
	Osimertinib	77 (63.6%)	66 (41–83)	(49, 28, 0)	Asian (40.3%)	(51, 26, 0)	(25, 48, 4)	8 (10.4%)
JO25567 (2014)(20) JapicCTI-111390	Erlotinib + Bev	75 (60%)	67 (59–73)	(42, 9, 24)*	Asian (100%)	(40, 35, 0)	(43, 32, 0)	None
	Erlotinib	77 (66%)	67 (69–73)	(45, 6, 26)*	Asian (100%)	(40, 37, 0)	(41, 36, 0)	None
NEJ026 (2019)(22) UMIN000017069	Erlotinib + Bev	112 (63%)	67 (61–73)	(65, 47, 0)	Asian (100%)	(56, 56, 0)	(64, 48, 0)	36 (32%)
	Erlotinib	112 (65%)	68 (62–73)	(64, 48, 0)	Asian (100%)	(55, 57, 0)	(68, 42, 2)	36 (32%)
Stinchcombe et al. (2019)(19) NCT01532089	Erlotinib + Bev	43 (72%)	65 (31–84)	(25, 17, 1)	Non-Asian (96%)	(29, 14, 0)	(24, 19, 0)	11 (26%)
	Erlotinib	45 (69%)	63 (47–84)	(23, 22, 0)	Non-Asian (94%)	(30, 15, 0)	(19, 26, 0)	14 (31%)
WJOG8715L (2021)(23) UMIN000023761	Osimertinib + Bev	40 (60%)	68 (43–82)	(21, 19, 0)	Asian (100%)	(22, 18, 0)	(20, 20, 0)	12 (30%)
	Osimertinib	41 (59%)	70 (41–82)	(20, 21, 0)	Asian (100%)	(28, 13, 0)	(17, 24, 0)	9 (22%)
WJOG9717L (2022)(28) UMIN000030206	Osimertinib + Bev	61 (60.7%)	67 (59–74)	(38, 23, 0)	Asian (100%)	(35, 26, 0)	(32, 29, 0)	NA
	Osimertinib	61 (62.3%)	66 (60–74)	(30, 31, 0)	Asian (100%)	(36, 25, 0)	(34, 27, 0)	NA
Youngjoo Lee et al. (2022)(29) NCT03126799	Erlotinib + Bev	64 (68.8%)	31 (48.4%)#	(41, 23, 0)	Asian (100%)	(37, 27, 0)	(33, 31, 0)	29 (45.3%)
	Erlotinib	63 (63.5%)	24 (38.1%)#	(42, 21, 0)	Asian (100%)	(37. 26, 0)	(28, 35, 0)	30 (47.6%)

*indicates (never smoker, former light smoker, other).
#indicates number of participants who aged ≥65 years (percentage%).

compared combination EGFR-TKI and bevacizumab therapy with EGFR-TKI monotherapy in treating advanced NSCLC (Wang et al., 2021); studies included patients with EGFR mutations (Jorissen et al., 2003); with at least one of the following reported outcomes: overall survival (OS), progression-free survival (PFS), objective response rate (ORR) and treatment-related adverse events with a grade equal or more than 3 (grade≥3 TRAEs) (Midha et al., 2015); studies with a sample size of at least 40 patients. For the overlapping reports obtained from the same group of patients, the latest and most complete reports were included. Duplicate publications, review articles, meta-analyses, editorials, case reports, letters, animal or cellular experiments and studies with incomplete data were excluded.

Data extraction

Data extraction was performed independently by two investigators according to the predefined criteria. The information extracted from each study was as follows: the name of study, year of publication, trial number and design, ethnicity involved, sample size (female%), treatment regimens, follow-up

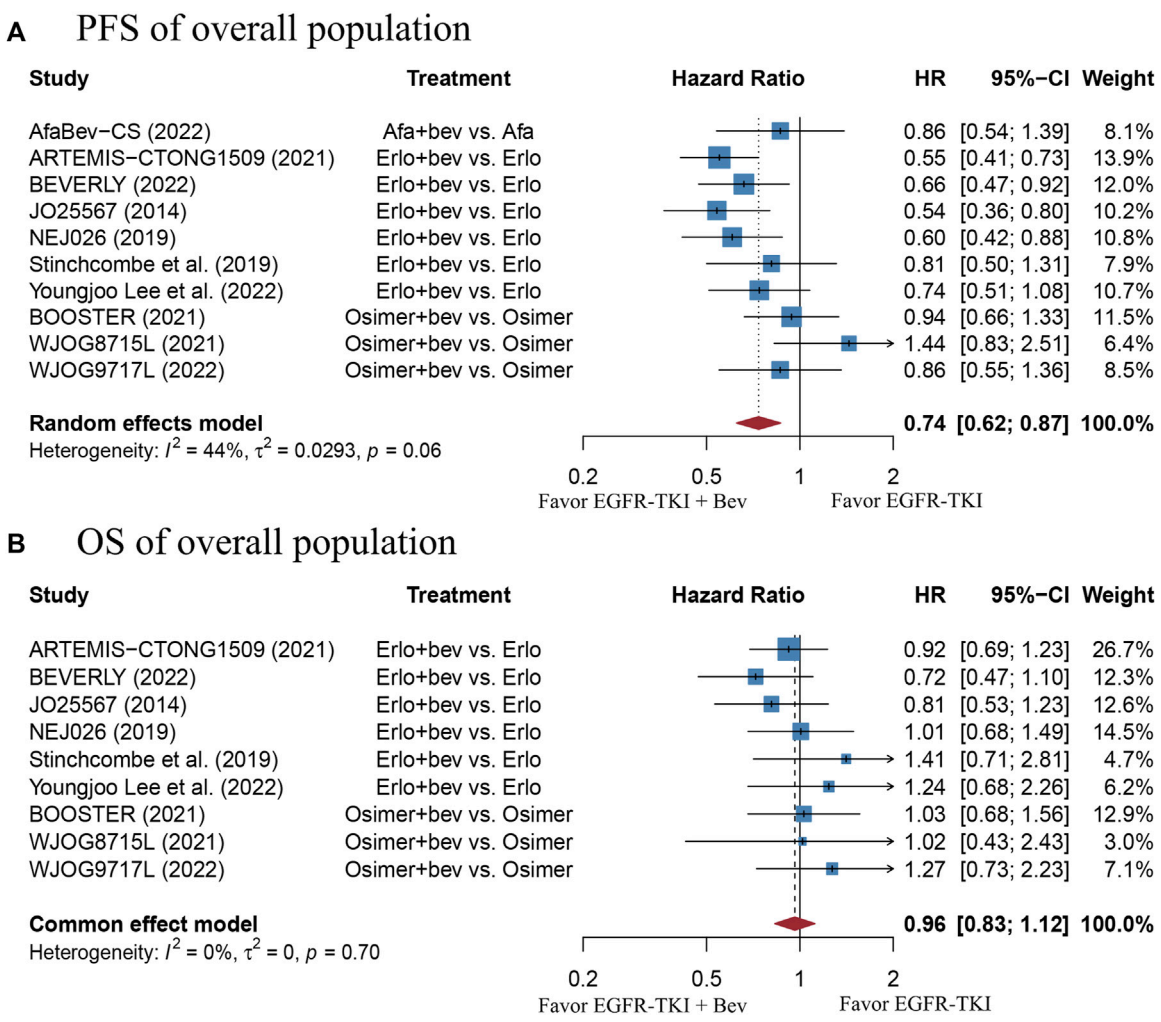

FIGURE 2
(A) Forest plot of HRs for PFS in the overall population. **(B)** Forest plot of HRs for OS in the overall population. Afa, Afatinib; Bev, Bevacizumab; Erlo, Erlotinib; Osimer, Osimertinib; CI, confidence interval.

time, age (median, range, years), Eastern Cooperative Oncology Group performance status (ECOG PS), smoking status, baseline central nervous system (CNS) metastasis condition, pathological features, EGFR mutation status, outcomes including PFS, OS, ORR and grade≥3 TRAEs. A third investigator was consulted when there were any disagreements during the process, and the discrepancies were resolved by discussion.

Quality assessment

The quality assessment of included trials was conducted independently by two investigators. The quality of RCT was evaluated according to the Cochrane Collaboration tool, with a total of 6 items included: selection bias, performance bias, detection bias, attrition bias, reporting bias, and other bias (Supplementary Figure S1). There are three levels for each item, that is, a high, low or unclear risk of bias. A third investigator was consulted when there were any disagreements during the process, and the discrepancies were resolved by discussion.

Statistical analysis

R software (version 4.1.0) with package meta was adopted to perform meta-analysis. The primary outcomes were OS and PFS, and the secondary outcomes were ORR and grade≥3 TRAEs. Hazard ratios (HR) with 95% CIs for OS and PFS, odds ratios (OR) with 95% CIs for ORR and grade≥3 TRAEs were extracted from the original report.

For each outcome, statistical heterogeneity was evaluated using the Cochran's Q test and the I^2 measure. An I^2 value greater than 50% or p-value equal or less than 0.1 is generally considered to indicate a substantial level of heterogeneity, which requires a random effects model for pooled analysis and initiates subsequent sensitivity analysis to identify the source. Otherwise, a fixed effects model was adopted. The Egger regression test with a funnel plot was used to evaluate the publication bias, and a p-value of less than 0.10 was considered to indicate significant asymmetry and publication bias. When there was publication bias, trim-and-fill method was used for data correction. Subgroup analyses were conducted with the following stratifications: gender, age, baseline

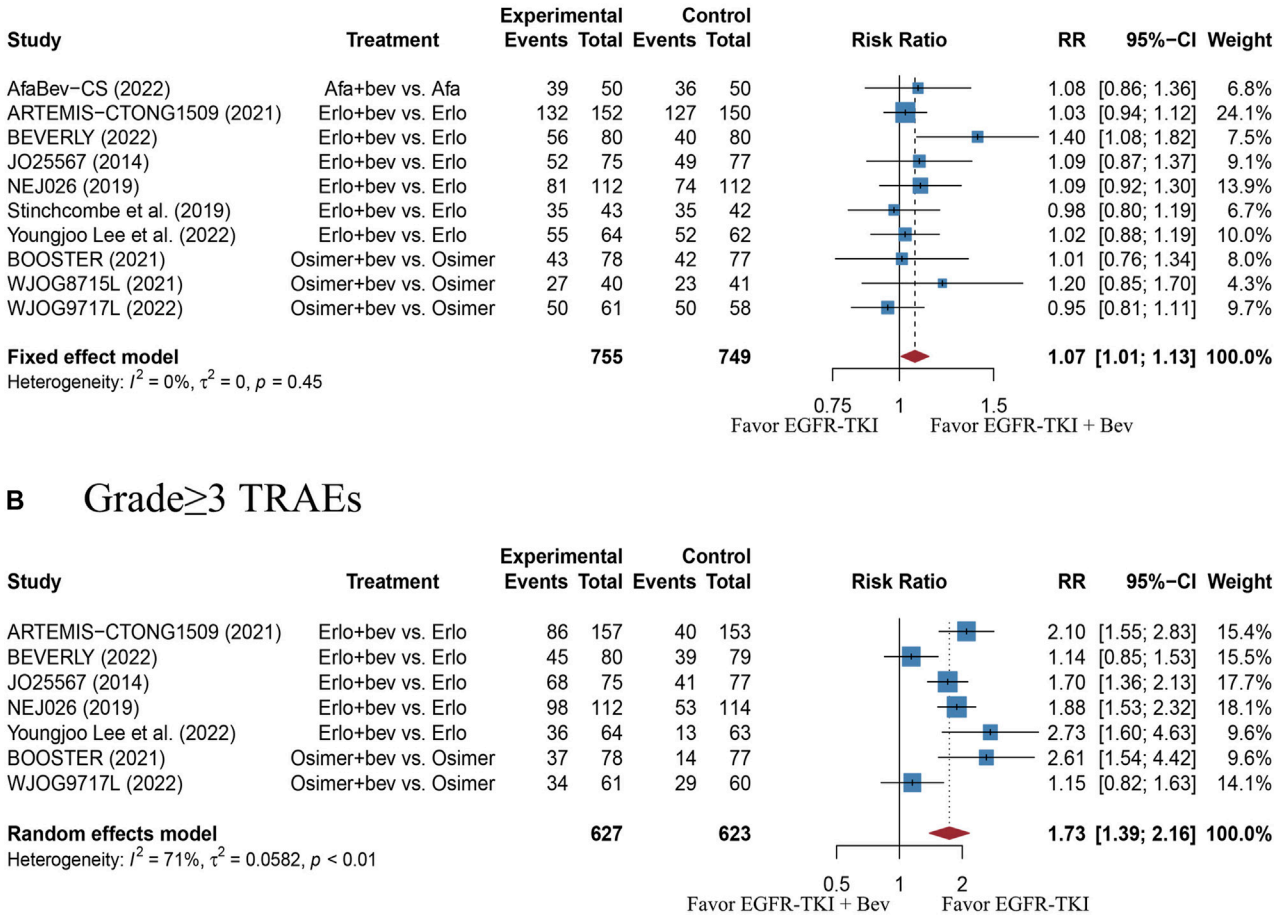

FIGURE 3
(A) Forest plot of RRs for ORR in the overall population. **(B)** Forest plot of RRs for grade≥3 TRAEs in the overall population. Afa, Afatinib; Bev, Bevacizumab; Erlo, Erlotinib; Osimer, Osimertinib; CI, confidence interval.

CNS metastasis, EGFR mutation type, smoking status, different type of EGFR-TKI, treatment line, ethnicity, and ECOG PS.

Results

Study selection and characteristics

We identified 797 records from the databases. After excluding 153 duplicates and 604 reports for irrelevant titles and abstracts, a total of 40 studies were reviewed for full-text assessment. Finally, 14 studies from 10 trials were included in our work (Seto et al., 2014; Kato et al., 2018; Saito et al., 2019; Stinchcombe et al., 2019; Akamatsu et al., 2021; Soo et al., 2021; Yamamoto et al., 2021; Zhou et al., 2021; Ishikawa et al., 2022; Kawashima et al., 2022; Kenmotsu et al., 2022; Lee et al., 2022; Nakamura et al., 2022; Piccirillo et al., 2022), with 1 trial only reported in conference abstract (Ishikawa et al., 2022) (Figure 1).

The detailed information of the 14 studies were shown in Table 1 and Table 2. A total of 1520 patients were included in our work, with 760 in the combination therapy group and 760 in the monotherapy group. One out of 10 trials had evaluated the efficacy of afatinib plus bevacizumab as compared with afatinib alone (Ishikawa et al., 2022), 6 had compared erlotinib plus bevacizumab with erlotinib alone (Seto et al., 2014; Kato et al., 2018; Saito et al., 2019; Stinchcombe et al., 2019; Yamamoto et al., 2021; Zhou et al., 2021; Kawashima et al., 2022; Lee et al., 2022; Piccirillo et al., 2022), and 3 had compared osimertinib plus bevacizumab with osimertinib monotherapy (Akamatsu et al., 2021; Kenmotsu et al., 2022; Nakamura et al., 2022). There were 3 phase III RCTs and 7 phase II RCTs. The majority of the included patient population was Asian. There were 8 RCTs adopted the EGFR-TKI regimen as first-line treatment (Seto et al., 2014; Saito et al., 2019; Stinchcombe et al., 2019; Zhou et al., 2021; Ishikawa et al., 2022; Kenmotsu et al., 2022; Lee et al., 2022; Piccirillo et al., 2022). Most patients were ECOG PS 0-1.

Overall population

There were 10 studies involving 1520 patients with EGFR-mutant advanced NSCLC eligible for the pooling analysis of PFS. The pooled PFS result derived from a random-effect model showed that the

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TABLE 3 Subgroup analyses of progression-free survival and overall survival.

Subgroup	Studies (patients, n)	HR for PFS (95%CI)	Heterogeneity *p*-value, I^2 (%)	Studies (patients, n)	HR for OS (95%CI)	Heterogeneity *p*-value, I^2 (%)
Gender						
Male	8 (494)	0.63 (0.51–0.78)	$p = 0.2$, $I^2 = 29\%$	4 (253)	0.92 (0.65–1.3)	$p = 0.29$, $I^2 = 21\%$
Female	8 (838)	0.76 (0.59–0.97)	$p = 0.06$, $I^2 = 48\%$	4 (438)	0.86 (0.66–1.12)	$p = 0.44$, $I^2 = 0\%$
Age (years)						
<75	5 (770)	0.69 (0.52–0.91)	$p = 0.09$, $I^2 = 50\%$	NA	NA	NA
≥75	4 (114)	0.6 (0.33–1.09)	$p = 0.26$, $I^2 = 26\%$	NA	NA	NA
ECOG PS						
0	8 (568)	0.68 (0.55–0.84)	$p = 0.15$, $I^2 = 35\%$	4 (362)	0.86 (0.63–1.18)	$p = 0.92$, $I^2 = 0\%$
1	8 (756)	0.71 (0.59–0.84)	$p = 0.49$, $I^2 = 0\%$	4 (321)	0.87 (0.66–1.16)	$p = 0.39$, $I^2 = 0\%$
Baseline CNS metastasis						
Yes	5 (284)	0.63 (0.47–0.85)	$p = 0.58$, $I^2 = 0\%$	NA	NA	NA
No	7 (873)	0.70 (0.56–0.88)	$p = 0.09$, $I^2 = 45\%$	NA	NA	NA
Smoking status						
Never-smoker	7 (599)	0.9 (0.66–1.24)	$p = 0.03$, $I^2 = 58\%$	4 (407)	1.05 (0.8–1.38)	$p = 0.39$, $I^2 = 0\%$
Smoker	7 (409)	0.59 (0.46–0.74)	$p = 0.43$, $I^2 = 0\%$	4 (271)	0.68 (0.48–0.95)	$p = 0.15$, $I^2 = 43\%$
EGFR mutation type						
19del	7 (694)	0.68 (0.57–0.82)	$p = 0.35$, $I^2 = 11\%$	5 (549)	1.03 (0.78–1.35)	$p = 0.77$, $I^2 = 0\%$
L858R	7 (551)	0.67 (0.54–0.83)	$p = 0.43$, $I^2 = 0\%$	5 (447)	0.85 (0.63–1.14)	$p = 0.63$, $I^2 = 0\%$
Ethnicity						
Asian	8 (1180)	0.71 (0.58–0.87)	$p = 0.07$, $I^2 = 46\%$	7 (1080)	0.96 (0.81–1.15)	$p = 0.84$, $I^2 = 0\%$
Non-Asian	3 (340)	0.84 (0.59–1.19)	$p = 0.12$, $I^2 = 52\%$	3 (340)	1.03 (0.67–1.58)	$p = 0.14$, $I^2 = 50\%$
Different type of EGFR-TKI						
Afatinib	1 (100)	0.86 (0.54–1.39)	NA	NA	NA	NA
Erlotinib	6 (1062)	0.63 (0.54–0.73)	$p = 0.65$, $I^2 = 0\%$	6 (1062)	0.93 (0.78–1.1)	$p = 0.51$, $I^2 = 0\%$
Osimertinib	3 (358)	1 (0.78–1.28)	$p = 0.34$, $I^2 = 8\%$	3 (358)	1.1 (0.8–1.5)	$p = 0.83$, $I^2 = 0\%$
Treatment line						
First-line	8 (1284)	0.66 (0.58–0.76)	$p = 0.49$, $I^2 = 0\%$	7 (1184)	0.95 (0.81–1.12)	$p = 0.5$, $I^2 = 0\%$
Non-first-line	2 (291)	1.06 (0.79–1.43)	$p = 0.2$, $I^2 = 38\%$	2 (291)	1.03 (0.71–1.5)	$p = 0.98$, $I^2 = 0\%$

combination therapy group had a significantly longer PFS as compared with the EGFR-TKI monotherapy group (HR = 0.74, 95% CI: 0.62–0.87, Cochran's Q $p = 0.06$, $I^2 = 44\%$; Figure 2A). The funnel plot and Egger's test both demonstrated publication bias (Supplementary Figure S2A, $p = 0.0227$). Thus, trim-and-fill method was adopted. The data after correction also suggested significant PFS benefit in the combination therapy group (HR = 0.655, 95% CI: 0.5439–0.7889; Supplementary Figure S2B). Sensitivity analysis showed that removal of any study did not affect the pooled HR, which indicates stability of the result (Supplementary Figure S3A).

A total of 9 studies including 1420 patients with EGFR-mutant advanced NSCLC were enrolled for the pooling analysis of OS. The pooled HR was 0.96 (95% CI: 0.83–1.12), with no heterogeneity (Cochran's Q $p = 0.7$, $I^2 = 0\%$; Figure 2B), suggesting that there was no significant difference in OS between the combination therapy group and EGFR-TKI monotherapy group. The funnel plot and Egger's test showed no publication bias (Supplementary Figure S2C, $p = 0.1486$). Sensitivity analysis showed that removal of any study did not affect the pooled HR, which indicates stability of the result (Supplementary Figure S3B).

There were 10 studies with 1520 EGFR-mutant advanced NSCLC patients provided the ORR outcome. The pooled RR was 1.07 (95% CI: 1.01–1.13), with no heterogeneity (Cochran's Q $p = 0.45$, $I^2 = 0\%$; Figure 3A), indicating a slightly better response in the

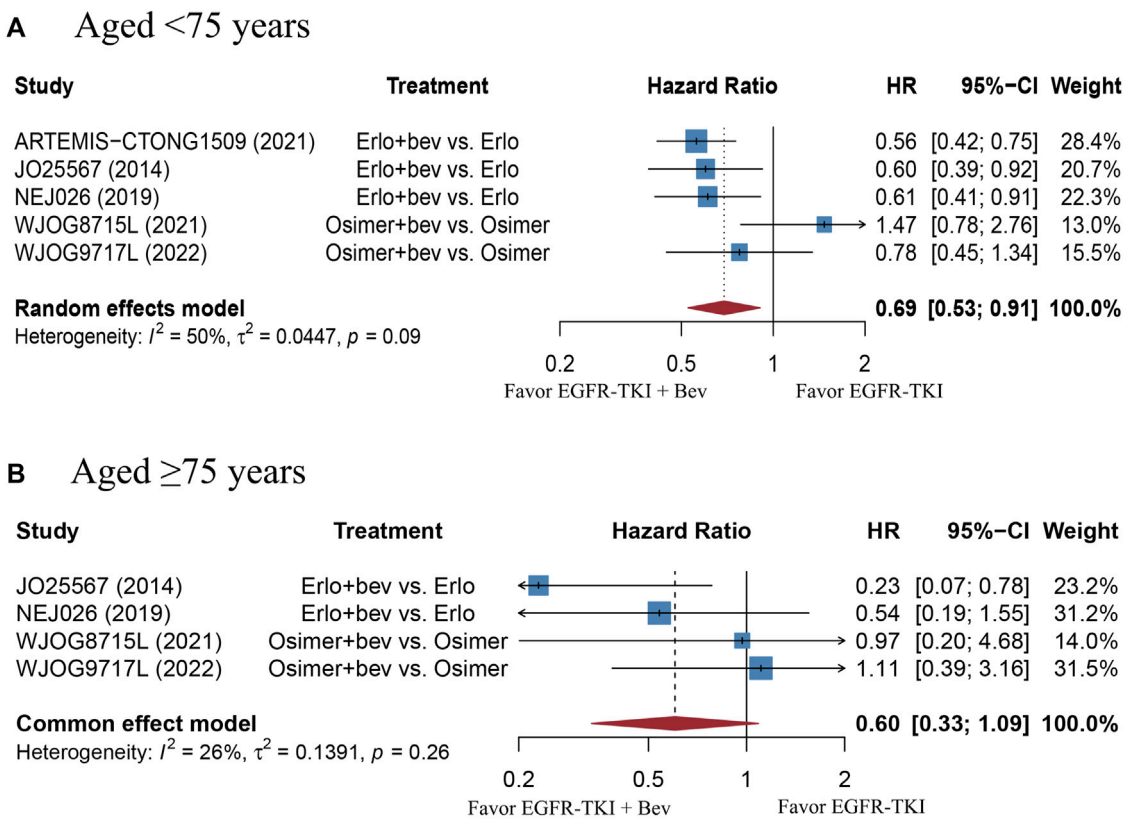

FIGURE 4
(A) Forest plot of HRs for PFS in patients aged less than 75 years old. **(B)** Forest plot of HRs for OS in patients aged equal or more than 75 years old. Afa, Afatinib; Bev, Bevacizumab; Erlo, Erlotinib; Osimer, Osimertinib; CI, confidence interval.

combination therapy group, as compared with the EGFR-TKI monotherapy group. The funnel plot and Egger's test showed no publication bias (Supplementary Figure S2D, p = 0.1524). Nevertheless, sensitivity analysis showed that removal of the BEVERLY research would affect the pooled RR, which indicates instability of the result (Supplementary Figure S3C). This data should be interpreted with caution.

There were 7 studies with 1250 EGFR-mutant advanced NSCLC patients reported data on grade≥3 TRAEs. The pooled RR was 1.73 (95% CI: 1.39–2.16), with high heterogeneity (Cochran's Q $p < 0.01$, I^2 = 71%; Figure 3B), which suggests a significantly higher risk of grade≥3 TRAEs with combination therapy, as compared with the EGFR-TKI monotherapy. The funnel plot and Egger's test showed no publication bias (Supplementary Figure S2E, p = 0.6441). Sensitivity analysis showed that removal of any study did not affect the pooled RR, indicating stability of the result (Supplementary Figure S3D). Moreover, the most reported grade≥3 TRAEs were listed in Supplementary Table S2. Of those, the increased risks of hypertension, proteinuria and rash in the combination therapy group were statistically significant, as compared with monotherapy group.

Subgroup analyses

Subgroup analyses of PFS and OS were conducted with the following stratifications: gender, age, baseline CNS metastasis condition, EGFR mutation type, smoking status, different type of EGFR-TKI, treatment line, ethnicity, and ECOG PS (Table 3).

The stratified analysis showed that addition of bevacizumab to EGFR-TKI therapy could significantly improve the PFS for all EGFR-mutant advanced NSCLC patients irrespective of the differences in gender, EGFR mutation type, ECOG PS, and baseline CNS metastasis (Table 3). However, significant PFS benefit of combination therapy was noticed in patients with age below 75 years (HR = 0.69, 95% CI: 0.52–0.91, Cochran's Q p = 0.09, I^2 = 50%; Figures 4A,B), in the smoker population (HR = 0.59, 95% CI: 0.46–0.74, Cochran's Q p = 0.43, I^2 = 0%; Figures 5A,B), and in the Asian population (HR = 0.71, 95% CI: 0.58–0.87, Cochran's Q p = 0.07, I^2 = 46%; Figures 6A,B). Moreover, patients treated with erlotinib and bevacizumab combination therapy yielded remarkably better PFS (HR = 0.63, 95% CI: 0.54–0.73, Cochran's Q p = 0.65, I^2 = 0%; Figure 7), whereas those treated with osimertinib or afatinib and bevacizumab had comparable efficacy with those treated with EGFR-TKI monotherapy (For osimertinib, HR = 1, 95% CI: 0.78–1.28, Cochran's Q p = 0.34, I^2 = 8%; Figure 7). Further analyses revealed that EGFR-TKI and bevacizumab had significantly better PFS outcome when adopted as first-line treatment (HR = 0.66, 95% CI: 0.58–0.76, Cochran's Q p = 0.49, I^2 = 0%; Figure 8).

Adding bevacizumab to EGFR-TKI therapy did not affect the OS for all EGFR-mutant advanced NSCLC patients, regardless of their gender, EGFR mutation type, different type of EGFR-TKI, treatment

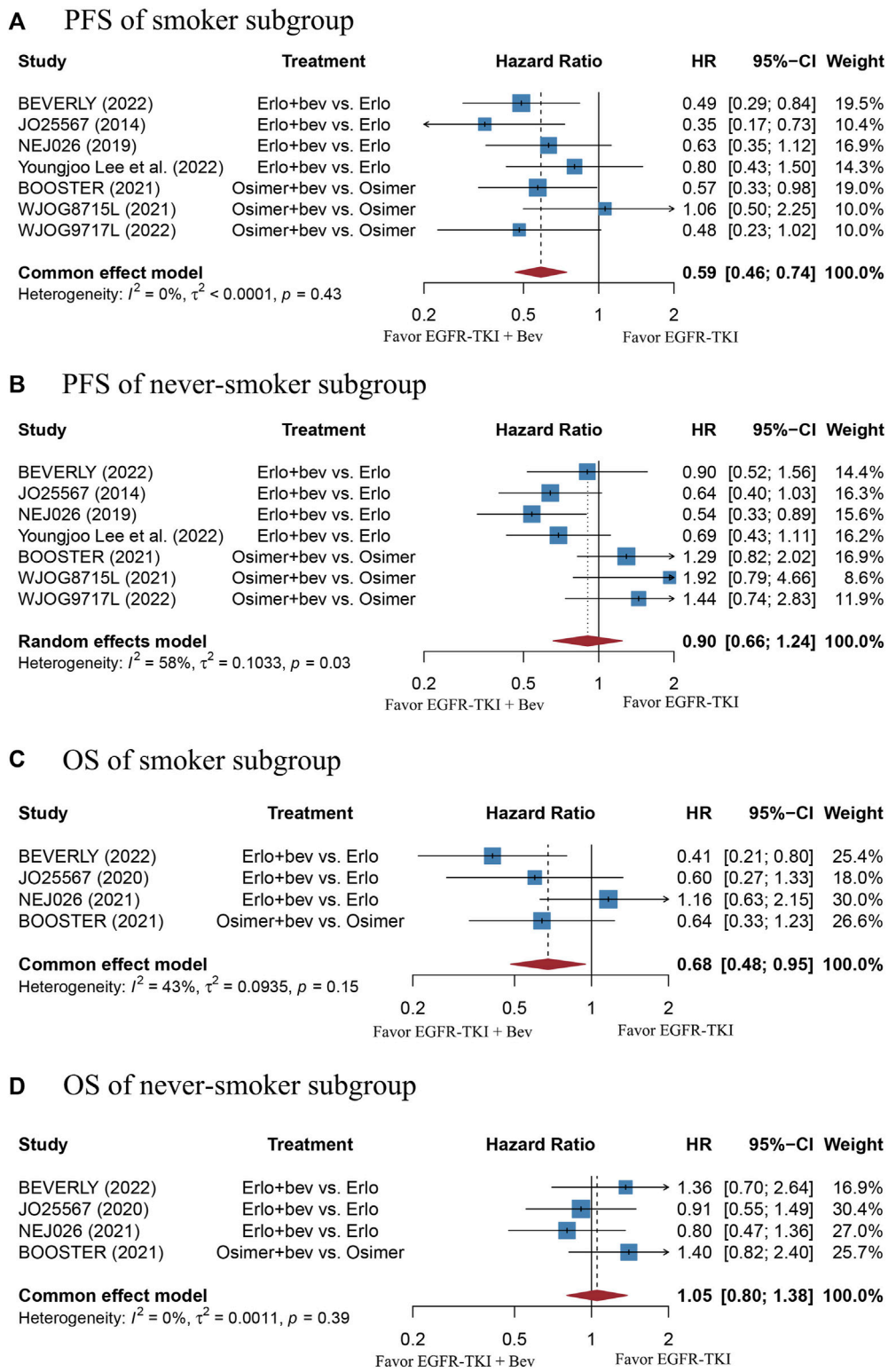

FIGURE 5
(A) Forest plot of HRs for PFS in smoker subgroup. **(B)** Forest plot of HRs for PFS in never-smoker subgroup. **(C)** Forest plot of HRs for OS in smoker subgroup. **(D)** Forest plot of HRs for OS in never-smoker subgroup. Note: There were 13 former light smokers from the NEJ026 study excluded from the analysis. The 15 former light smokers from the JO25567 study were included in the never-smoker subgroup. Afa, Afatinib; Bev, Bevacizumab; Erlo, Erlotinib; Osimer, Osimertinib; CI, confidence interval.

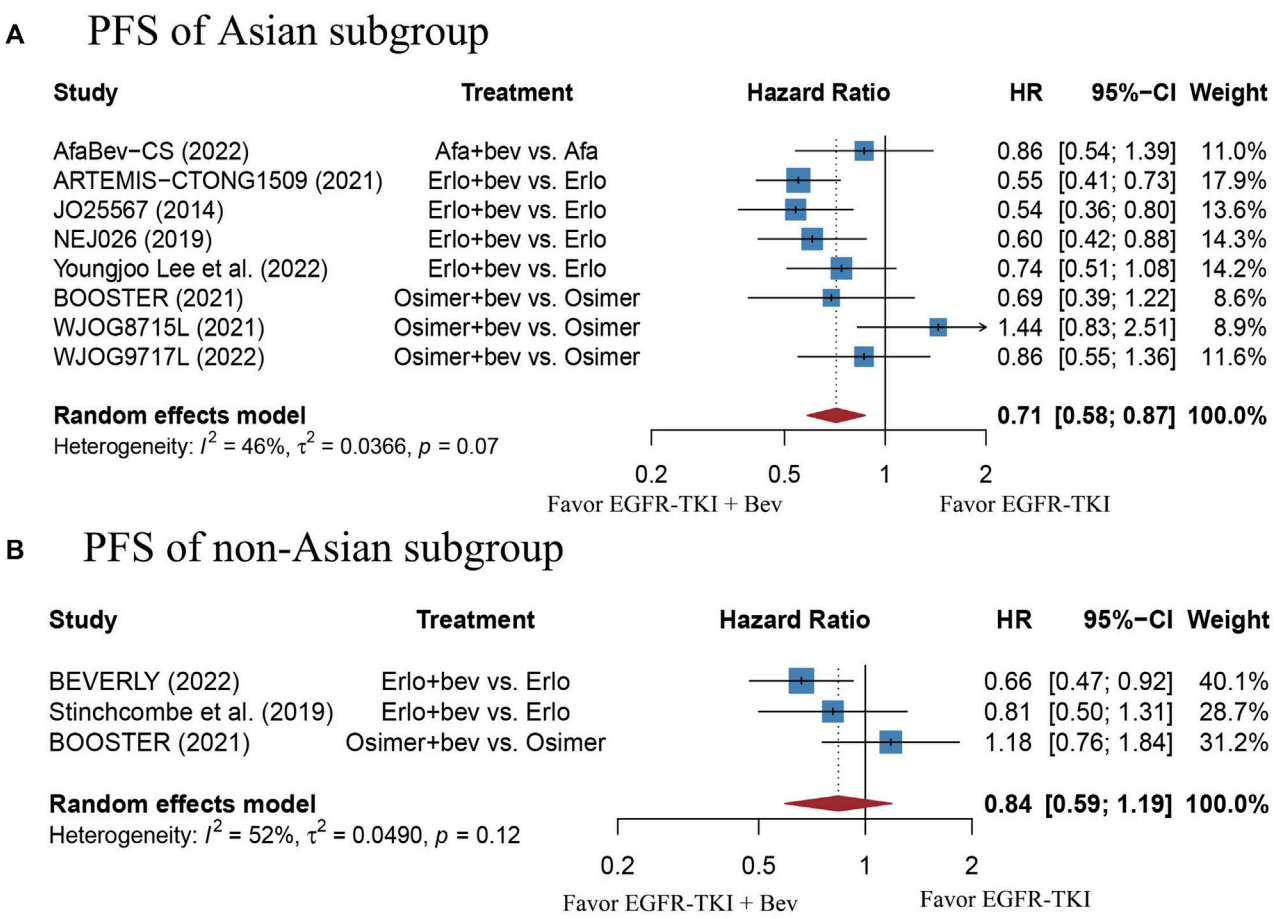

FIGURE 6
(A) Forest plot of HRs for PFS in Asian subgroup. **(B)** Forest plot of HRs for PFS in non-Asian subgroup. Note: The BEVERLY study conducted in Italian centers and the work proposed by Stinchcombe et al. including mostly non-Asian people were both categorized as non-Asian group. Afa, Afatinib; Bev, Bevacizumab; Erlo, Erlotinib; Osimer, Osimertinib; CI, confidence interval.

line, and ECOG PS (Table 3). Interestingly, significant OS benefit of combination therapy was observed in the smoker subgroup, with no heterogeneity (HR = 0.68, 95%CI: 0.48–0.95, Cochran's Q p = 0.15, I^2 = 43%; Figures 5C,D).

Discussion

The results of this meta-analysis showed that adding bevacizumab to EGFR-TKI therapy provided significantly better PFS and ORR results for NSCLC patients harboring EGFR mutations, though this benefit failed to translate into prolonging OS. The subgroup analyses stratified by patients' clinical features also proved that EGFR-TKI and bevacizumab combination therapy consistently resulted in longer PFS regardless of the gender, ECOG PS, baseline CNS metastasis and EGFR mutation type. Interestingly, in the smoker subgroup (former or current smoker), addition of bevacizumab to EGFR-TKI could significantly prolong the PFS and OS. Moreover, as compared with those aged equal or more than 75 years, combination therapy provided with significantly favorable PFS results for EGFR-mutant advanced NSCLC patients who aged less than 75 years.

VEGF, a family of polypeptide growth factors, mainly included VEGF-A, -B, -C and -D (32). Of those, VEGF-A is the most investigated variant, which primarily binds to VEGF receptor 1 and 2, thus inducing angiogenesis (Ferrara and Adamis, 2016). Bevacizumab, a humanized monoclonal antibody directed against VEGF-A, has been approved for the treatment of NSCLC globally. Given that the VEGF and EGFR pathways share common downstream signaling pathway that regulate cellular proliferation, it is suggested that EGFR-mutant tumors are more VEGF-dependent, thus dual inhibition of EGFR and VEGF might yield better antitumor effects (Abid et al., 2004; Le et al., 2021). In addition, it has been found that VEGF contributes to the acquired EGFR-TKI resistance, which supports the hypothesis that dual inhibition of EGFR and VEGF could delay resistance to EGFR-TKI, thus prolonging antitumor activity (Byers and Heymach, 2007; Le et al., 2021).

Subgroup analyses showed that the PFS benefit was consistently observed in EGFR-mutant advanced NSCLC patients of different gender (male or female), patients with different ECOG PS (0 or 1), baseline CNS metastasis (presence or absence) and EGFR mutation type (19del or 21L858R). The finding is echoed with the same subgroup analyses in the study of Deng et al., 2021. In addition, we

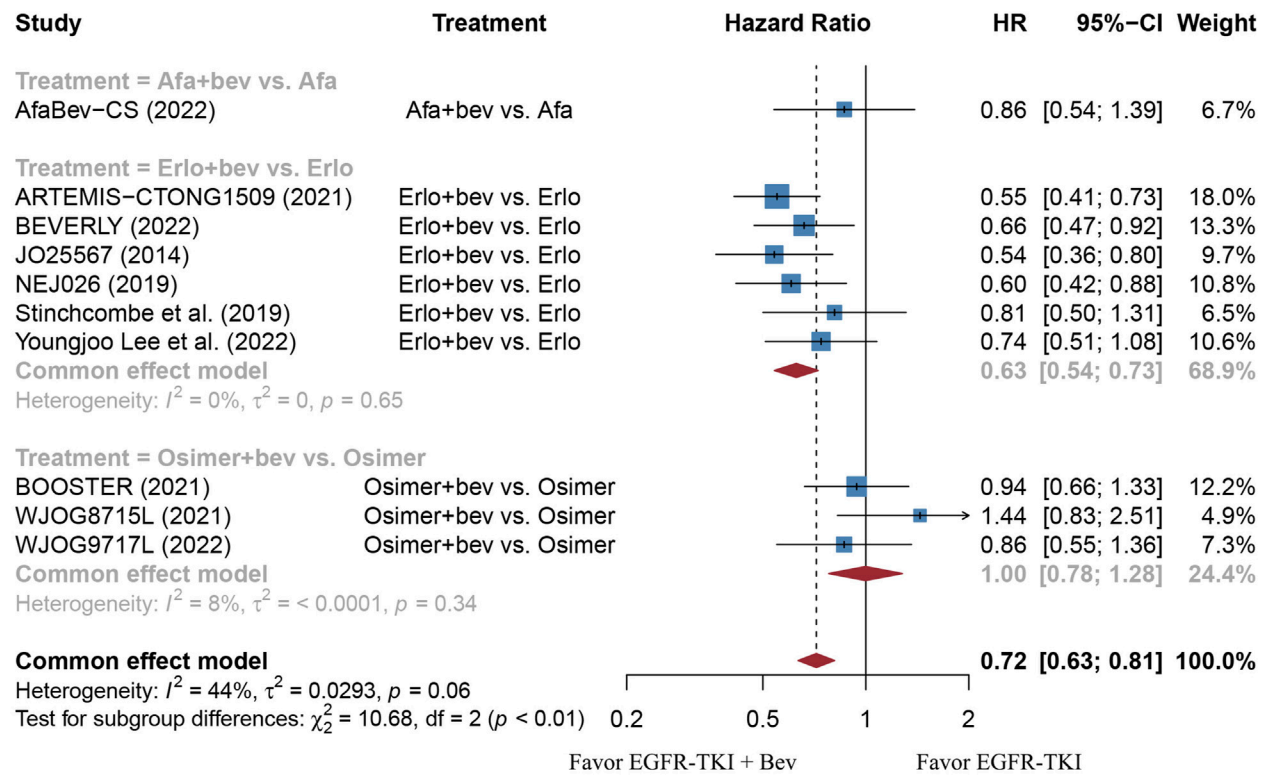

FIGURE 7
Forest plot of HRs for PFS based on different types of EGFR-TKI. Afa, Afatinib; Bev, Bevacizumab; Erlo, Erlotinib; Osimer, Osimertinib; CI, confidence interval.

found that combination bevacizumab and EGFR-TKI therapy significantly improved the PFS and OS result in smokers rather than those who never smoked, which is in line with the findings of Dafni et al., 2022. One possible explanation of this phenomenon is that TP53 mutation triggered by cigarette exposure would lead to increased sensitivity to anti-VEGF therapy (Schwaederlé et al., 2015). Moreover, we also noticed a significantly improved PFS in patients younger than 75 years old, as compared with those aged equal or more than 75 years. This finding is contradictory to that of Deng et al. (Deng et al., 2021). Nevertheless, it should be noted that the sample size of patients who aged equal or more than 75 years were too small in the ARTEMIS-CTONG1509 study and the PFS of the population could not be calculated, the number of patients aged equal or more than 75 years included was much less than those aged less than 75 years. In terms of different types of EGFR-TKI, our work included trials using all three generations of EGFR-TKI. Our data found that patients treated with erlotinib and bevacizumab combination therapy resulted in significantly better PFS than monotherapy, whereas the regimen involving osimertinib did not. The result may be partially explained by the fact that osimertinib and bevacizumab combination therapy adopted in both BOOSTER and WJOG8715L trials were used as non-first-line treatment. In the WJOG9717L trial, in which osimertinib and bevacizumab combination therapy was used in un-treated EGFR-mutant advanced NSCLC patients, bevacizumab was administered with a median duration of 33.4 weeks, which is shorter than that used with erlotinib (11–12 months) (Kenmotsu et al., 2022). There is another clinical trial (NCT04181060) currently evaluating the efficacy of osimertinib and bevacizumab combination therapy in un-treated EGFR-mutant advanced NSCLC patients, and the results are anticipated. Currently, most published work had focused on the Asian population. Our data showed that Asian population experienced significantly prolonged PFS than the non-Asian group. However, it should be noted that the sample size of non-Asian population is limited. There are several ongoing RCTs of EGFR-TKI with or without bevacizumab in EGFR-mutant advanced NSCLC that had primarily included non-Asian population (NCT04181060, NCT02971501), and the results are anticipated.

Noteworthy, several studies aimed to investigate the clinical value of multi-drugs therapy in treatment-naïve EGFR-mutant advanced NSCLC patients. The recently published FLAURA2 study confirmed significantly prolonged PFS in EGFR-mutant advanced NSCLC patients treated with osimertinib and chemotherapy, as compared with osimertinib alone (median PFS 25.5 months vs. 16.7 months, HR = 0.62, p < 0.001) (Planchard et al., 2023). The MARIPOSA study proved the superiority of amivantamab (with dual activity against EGFR and MET) and Lazertinib (a third-generation EGFR-TKI with CNS permeability) combination therapy in un-treated EGFR-mutant advanced NSCLC patients, as compared with osimertinib alone (median PFS 23.7 months vs. 16.6 months, HR = 0.7, p < 0.001) (Soria et al., 2013). The updated median PFS of the osimertinib monotherapy arm in the WJO9717L study was 20.2 months, which is longer than that reported in the FLAURA2 and MARIPOSA study. The reason

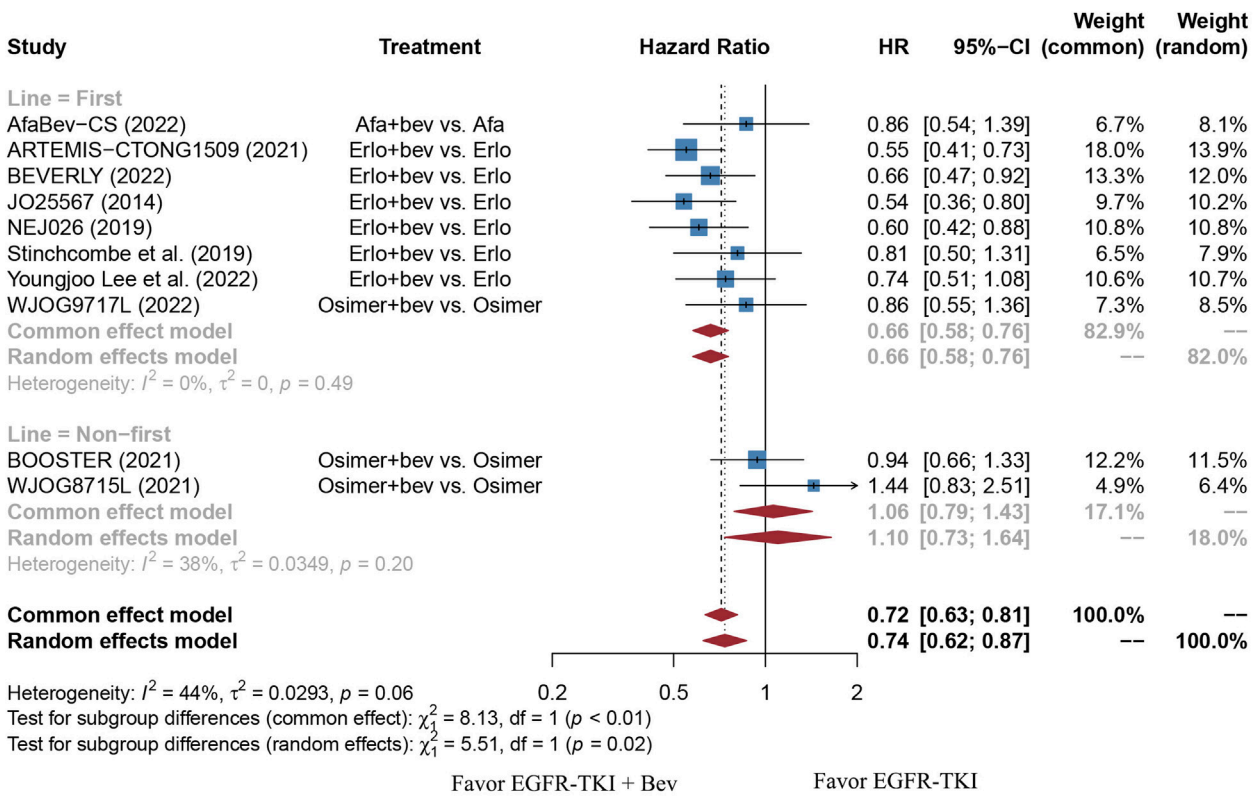

FIGURE 8
Forest plot of HRs for PFS based on different treatment lines. Afa, Afatinib; Bev, Bevacizumab; Erlo, Erlotinib; Osimer, Osimertinib; CI, confidence interval.

may be that both FLAURA2 and MARIPOSA study had included more patients with CNS metastasis at baseline. With the emerging evidence of various combination therapy, the optimal choice for EGFR-mutant advanced NSCLC patients awaits further exploration.

However, the increased risk of combination therapy is non-neglectable. The most frequently observed grade≥3 TRAEs were hypertension, proteinuria, thrombotic events, rash, diarrhea and increased aminotransferase, which were similar to the established profiles of bevacizumab and EGFR-TKI, with no new safety concerns. Though it had been reported that the adverse effects of combination therapy were manageable (Kato et al., 2018), combination therapy of bevacizumab and EGFR-TKI should be applied with caution, and the occurrence of adverse events should be monitored carefully.

To the best of our knowledge, this is the meta-analysis that had included the most recently published RCTs comparing the clinical efficacy of combination therapy of bevacizumab and EGFR-TKI with EGFR-TKI monotherapy, and it is also the first meta-analysis that had performed subgroup analyses for both PFS and OS outcomes. However, some limitations should be taken under consideration. First, the majority of included trials had only involved Asian patients, and the non-Asian population is limited, which may affect the subgroup comparison between Asian group and non-Asian group. Second, the OS data of the AfaBev-CS study is immature and the subgroup analyses result are not reported, thus we failed to include the information in our work.

Conclusion

Addition of bevacizumab to EGFR-TKI therapy provided significantly better PFS and ORR results for NSCLC patients harboring EGFR mutations, but no obvious OS benefit was observed and the risk of grade≥3 AEs was higher. Patients who ever smoked, aged <75 years old, and the Asian population might benefit more from the combination regimen, whereas gender, ECOG PS, baseline CNS metastasis and EGFR mutation type did not lead to significant differences.

Data availability statement

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

Author contributions

HZ, XQ, and HL contributed to the study conceptualization and design. HZ, YZ, and XY contributed to data acquisition and formal analysis. XQ, JT, and WC contributed to manuscript writing. HL and SH contributed to manuscript revision and study supervision. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1238579/full#supplementary-material

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Treatment decision for recurrences in non-small cell lung cancer during or after adjuvant osimertinib: an international Delphi consensus report

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Introduction: Osimertinib is recommended by major guidelines for use in the adjuvant setting in patients with EGFR mutation-positive NSCLC following the significant improvement in disease-free survival observed in the Phase III ADAURA trials. Due to limited real-world data in the adjuvant setting, little guidance exists on how to approach potential recurrences either during or after the completion of the treatment. This study aimed to reach a broad consensus on key treatment decision criteria in the events of recurrence.

Methods: To reach a broad consensus, a modified Delphi panel study was conducted consisting of two rounds of surveys, followed by two consensus meetings and a final offline review of key statements. An international panel of experts in the field of NSCLC (n=12) was used to provide clinical insights regarding patient management at various stages of NSCLC disease including patient monitoring, diagnostics, and treatment approach for specific recurrence scenarios. This study tested recurrences occurring 1) within or outside the central nervous system (CNS), 2) during or after the adjuvant-osimertinib regimen in NSCLC disease which is 3) amenable or not amenable to local consolidative therapy.

Results: Panellists agreed on various aspects of patient monitoring and diagnostics including the use of standard techniques (e.g., CT, MRI) and tumour biomarker assessment using tissue and liquid biopsies. Consensus was reached on 6 statements describing treatment considerations for the specific NSCLC recurrence scenarios. Panellists agreed on the value of osimertinib as a monotherapy or as part of the overall treatment strategy within the probed recurrence scenarios and acknowledged that more clinical evidence is required before precise recommendations for specific patient populations can be made.

Discussion: This study provides a qualitative expert opinion framework for clinicians to consider within their treatment decision-making when faced with recurrence during or after adjuvant-osimertinib treatment.

KEYWORDS

osimertinib, non-small cell lung cancer, adjuvant treatment, EGFR mutation, recurrence, treatment sequencing

1 Introduction

A significant number of NSCLC patients harbor *EGFR* driver mutations (*EGFRm* NSCLC) which activate *EGFR* tyrosine kinase to have a ligand-independent activity, resulting in tumorigenesis (1–3). In US plus Europe and Asia, *EGFRm* NSCLC patients account for ~10-15% and ~30-50% of all NSCLC cases, respectively (4). The two most common *EGFR* mutations are short in-frame deletions of exon 19 and a point mutation in exon 21 which result in the substitution of leucine by arginine at codon 858 (L858R), together, accounting for ~85% of all *EGFR* mutations in NSCLC (4). *EGFR* tyrosine kinase inhibitors (*EGFR*-TKIs) have been shown to significantly improve disease-free survival (DFS) in patients with resected early-stage *EGFRm* NSCLC (5, 6). Osimertinib is a third-generation *EGFR*-TKI approved in many countries around the world for both *EGFR*-TKI sensitizing (exon 19 deletion & L858R point mutation in exon 21) and T790M resistance mutations in advanced stage NSCLC patients (7). In 2020, primary analysis of the pivotal phase 3 ADAURA trial demonstrated a substantial DFS benefit in patients with EGFR-mutated NSCLC who underwent complete tumor resection, with hazard ratios of 0.17 (99% CI 0.11 to 0.26; $p < 0.001$) for stage II to IIIA disease and 0.20 (99% CI 0.14 to 0.30; $p < 0.001$) for stage IB to IIIA disease compared to placebo. Due to the significant improvement in DFS, the independent data monitoring committee recommended reporting the trial results two years earlier than originally planned, allowing patients to continue in the trial (8). Updated data with an additional 2 years of follow-up continued to show a sustained DFS benefit (hazard ratios of 0.23 in stage II and IIIA disease and 0.27 in stage IB and IIIA disease, respectively), In addition, recurrences among all the patients with stage IB to IIIA disease were less frequent with osimertinib (93 patients [27%]) than with placebo (205 patients [60%]). Recurrences in the osimertinib group included distant metastases only (45 patients [13%]), local/regional only (42 patients [12%]), as well as both local/regional and distance (6 patients [2%]) (9).

Most recently, published data on overall survival (OS) in the overall population (patients with stage IB to IIIA disease) report an OS HR of 0.49 (95% CI 0.34 to 0.70; $p < 0.0001$) with a 5-year OS rate of 88% with osimertinib vs 78% with placebo. In stage II–IIIA disease, OS HR was reported to be 0.49 (95% CI 0.33 to 0.73; $p=0.0001$) and the 5-year OS rate was 85% with osimertinib vs 73% with placebo. The median OS was not reached in either population or treatment group (10).

While adjuvant-osimertinib demonstrated an unprecedented patient benefit in terms of OS improvements, there is a need to understand better the optimal management of patients who show tumor recurrence either during or after the completion of the adjuvant-osimertinib regimen. Given the anticipated emergence of a patient population with disease relapse following adjuvant osimertinib treatment, and the absence of real-world data or trial data, creating formal guidelines on how to approach and manage recurrent patients either during or after completion of adjuvant-osimertinib is not yet possible. The knowledge gap regarding the appropriate approach for patient monitoring, diagnostics, and treatment sequencing decisions in cases of various recurrence scenarios can be bridged via clinical consensus studies. Recent

Abbreviations: CNS, central nervous system; CT, computerized tomography; DFS, disease-free survival; EGFRm, Epidermal growth factor receptor mutation; HR, hazard ratio; HRQoL, Health-related quality of life; MDT, multi-disciplinary team; MRD, minimal residual disease; MRI, magnetic resonance imaging; NGS, next-generation sequencing); OS, overall survival; PET, positron emission tomography; SBRT, stereotactic body radiation therapy; SRS, stereotactic radiosurgery; TKI, tyrosine kinase inhibitor; NSCLC,non-small-cell lung cancer; WBRT, whole-brain radiotherapy.

consensus studies echoed the need for further clinical trial data to create formal guidelines (11, 12) and outlined the appropriate treatment options (including osimertinib) for different recurrence scenarios (12). In this consensus paper, we discuss the key clinical factors that can be considered during treatment decision-making as well as clinical value of osimertinib for various recurrence scenarios.

2 Materials and methods

The study utilized a modified Delphi method which included two rounds of surveys, followed by two consensus meetings and a final offline review by an expert panel. The key topics addressed in this study are listed in Table 1.

2.1 Panel selection

In this study, an international panel of experts was recruited with significant expertise in NSCLC as well as patient management with *EGFR*-TKIs or other systemic therapies (n=12). Experts who fulfil the following criteria were selected as panelists in this study: a physician specializing in NSCLC (medical oncologist or thoracic surgeon); based in a specialist lung cancer treatment and research center; significant years of experience in practice since completing residence/fellowship; over 60% of combined professional time dedicated to clinical practice and research activities related to NSCLC; regularly treating and managing patients across all stages of NSCLC (stage I-III); active advisor/member of a national or international society for lung cancer with participation in guideline creation for NSCLC in the last 5 years; has published on the topic of stage I-III NSCLC in international peer-reviewed journals within the last 5 years.

The steering committee consisted of two medical oncologists and one thoracic surgeon from different geographies (Asia, Europe, and the USA) to ensure geographic as well as different clinical expertise were incorporated in the development of materials.

TABLE 1 Key topics addressed in this study[i].

1	**General treatment decision influencers within NSCLC and recurrence scenarios**: Patient characteristics and history including age, smoking status, the initial stage of the disease, and the subsequent treatment methodology
2	**Patient monitoring approach within adjuvant setting:** Different techniques used and the monitoring frequency both during and after the completion of the adjuvant-osimertinib regimen
3	**Diagnostic approach upon recurrence suspicion:** Different diagnostic approaches and their potential impact on the ongoing adjuvant-osimertinib regimen
4	**Treatment and management approaches for CNS and ex-CNS recurrence:** Treatment decision-making process considering recurrence type, timing, and other clinically relevant factors[ii]
5	**Variation in patient management based on the recurrence scenarios:** Potential differences in the patient monitoring, diagnostic and/or treatment approaches for the following distinct recurrence scenarios: i. Ex-CNS or CNS recurrences during the adjuvant-osimertinib regimen that are amenable to local consolidative therapy[iii] ii. Ex-CNS or CNS recurrences post-adjuvant osimertinib regimen that are amenable to local consolidative therapy iii. Ex-CNS or CNS recurrences during the adjuvant-osimertinib regimen that are not amenable to local consolidative therapy iv. Ex-CNS or CNS recurrences post-adjuvant-osimertinib regimen that are not amenable to local consolidative therapy

[i]The table displays the key topics related to disease recurrence events during or after adjuvant osimertinib treatment for EGFRm NSCLC.
[ii]Clinical considerations of treatment sequencing decisions and potential geographic differences in the recommended treatment sequencing were also captured.
[iii]Local consolidative therapy includes surgery, ablation (percutaneous/endoscopic ablations including thermal/cryo ablation), radiotherapy, conformal radiotherapy, or stereotactic body radiation therapy (SBRT).

2.2 Delphi methodology and statement development

Both surveys were composed of a series of open and close ended question and shared via email. All materials tested in the study were co-developed and reviewed by the steering committee. In Survey 1, panelists were presented with six hypothetical patient case studies representing distinct real-world *EFGRm* NSCLC patients who recur during or after the treatment with adjuvant-osimertinib and were surveyed on their approach to patient monitoring, diagnostic workup, and treatment sequencing (see Supplementary Data 1 – Table 1 for hypothetical patient cases). Most questions were asked in an open-ended style to capture individual approach as well as clinical considerations. After analysis, topics that reached clinical consensus were reported back in Survey 2 as anonymized consolidated feedback and those that did not reach consensus were further probed using new clinical statements based on insights from Survey 1 (see Supplementary Data 2 for both survey 1 and 2). The insights gathered from Survey 2 were analyzed to find topics of clinical consensus. Statements or insights where clinical consensus was not achieved were brought forward to a series of consensus meetings (see Supplementary Data 1 – Table 2).

Two virtual consensus meetings were held where a final set of statements were discussed and amended live during the meeting. The level of consensus on the amended statements was probed through anonymous polls. The statement modification process was iterated until an overall clinical consensus (≥80% panelist agreement) was achieved for all six statements (see Supplementary Data 1 – Table 3 for the evolution of the survey statements pre- and post-consensus meeting). The final set of consensus statements were shared with all panelists for offline review and to capture their final level of agreement.

2.3 Defining consensus

Closed statements were ranked on the Likert scale of 1 to 9, where 1 equals "strongly disagree with the statement," and 9 equals "strongly agree with the statement,". Likert scale rating of 7 or higher for a given statement from a minimum of 80% of panelists was defined as a threshold for a consensus on the statement. In contrast, a rating of 3 or lower for a given statement from a minimum of 80% of panelists indicated consensus had been reached of disagreement with the statement.

2.4 Limiting bias

To limit bias, all surveys were conducted anonymously, and identity of experts was revealed only during the consensus meetings. Furthermore, results from rating exercises were provided as median scores and anonymous votes were held to finalize the consensus statements. In addition, offline review of the final statements was conducted individually without revealing the level of agreement from other panelists.

An independent third-party vendor, Charles River Associates (CRA) designed the survey, moderated the consensus meetings, analyzed the data and supported the manuscript development. The sponsor of the study (AstraZeneca Ltd.) did not participate in consensus meetings.

3 Results

Surveys 1 and 2 were used to gather a greater understanding of the factors influencing treatment sequencing within the adjuvant-osimertinib setting, including recurrence type and timing. A summary of the key insights gathered from Surveys 1 and 2 is shown in Table 2.

3.1 Survey 1

In Survey 1, six distinct hypothetical NSCLC patient case studies were used to understand how panelists approach management of different patient types and understand the potential differences under varying recurrence scenarios. Patients within the case studies had varying *EGFRm* mutations, ethnicities (Asian/non-Asian), age groups (40-65 years old), smoking status, past experience with adjuvant chemotherapies, recurrence either during or after adjuvant-osimertinib and details of the recurrence. All patients within the case studies were given a performance status (PS) score of 1.

Overall, panelists agreed that all the hypothetical patient cases are representative of real-world profiles and the use of adjuvant-osimertinib in these hypothetical patient cases is approved under the ADAURA label. Panelists also agreed on the monitoring and diagnostic approaches laid out, albeit frequency of monitoring and how molecular analysis is conducted remained unclear. Moreover, although some panelists mentioned the use of monitoring techniques such as minimal residual disease (MRD) tests and blood tests (e.g., CEA - carcinoembryonic antigen), it was unclear if these techniques would be used for all patients or in specific circumstances. Finally, there was a lack of agreement on the value of liquid biopsy within the diagnostic process. Overall, despite no diagnostic procedural differences between oligometastatic and disseminated recurrences being reported there remained a lack of clarity as to whether adjuvant-osimertinib regimen should be continued during diagnostic workup and up until a new treatment strategy is confirmed.

TABLE 2 Survey 1 and Survey 2 outcomes – Major insights with an overall panellists' agreement.

Survey 1 and 2 Outcomes			
Key topic	Insights		Agreement reached
Patient monitoring during or after adjuvant-osimertinib regimen	1	Patients monitoring during or after ADAURA regimen is conducted using CT scan and brain MRI in line with local guidelines.	Survey 1
	2	Additional patient monitoring techniques like MRD and blood tests (e.g., Carcinoembryonic antigen assay) can be used as add-on techniques but caution is recommended as currently there is no clinically validated MRD assay	Survey 2
	3	Tissue biopsy is performed for histological and molecular analysis to look for actionable targets (e.g., EGFR, PDL-1, others) in all feasible cases	Survey 1
	4	Liquid biopsies are performed when tissue biopsy is difficult or as a second diagnostic method. Factors such as patient or doctor preference for non-invasive procedure also influence the decision to perform liquid biopsies but they have secondary priority	Survey 2
	5	NGS or other molecular analysis procedures are always performed if progression is observed	Survey 2
	6	In case of recurrence, osimertinib is used during the entire diagnostic workup and up until a new treatment regimen is decided • Use of osimertinib can help avoid disease flare (as observed in metastatic setting) and decrease the risk of progression in the CNS • Osimertinib might be part of the new treatment regimen depending on the re-biopsy results	Survey 2
Treatment approach: ex-CNS and/or CNS recurrence (amenable to local consolidative therapy)	7	Treatment for oligometastatic distant recurrence includes local consolidative therapies – surgery, percutaneous/endoscopic ablations including thermal/cryo ablation, radiotherapy, conformal radiotherapy, or stereotactic body radiation therapy (SBRT)	Survey 1
	8	Treatment strategies for CNS recurrence include combination of local consolidative therapies (surgery or radiotherapy) with systemic therapies • Surgery will be performed only when anatomically feasible • Stereotactic radiosurgery (SRS) and whole-brain radiation therapy (WBRT) are the preferred radiotherapy options for oligometastatic and disseminated	Survey 1

(Continued)

TABLE 2 Continued

Survey 1 and 2 Outcomes			
Key topic	Insights		Agreement reached
		CNS recurrences, respectively • Continuation of osimertinib is a treatment option depending on the recurrence histology	
	9	In case of recurrence during the adjuvant osimertinib regimen that is amenable to local consolidative therapy, local consolidative therapy is performed in parallel to continuation of adjuvant osimertinib use, if possible	Survey 2
	10	In case of recurrence after the adjuvant osimertinib regimen is completed, local consolidative therapy is performed in parallel to rechallenge with osimertinib, if possible	Survey 2
Treatment approach: ex-CNS and/or CNS recurrence (not amenable to local consolidative therapy)	11	Treatment approach for disseminated distant recurrence involves systemic therapies based on the newly obtained histology. Systemic therapies mentioned include targeted drugs against the resistance mechanism (other TKIs), chemotherapy, immunotherapy, and combinational approach (e.g., chemo + TKI, chemo + immunotherapy)[i]	Survey 1
	12	In case of ex-CNS or CNS recurrence during the adjuvant osimertinib regimen and local consolidative therapy is not an option, systemic therapy based on the re-biopsy result is recommended with potential to include osimertinib depending on patient case	Survey 2
	13	In case of recurrence ex-CNS and/or CNS after completion of adjuvant osimertinib regimen, therapeutic approach will be based on re-biopsy results and in conjunction with the MDT with potential to include osimertinib depending on patient case	Survey 2

[i]Combination treatment approach with osimertinib and immunotherapy was flagged as unsuitable by KEEs and additional caution was recommended in case of sequential use.

Panelists agreed on treatment approaches for local and distant oligometastatic CNS/ex-CNS recurrence as well as disseminated CNS/ex-CNS recurrence. However, treatment sequencing decision for both ex-CNS and CNS recurrences and the associated driving factors were unclear. No consensus was observed on how patients treated with the preferred initial treatment (e.g., ablative therapy) are therapeutically followed up and to what extent osimertinib would be considered a therapeutic option.

3.2 Survey 2

Gaps identified in Survey 1 were explored in Survey 2, where agreement was reached on all outstanding aspects of diagnosis and monitoring. Panelists were presented with different scenarios to better understand the treatment sequencing drivers and how adjuvant-osimertinib would be considered in the overall treatment strategy. These scenarios focused on understanding the impact of recurrence location (CNS versus ex-CNS), amenability to local consolidative therapy, timing (during or after adjuvant-osimertinib regimen) as well as time points (3, 6, 18 months after initiation of adjuvant-osimertinib versus 3, 12 and 36 months after completion with adjuvant-osimertinib regimen).

While panelists agreed that continuing with adjuvant-osimertinib is clinically suitable during the diagnostic process and can be considered within treatment decision-making in the described CNS/ex-CNS recurrence scenarios, how its use would be decided remained unclear. Furthermore, there was no consensus on how long treatment with osimertinib would continue within recurrence scenarios and what considerations would influence this decision. Finally, some panelists suggested temporarily pausing the adjuvant-osimertinib regimen upon recurrence and resuming after ablative therapy, but the recommended pause duration and its applicability to all ablative therapy procedures are not clear. For recurrences after completing the adjuvant-osimertinib regimen, a combination of ablative therapy and osimertinib rechallenge was proposed, but it remains unclear whether the rechallenge would be performed alongside ablative therapy or after its completion.

3.3 Consensus meeting

Gaps in key treatment decision factors and the value of osimertinib across tested recurrence scenarios were addressed during 2 virtual consensus meetings, where experts amended wording of original proposed statements in line with best clinical practice and experience given limited randomized evidence. A summary of the statements that reached a consensus is shown in Table 3.

4 Discussion

A lack of clinical guidance on how to treat recurrences during or after the completion of the adjuvant-osimertinib regimen has been raised in recent publications (11, 12). Our Delphi consensus study surveyed an international panel of experts on patient-specific as well as recurrence scenario-specific considerations when making treatment decisions in the absence of extensive clinical data and formalized guidelines.

Our study approach enables experts to share their own opinions based on clinical experience, and, where relevant, knowledge of the ADAURA phase 3 data, with the aim of fostering the integration of these viewpoints. Overall, our research shows a high consensus

TABLE 3 Final consensus statements with an overall panelist agreement.

Recurrence scenarios		Final Consensus Statement	Panelist Agreement
ex-CNS or CNS recurrence amenable to local consolidative therapy	1	If a patient experiences an ex-CNS or CNS recurrence amenable to local consolidative therapy during the adjuvant-osimertinib regimen, in absence of existing evidence, I would use the adjuvant-osimertinib regimen in parallel with local consolidative therapy, considering the risk of toxicities, site of recurrence, method of local consolidative therapy and after discussion with the MDT	**Consensus reached** 11 out of 12 panelists agreed
	2	If a patient experiences an ex-CNS or CNS recurrence amenable to local consolidative therapy after completion of the adjuvant-osimertinib regimen, I would consider rechallenging with osimertinib as an option after local consolidative therapy, after discussion with MDT	**Consensus reached** 10 out of 12 panelists agreed
ex-CNS and/or CNS recurrence during adjuvant-osimertinib regimen not amenable to local consolidative therapy	3	If a patient experiences CNS-only recurrence not amenable to local consolidative therapy during the adjuvant-osimertinib regimen, I would consider continuing with osimertinib as a part of the overall treatment strategy when a patient has asymptomatic progression, if fits with the best clinical practice	**Consensus reached** 10 out of 12 panelists agreed
	4	In the absence of randomized evidence, if a patient experiences ex-CNS recurrence not amenable to local consolidative therapy during the adjuvant-osimertinib regimen, I would consider continuation with osimertinib as a part of the overall treatment strategy if a patient has asymptomatic progression and if fits with the current guideline recommendations	**Consensus reached** 10 out of 12 panelists agreed
ex-CNS and/or CNS recurrence post-adjuvant-osimertinib regimen not amenable to local consolidative therapy	5	If a patient experiences an ex-CNS and/or CNS recurrence after completion of the adjuvant-osimertinib regimen, and local consolidative therapy is not an option, I would rechallenge with monotherapy osimertinib or osimertinib as a part of the overall treatment strategy irrespective of when the recurrence happens after treatment completion, if fits with the best clinical practice and is supported by re-biopsy in all feasible cases	**Consensus reached** 12 out of 12 panelists agreed
All ex-CNS and/or CNS recurrence types	6	If a patient experiences an ex-CNS and/or CNS recurrence during or after the completion of the adjuvant-osimertinib regimen and I choose to continue with osimertinib as part of the overall treatment strategy, I would continue the osimertinib therapy until the clinical situation mandates a change or stop in therapy	**Consensus reached** 11 out of 12 panelists agreed

regarding patient considerations and approaches to various recurrence scenarios. Statements and considerations that garnered consensus can serve as valuable guidance in clinical practice when combined with a tailored patient approach and recommendations from multidisciplinary teams.

4.1 Key considerations for adjuvant-osimertinib and *EGFRm* NSCLC patient monitoring & diagnostic work-up

Our results show that the decision to use adjuvant-osimertinib is not driven by patient's gender, ethnicity, age, smoking or alcohol use status, or previous treatment experience with adjuvant-chemotherapy. However, the decision to use adjuvant-osimertinib might change if a patient has poor PS score (PS >1), has high comorbidities or is likely to be less compliant with the dosing regimen.

Furthermore, patients receiving adjuvant-osimertinib should be monitored using local guidelines which broadly align with international guidelines such as ESMO (Table 2 – statement 1).

In our study, tissue biopsy is recommended in all feasible cases in case of progression to assess the suitability of different treatment options based on tumor biomarker/mutation profile. The diagnostic result from liquid biopsy was highlighted to be less sensitive and contingent on site and burden of the recurrence (Table 2 – statements 3-5). Therefore, only when tissue biopsy is not possible should liquid biopsy be used as it is unable to capture histologic transformation and has limited sensitivity from amplifications and fusions (12). Moreover, liquid biopsies may give false negative or positive results, especially in cases of low volume disease with lower than threshold circulating ctDNA (13). These recommendations are echoed in the ESMO expert consensus on the management of *EGFRm* NSCLC (12). The panel's consensus is to continue use of adjuvant-osimertinib during the diagnostic workup and up until a new treatment regimen is decided for patients who present with recurrence during the adjuvant-osimertinib regimen. The continued use of osimertinib can help avoid disease flare, a well-established scenario for metastatic disease, and to additionally protect against CNS progression (Table 2 – statement 6) (9, 14, 15).

4.2 Key considerations in treatment sequencing for adjuvant-osimertinib recurrence scenarios

Across all tested scenarios, treatment decisions are taken on a patient-by-patient basis and taking input from the multi-disciplinary team (MDT), aligning with major guidelines (16–21). The following key clinical criteria should be considered during the treatment sequencing decision process (Table 2 – statements 7-13):

- Pattern and location – Number of lesions, size and location determine if the recurrence is oligometastatic and amenable to local consolidative therapy or disseminated and requires

treatment with a systemic therapy. Furthermore, limited metastases which may include lesions in contralateral lung, lymph node, CNS or other organs could be managed with a combination of local consolidative therapy and a systemic therapy. Panelists did not rule out the treatment value of osimertinib in various recurrence scenarios solely based on pattern and location.

- Timing of recurrence – whether and when recurrence occurs during or after the adjuvant-osimertinib regimen can indicate if the recurrent lesion is sensitive or resistant to osimertinib in addition to genetic profiling. Recurrence during the adjuvant-osimertinib regimen (especially ex-CNS) might be a sign of acquired resistance to osimertinib, while recurrence after the completion of the regimen might indicate a disease flare post-treatment cessation.
- Molecular characterization of the recurrence – Confirming molecular histology or biomarker profile informs the presence of any actionable target and is necessary when considering further treatment with osimertinib.

It is important to note here that while panelists acknowledge osimertinib to be a valuable treatment option for patients with complex clinical presentations, including the emergence of distinct resistance mechanisms (e.g., MET amplification, PIK3CA mutation, BRAF mutation) and/or progression to acquire other genetic alterations (e.g., ALK mutation), these specific recurrence scenarios were not explored in this study.

4.2.1 Therapeutic value of osimertinib: ex-CNS or CNS recurrences during the adjuvant-osimertinib regimen that are amenable to local consolidative therapy

For ex-CNS or CNS recurrences which occur during the adjuvant-osimertinib regimen and are amenable to local consolidative therapy (surgery, ablation, or radiotherapy), the panel recommended to continue adjuvant-osimertinib treatment along with local consolidative therapy if found clinically suitable (Table 3 – consensus statement 1). A consensus was achieved in pausing osimertinib regimen during radiotherapy due to severe toxicity risk in case of combined use, however, length of pause was not specified. Pausing the use of therapies such as osimertinib during radiotherapy was also recently highlighted in published consensus recommendations by the EORTC-ESTRO OligoCare consortium, where a consensus was reached to not perform SBRT within one week of the administration of anti-EGFR antibody (22).

However, in the absence of safety data in combining local consolidative therapy with osimertinib treatment, recommendations on pausing or continuing with osimertinib during ablative or surgical procedures could not be reached and instead in the absence of treatment guidelines, the decision to pause osimertinib should be based on treatment experience from the metastatic setting, i.e., considering the extent of recurrence including location, size and number of the lesions (note that the treatment guidelines for metastatic settings are also not yet established). Additionally, caution was recommended when patients are given other drugs (e.g., prophylactic antibiotics before local consolidative therapy) that could show drug-drug interactions with osimertinib.

4.2.2 Therapeutic value of osimertinib: ex-CNS or CNS recurrences post-adjuvant osimertinib regimen that are amenable or not amendable to local consolidative therapy

While the place of osimertinib within the treatment strategy is dependent on multiple factors (see Section 4.2), the treatment value of rechallenging with osimertinib was thought to increase as duration between time to recurrence and completion of the adjuvant-osimertinib regimen increases. However, no consensus was achieved on the minimum recurrence free time (3, 12 or 36 months) to consider osimertinib for rechallenge.

In cases of post-adjuvant osimertinib regimen recurrence scenarios that are amenable to local consolidative therapy and rechallenging with osimertinib is suitable, the consensus is to rechallenge with osimertinib after the completion of the local consolidative therapy (Table 3 – consensus statement 2), especially in the case of radiotherapy where parallel use of osimertinib is not recommended due to the risk of toxicities. However, it is still to be determined whether stand-alone local consolidative therapy is sufficient and has curative potential in some patient cases or whether it should always be followed with osimertinib rechallenge to prevent potential distant metastasis.

In case of post-adjuvant osimertinib regimen recurrence scenarios that are not amenable to local consolidative therapy, rechallenge with osimertinib may be of high clinical value and should therefore be considered either as osimertinib monotherapy or as a part of treatment strategy involving other therapies (Table 3 – consensus statement 5).

4.2.3 Therapeutic value of osimertinib: ex-CNS or CNS recurrences during the adjuvant-osimertinib regimen that are not amenable to local consolidative therapy

Our study found that ex-CNS recurrence in the first 6 months is an indication of treatment failure of adjuvant-osimertinib, requiring change of treatment to either chemotherapy, other targeted therapies, or a combination of both. However, for later recurrences, continuation of osimertinib as a part of a broad treatment strategy could be potentially valuable (Table 3 – consensus statement 4) as it may prevent brain metastases, especially in case of ex-CNS recurrence.

For CNS-only recurrences, the consensus is that continuation of osimertinib as a part of a broad treatment strategy could be an effective treatment option (Table 3 – consensus statement 3). For indolent CNS recurrences, continuation of osimertinib monotherapy could also be a valuable treatment option, given the CNS recurrence could stem from underexposure to osimertinib. However, further evidence is needed on the appropriate osimertinib dosing regimen and/or combination with other therapies before these treatment approaches are considered outside clinical trials. The use of osimertinib for both ex-CNS and CNS-only recurrence was identified to be more suitable for patients with asymptomatic progression which indicates that the tumor growth is gradual and the risk of resistance to osimertinib is relatively lower than within symptomatic progression (Table 3 – consensus statements 3 & 4).

4.2.4 Osimertinib treatment duration for all indicated CNS and/or ex-CNS recurrences

In cases where osimertinib was considered as a treatment option within the recurrence scenarios, the consensus was that treatment would continue until disease progression or toxicities were observed, or patient quality of life deteriorated. For a low progression risk patient (e.g., indolent oligometastatic progression), the duration of osimertinib treatment should be limited and a decision to stop treatment should be taken after monitoring the efficacy and patient's health profile and considering the safety and tolerance profile of osimertinib and patient wishes (Table 3 – consensus statement 6). For a high progression risk patient, the treatment duration would be longer compared to a low progression risk patient with an aim to avoid any residual disease flare after osimertinib treatment cessation.

5 Conclusion

Outcomes from the phase 3 ADAURA trial show significant improvements in DFS and OS for EGFR-mutated NSCLC patients with stage IB to IIIA disease. Nonetheless, patterns of recurrence were reported within the osimertinib group, although lower compared with placebo (9, 10). In the absence of clinical guidance on how to treat recurrences during or after the completion of the adjuvant-osimertinib regimen, our consensus study offers a qualitative framework for clinicians in such scenarios, drawing from international expert consensus. While recognizing the importance of additional clinical data from trials and real-world settings, the study provides broader treatment considerations. It also considers osimertinib's efficacy, as supported by FLAURA, AURA3, and ADAURA trials (7, 9, 23), with panelists acknowledging its potential benefits across various recurrence scenarios, including oligometastatic CNS recurrences and CNS metastases. In addition, panelists acknowledged the potential treatment value of combination therapies using osimertinib with local consolidative therapy, chemotherapy, and other systemic therapies; however, further efficacy and safety data is needed. The suitability of each combination approach under different recurrence scenarios was not tested in this study indicating the need for more discussion on clinical experience in the absence of concrete data.

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.

Author contributions

MM: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. AS: Conceptualization, Formal Analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. CM: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. NL: Writing – review & editing. CN: Writing – review & editing. DP: Writing – review & editing. SP: Writing – review & editing. JR: Writing – review & editing. ES: Writing – review & editing. RS: Writing – review & editing. MT: Writing – review & editing. FY: Writing – review & editing. BS: Supervision, Writing – review & editing. CG: Supervision, Writing – review & editing. Y-LW: Supervision, Writing – review & editing.

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Conflict of interest

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2023.1330468/full#supplementary-material

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Case report: Target and immunotherapy of a lung adenocarcinoma with enteric differentiation, *EGFR* mutation, and high microsatellite instability

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Background: Pulmonary enteric adenocarcinoma (PEAC) is a rare histological subtype of non-small-cell lung cancer (NSCLC) with a predominant (>50%) enteric differentiation component. The frequency of high microsatellite instability (MSI-H) is very low in lung cancer. EGFR tyrosine kinase inhibitors and immunotherapy are standard treatment for NSCLC patients, but their effectiveness in lung adenocarcinoma with pulmonary enteric differentiation is unknown.

Case presentation: This report describes a 66-year-old man who was initially diagnosed with metastatic lung adenocarcinoma with *EGFR* mutation based on pleural fluid. A lung biopsy was obtained after 17 months of first-line icotinib treatment. Histological analysis of biopsy samples and endoscopic examination resulted in a diagnosis of adenocarcinoma with enteric differentiation. Next-generation sequencing of 1,021 genes showed *EGFR* E19del, T790M, and MSI-H, while immunohistochemical assay showed proficient expression of mismatch repair (MMR) proteins. Consequently, the patient was treated with osimertinib and had a progression-free survival (PFS) of 3 months. His treatment was changed to chemotherapy with/without bevacizumab for 6.5 months. Then, the patient was treated with one cycle of camrelizumab monotherapy and camrelizumab plus chemotherapy, respectively. The tumor continued to grow, and the patient suffered pneumonia, pulmonary fungal infections, and increased hemoptysis. He received gefitinib and everolimus and died 2 months later and had an overall survival of 30 months.

Conclusion: In summary, our case describes a rare pulmonary enteric adenocarcinoma with an *EGFR*-activating mutation and MSI-H, responding to an EGFR tyrosine kinase inhibitor and poorly benefiting from an immune checkpoint inhibitor.

KEYWORDS

pulmonary enteric adenocarcinoma, *EGFR*, MSI-H, immune checkpoint inhibitor, pMMR

Introduction

Pulmonary enteric adenocarcinoma (PEAC) is a rare variant of lung adenocarcinoma. According to the 2015 World Health Organization (WHO) classification, PEAC has been defined as primary pulmonary adenocarcinoma with more than 50% of intestinal differentiation components, and the tumor cells should be positive for at least one immunohistochemical marker of enteric differentiation, including CK20, CDX2, and MUC2 (1). The pathogenesis of PEAC and specific treatment plans have not been fully determined. At present, the treatment strategy for PEAC is similar to that of lung adenocarcinoma. The strategy methods derived from the literature for PEAC are mainly surgery and chemotherapy. Although several case reports have described immunotherapies in patients with PEAC, few have reported targeted therapy (2).

KRAS and DNA mismatch repair (MMR) genes are more frequently mutated in PEAC compared with those in other lung adenocarcinomas (3, 4). The high frequency of MMR mutation rates may facilitate the possibility of checkpoint-blocking immunotherapy for PEAC patients. The positive rate of *EGFR* mutations is approximately 16.7%, much lower than that of *KRAS* (3, 4). Few reports to date have described the effects of treatment with sequential *EGFR* tyrosine kinase inhibitors in PEAC. The present report describes the targets and immunotherapy of a lung adenocarcinoma with enteric differentiation, *EGFR*-activating mutation, and high microsatellite instability (MSI-H).

Case report

In February 2019, a 66-year-old man visited the First Affiliated Hospital of Guangxi Medical University due to a 2-month history of cough, sputum, and shortness of breath. The chest computed tomography (CT) scan showed a suspicious central right lung mass, with hilum of right lung, bilateral lung, and mediastinal lymph node metastasis; right-sided pleural effusion; and a small amount of pericardial effusion and atelectasis of the middle and lower lobes of the right lung (Figure 1A). The patient was a former heavy smoker with a smoking index of 20 packs/year. Cytological examination of pleural fluid revealed the diagnosis of malignant pleural effusion with the tumor cells positive staining for NapsinA and thyroid transcription factor-1 (TTF-1; Figures 2A–C). The patient was diagnosed with a metastatic lung adenocarcinoma (cT4N2M1, stage IV).

The patient immediately received one cycle of pemetrexed disodium (500 mg/m^2) plus carboplatin (AUC 5) after diagnosis while waiting for the results of next-generation sequencing (NGS). Molecular analysis of pleural fluid by NGS based on a nine-gene panel was performed and showed the common *EGFR* exon 19

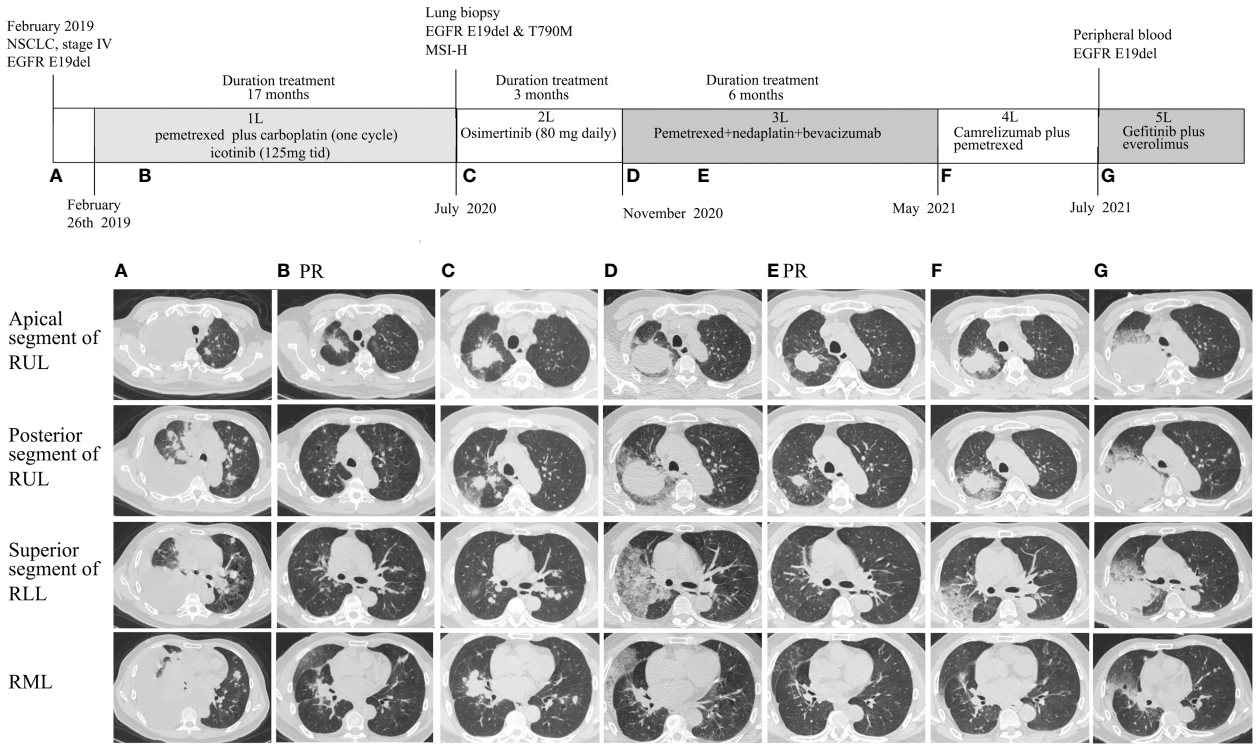

FIGURE 1
Radiographic evaluations of main lesions during the disease course. **(A)** Computed tomography (CT) scan at diagnosis. **(B)** CT scan showed lesions shrank after 1 month of first-line icotinib; **(C)** CT scan after resistance to icotinib. **(D)** CT scan after 3 months of osimertinib treatment; **(E)** CT scan before the second cycle of nedaplatin–pemetrexed–bevacizumab showing partial response; **(F)** CT after 6 months of third-line therapy showing disease progression; **(G)** CT scan after 2 months of immunotherapy. RUL, right upper lobe; RLL, right lower lobe; RML, right middle lobe; PR, partial response.

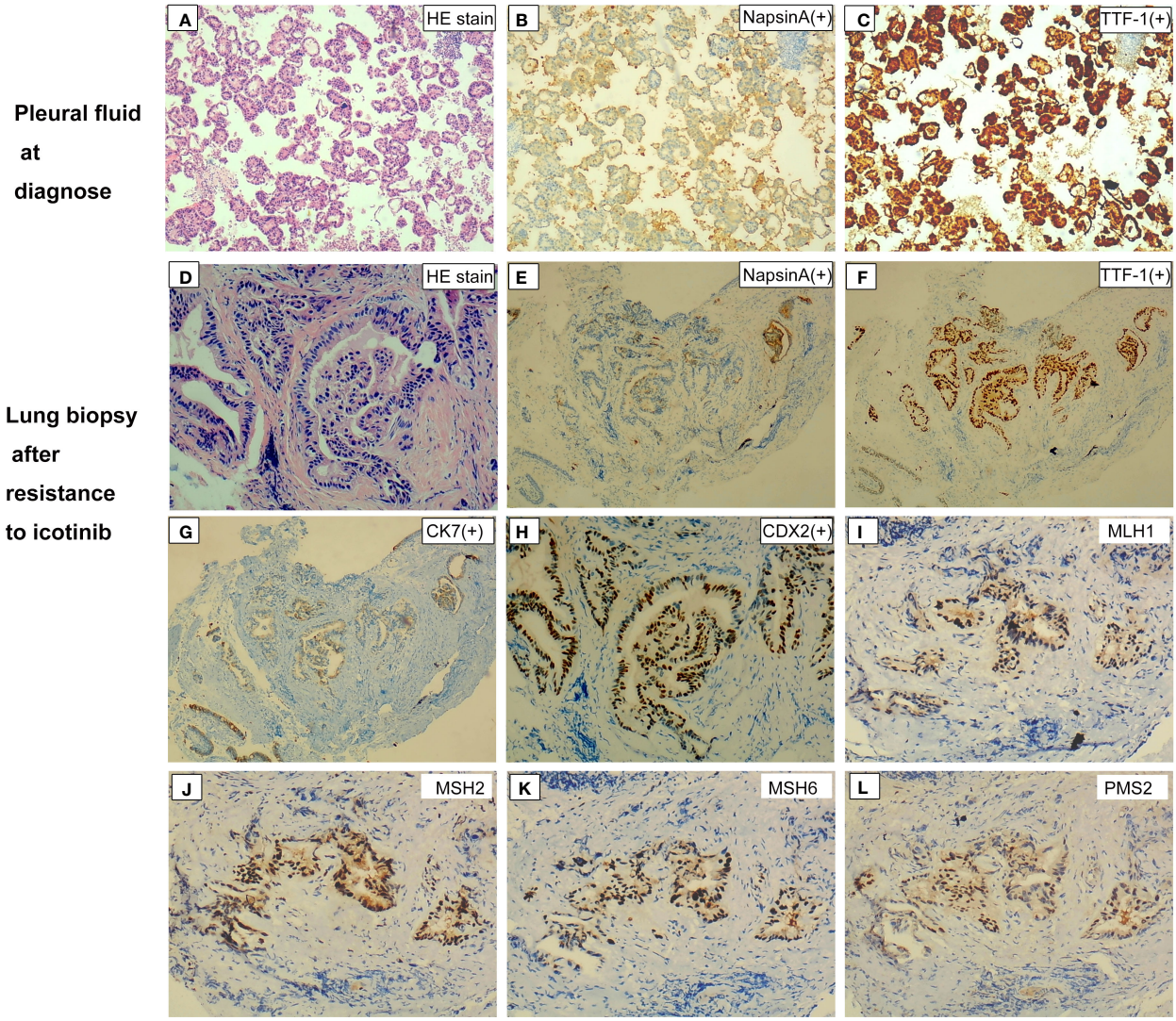

FIGURE 2
Hematoxylin and eosin staining and immunohistochemical findings of samples at different disease courses. **(A)** Circulating tumor cells in pleural fluid on hematoxylin and eosin staining at diagnosis. **(B, C)** Immunohistochemical staining was positive for NapsinA **(B)** and TTF-1 **(C)**. **(D)** Hematoxylin and eosin staining of lung biopsy after resistance to first-line icotinib showed cylindrical morphology and formed glandular tubular structures. **(E–H)** Immunohistochemical staining was positive for NapsinA **(E)**, TTF-1 **(F)**, CK7 **(G)**, and CK20 **(H)**. **(I–L)** Immunohistochemical staining was positive for four MMR proteins. Magnification ×40 **(A–C, E–G, I–L)**; magnification ×200 **(D, H)**. NapsinA, novel aspartic proteinase of the pepsin family A; TTF-1, thyroid transcription factor-1; CK, cytokeratin; CDX2, caudal-type homeobox transcription factor 2; MMR, mismatch repair.

mutation (p.E746_A750del). The patient then received continuous oral icotinib (125 mg tid). After 1 month, CT showed a partial response (PR; Figure 1B). The clinical and morphological response was confirmed after 6 months since icotinib treatment. The patient continued icotinib beyond slow progression after 17 months of icotinib. The tumor continued to grow slowly over 3 months (Figure 1C), and a lung biopsy was obtained. The lung biopsy showed enteric differentiation components, which exceeded 50% (Figure 2D). Unfortunately, cell block at diagnosis was not available for further examination. Abdominal CT and colonoscopy examination revealed no evidence of gastrointestinal disease. Immunohistochemical (IHC) assays showed that the tumor was positive for TTF-1, NapsinA, CK7, and CDX2 (Figures 2D–H) and negative for programmed death ligand 1 (PD-L1; SP263 antibody), resulting in a diagnosis of an intestinal-type adenocarcinoma of the lung. IHC analysis for DNA mismatch repair proteins (MLH1, PMS2, MSH2, MSH6) revealed that the patient had proficient DNA mismatch repair (pMMR) positive for all microsatellite markers (Figures 2I–L). To identify actionable mutations, the tumor biopsy specimen was sequenced by capture-based next-generation DNA sequencing with a panel containing 1,021 cancer-related genes [(5) Beijing Geneplus Technology Co., Ltd.]. The mean effective depth of coverage of the sequence was 800×. A total of 35 somatic mutations were identified, including *EGFR* E19del, an acquired *EGFR* T790M mutation, and *MLH1* (c.332C>T, p.A111V mutation) with the highest variant allele fraction (VAF) of 45.2%, MSI-H, and high tumor mutation burden (TMB-H; 29.76 Muts/Mb) (Supplementary Table 1). No germline mutation in the coding

region of *MSH2*, *MSH6*, *MLH1*, or *PMS2* was identified. The patient received osimertinib (80 mg daily), which allowed an improvement for 3 months until reassessment CT revealed pulmonary progression (Figure 1D), accompanied with increased hemoptysis and cough. From November 2020 to April 2021, he received two cycles of nedaplatin (180 mg)–pemetrexed (900 mg)–bevacizumab (600 mg) therapy, then four cycles of combination therapy with nedaplatin (180 mg) plus pemetrexed (900 mg), and one cycle of pemetrexed (900 mg) monotherapy. Pulmonary CT scans showed PR after first cycle of nedaplatin–pemetrexed–bevacizumab treatment and disease progression in May 2021 [progression-free survival (PFS) 6.5 months, Figures 1E, F]. Then, the patient received camrelizumab (anti-PD1, 200 mg) monotherapy (PFS 1 month) and camrelizumab (200 mg, q 3 weeks) plus pemetrexed (900 mg) therapy (Figure 1G). The patient's condition worsened with multiple complications, including hemoptysis, pneumonia, and pulmonary fungal infections. He received supporting treatment and underwent another next-generation DNA sequencing test, which included 73 cancer-related genes (Beijing Geneplus Technology Co., Ltd.) with peripheral blood. The NGS results of plasma circulating tumor DNA showed loss of the T790M mutation with sustained presence of the *EGFR* exon 19 mutation (4.6% abundance, Supplementary Table 1). The patient started to receive gefitinib (250 mg/day) and everolimus (10 mg/day) therapy. He died 2 months later and had an overall survival of 30 months.

Descriptions of adjuvant therapies or systemic therapies are available in 34 PEAC patients derived from the literature and the case report reported above. The treatment regimens are summarized in Supplementary Table 2 (6–19). Two cases with early-stage diseases received adjuvant chemotherapy with a regimen of unspecified drugs. As for patients with advanced disease, 22/34 received lung cancer-oriented chemotherapy, 2/34 received immunotherapy plus platinum-containing chemotherapy, 1/34 received icotinib without *EGFR* mutation and then nivolumab monotherapy, and 6/34 patients received colorectal cancer-oriented chemotherapy.

Discussion

PEAC is a rare subtype of non-small-cell lung cancer (NSCLC), which was first reported by Tsao and Fraser as a case in 1991 (20). To date, over 200 cases have been reported (21). PEAC was officially defined as a rare variant of invasive lung adenocarcinoma by the WHO classification in 2015, with more than 50% of intestinal differentiation components, and the tumor cells should be positive for at least one immunohistochemical marker of enteric differentiation (1). The present case was first diagnosed as lung adenocarcinoma containing an *EGFR* mutation using tumor cells from pleural fluid. Then, the patient was diagnosed with adenocarcinoma with intestinal differentiation components from lung biopsy after resistance to first-line icotinib treatment. It may be difficult to diagnose PEAC using circulating tumor cells or a small sample of lung biopsy. Given the fact that PEAC is a mixed histological subtype of lung adenocarcinoma, the molecular differences between intestinal differentiation components and other non-intestinal differentiation components remain unknown. *EGFR* in-frame deletion of exon 19 (E19del) was reported in pulmonary enteric adenocarcinoma (1/10) (22). *EGFR* mutation may present in ordinary lung adenocarcinoma components, which decreased with the use of icotinib, while the intestinal differentiation components were insensitive to icotinib and increased during treatment. More molecular studies about the origin and intratumor heterogeneity of PEAC are needed in the future.

Owing to the rarity of disease, the molecular characteristics of PEAC have not been comprehensively determined. Several studies have described the genomic landscape of PEAC. High *KRAS* and *MMR* gene mutation rates and a low *EGFR* mutation rate were observed in PEAC (3, 4). The present case had a common *EGFR* E19del mutation and had a PFS of 17 months on first-line icotinib treatment. Genomic sequencing of tumor biopsy after icotinib resistance revealed *EGFR* T790M mutation. The patient received a third-generation EGFR tyrosine kinase inhibitor (TKI), osimertinib, and achieved a PFS of 3 months. This study is the first to describe *EGFR* tyrosine kinase inhibitors in a PEAC patient with an *EGFR*-sensitive mutation. The first- and second-line target therapies indicated that a PEAC patient with an *EGFR*-activating mutation could also benefit from *EGFR*-TKI, and an NGS test after first-line TKI resistance could guide subsequent therapy.

At present, the treatment plan of PEAC has not been fully studied in previous literature. The present treatment strategy for advanced PEAC is the same as that of primary lung adenocarcinoma, including chemotherapy and radiotherapy and/or targeted therapy. Immunotherapy might be a useful treatment option for PEAC because of its high frequency of MMR mutation (21). According to IMpower150, the atezolizumab (anti-PD-L1) plus bevacizumab plus chemotherapy regimen showed a progression-free and overall survival benefit when compared with the standard-of-care bevacizumab plus chemotherapy regimen in EGFRm-TKI progressed patients (23). The ORIENT-31 trial consistently demonstrated that combination therapy of sintilimab plus chemotherapy with or without bevacizumab biosimilar IBI305 significantly improved PFS compared with chemotherapy alone in EGFRm-TKI progressed patients (24). To date, only two case studies have demonstrated checkpoint inhibitor therapy in PEAC and exhibited controversial results. A recently published case showed that a metastatic PEAC patient with a *KRAS* G12C mutation suffered hyperprogressive disease after one cycle of first-line paclitaxel plus carboplatin, along with sindilizumab (25). However, another recently published study demonstrated that primary and metastatic lesions were effectively treated by pembrolizumab plus carboplatin and pemetrexed in a PEAC with a *KRAS* G12D mutation (26). The discrepancy of clinical benefits between the two PEAC cases receiving first-line chemoimmunotherapy might be explained by the different NGS panels used and metastatic status, and the latter case received palliative radiation for bone metastases. The effectiveness of combination therapy with a checkpoint inhibitor and chemotherapy in PEAC remained uncertain.

Our patient only received a bevacizumab plus chemotherapy regimen, without combination with a checkpoint inhibitor after progressing on treatment with icotinib and osimertinib. Then, the

patient changed to ICI monotherapy or chemoimmunotherapy after progressing on treatment with bevacizumab plus chemotherapy, but he benefited poorly from those treatments. The presence of an *EGFR* mutation and *PTEN* and *JAK1* truncation mutations were negative predictors of immunotherapy (27–29) and might account for the treatment failure of the case. Accumulation of clinical experience in immunotherapy is necessary for better treatment of this rare lung cancer. Our case indicates poor benefit from immunotherapy for PEAC with an EGFR-sensitive mutation and MSI-H in later-line settings.

As we know, MSI-H is a more common molecular feature observed in colorectal and endometrial cancer compared with other solid tumors, while few studies concerned MSI status in lung cancer. Patients with MSI-H are more likely to benefit from immunotherapy across cancers (30). Polymerase chain reaction (PCR)-, IHC-, and NGS-based MSI analyses were commonly used in most clinical laboratories. However, it has been reported that approximately 5% of colorectal cancers that display retention of all four MMR proteins may indeed be MSI-H, possibly due to the heterogeneous expression of MMR proteins, or proteins emanating from abrogated MMR genes were still detected by IHC (31). The *MLH1* c.332C>T mutation, a germline pathogenic mutation reported in colorectal cancers (32), was detected in the lung biopsy of our case with the highest VAF. Missense mutations of MMR genes in formalin-fixed paraffin-embedded (FFPE) tumor tissues were also detected in colorectal cancer cases with pMMR and MSI-H (31). In lung cancer, 0.17% (2/1,153) and 0.5% (66/12,484) patients were reported to be MSI-H via IHC and the NGS-based method, respectively (33, 34). Recent studies showed all tumor tissue samples were microsatellite stable (MSS) in PEAC according to PCR- (17 cases) or IHC-based (8 cases) MSI analysis. The present case was a rare lung adenocarcinoma with enteric differentiation, pMMR, and MSI-H. Further studies in cases with somatic MMR gene mutations and MSI-H may help elucidate the phenomena.

In summary, this is the first case to describe an *EGFR*-mutated lung adenocarcinoma that had enteric differentiation components, *EGFR* T790M, and MSI-H after resistance to first-line icotinib and responded poorly to osimertinib and immunotherapy. Few reports to date have described the sequential treatment of PEAC with EGFR-TKIs and immunotherapy. The findings observed in this patient, including diagnoses, treatments, and the association between clinical outcomes and driver genes, may lead to future studies on the origin, diagnosis, and treatment of patients with PEAC.

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 humans were approved by Ethics Committee of First Affiliated Hospital of Guangxi Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The 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

MY: Data curation, Investigation, Writing – original draft, Writing – review & editing. PY: Investigation, Writing – original draft, Writing – review & editing. ZH: Conceptualization, Writing – review & editing. JD: Conceptualization, Investigation, Writing – review & editing.

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Conflict of interest

Author PY is employed by the company Geneplus, Beijing.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1266304/full#supplementary-material

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The efficacy of furmonertinib in untreated advanced NSCLC patients with sensitive EGFR mutations in a real-world setting: a single institutional experience

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Background: Furmonertinib is the standard treatment option in the first-line setting for advanced non-small cell lung cancer (NSCLC) with sensitive epidermal growth factor receptor (EGFR) mutations in China. However, there are limited real-world data available.

Methods: We conducted a retrospective study at a single center, analyzing a cohort of 73 NSCLC patients who tested positive for EGFR mutations and were treated with furmonertinib as their initial therapy between August 2022 and December 2023. The primary endpoint was progression-free survival (PFS), with secondary endpoints including objective response rate (ORR), overall survival (OS), and safety profile.

Results: The median observation period was 9 months (95% confidence interval [CI], 8.0–20.0). The median PFS was 19.5 months (95% CI, 14.6–24.4). OS data were not yet mature. Univariate analysis showed no significant correlation between PFS and factors such as Eastern Cooperative Oncology Group performance status (ECOG PS) score, presence of brain or liver metastases, sex, age, EGFR mutation status, or number of metastatic sites. However, multivariate analysis indicated a potential trend toward extended PFS in patients younger than 65 years (p = 0.053, 95% CI, 0.10–1.02), although the p-value was only marginally significant. The most common adverse events were diarrhea (24%), anemia (36%), and liver injury (32%); however, only four cases experienced severe adverse events.

Conclusion: In a real-world setting, furmonertinib appears to be a favorable treatment option for EGFR-mutated patients. The manageable nature of adverse events further supports its use in clinical practice.

KEYWORDS

furmonertinib, non-small cell lung cancer, EGFR-mutated, epidermal growth factor receptor, real-world setting

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, with a mortality rate of 18% (1). In China, lung cancer is also the most common cancer in terms of incidence and is the leading cause of cancer-related mortality (2). Among the different subtypes of lung cancer, non-small cell lung cancer (NSCLC) is the most frequently diagnosed histological subtype at initial diagnosis, with adenocarcinoma being the most common subtype. Approximately 60% of NSCLC patients with adenocarcinoma harbor oncogenic driver mutations, with the epidermal growth factor receptor (EGFR) mutation being the most commonly found and targetable driver mutation in NSCLC (3).

Current standard treatment for NSCLC patients with EGFR-sensitive mutations, such as exon 19 deletion (19DEL) and substitution of lysine with arginine in exon 21 (21L858R), involves the use of EGFR–tyrosine kinase inhibitors (TKIs). These TKIs have been shown to prolong the survival of EGFR-mutated NSCLC patients (4–10). Currently, there are three generations of EGFR-TKIs approved for use in EGFR-mutated NSCLC. In China, a total of eight agents targeting EGFR mutations are approved. First-generation EGFR-TKIs have demonstrated an objective response rate (ORR) of approximately 60%–80% and a median progression-free survival (mPFS) of 8–13 months (4, 5, 10, 11). Second-generation agents targeting EGFR mutations have shown an extended mPFS of 11–16.5 months (6, 12). The third generation of EGFR-TKIs, including osimertinib, almonertinib, and furmonertinib, have shown even better efficacy with an mPFS of 18.9–20.8 months (7–9). Due to their improved efficacy and ability to penetrate the brain, third-generation agents are now recommended as the preferred treatment option for EGFR-mutated NSCLC.

Furmonertinib, a third-generation EGFR-TKI, is an original drug developed by a Chinese pharmaceutical company. Furmonertinib is a potent irreversible inhibitor of EGFR that specifically targets mutations in the receptor. It is effective against two types of mutations: resistance mutations (T790M) and activating mutations (L858R and exon 19 deletions). Furmonertinib is more effective at inhibiting tumor cells with these mutations compared to cells with the normal, wild-type EGFR. In the FURLONG study, a randomized Phase III trial that included previously untreated advanced NSCLC patients with EGFR-sensitive mutations, furmonertinib demonstrated superior PFS compared to gefitinib (20.8 versus 11.1 months, hazard ratio [HR] 0.44, 95% confidence interval [CI], 0.34–0.58, $p < 0.0001$) (9). The PFS achieved with furmonertinib was numerically longer than that observed with other third-generation EGFR-TKIs such as osimertinib and almonertinib, making it the longest achieved PFS to date. It is worth noting that the FURLONG study was conducted in China, exclusively including Chinese NSCLC patients. This suggests that furmonertinib may be particularly well-suited for Chinese NSCLC patients with EGFR mutations. However, the superiority of furmonertinib in terms of overall survival (OS) remains unreported due to the immaturity of OS data. Thus, real-world evidence regarding furmonertinib as a first-line treatment option for EGFR-mutated NSCLC patients, as well as clinically measurable prognostic factors, remains limited.

The objective of this report is to explore real-world data on the efficacy and safety of furmonertinib as a first-line treatment option in routine clinical practice.

Patients and methods

Study design

This retrospective study was conducted at a single center with the objective of examining the effectiveness of furmonertinib in patients diagnosed with previously untreated NSCLC harboring EGFR mutations. The study included patients treated at the First Affiliated Hospital of Zhengzhou University between October 1, 2021, and July 19, 2023.

Patients

Consecutive cases of advanced/metastatic NSCLC with EGFR mutations, who received furmonertinib as their initial treatment between October 2021 and July 2023 at the First Affiliated Hospital of Zhengzhou University, were included in this retrospective study. The data cutoff date was September 24, 2023. The key inclusion criteria were as follows: 1) patients aged 18 years or older; 2) patients pathologically confirmed with unresectable locally advanced (stage IIIB/C: unfit for radical surgery or local radiotherapy) or metastatic (stage IV) NSCLC with EGFR actionable genomic mutations according to the TNM staging system, American Joint Committee on Cancer (AJCC) 8th edition; 3) no previous treatments given, with a treatment interval of at least 12 months after radical mastectomy for those who received neoadjuvant or adjuvant therapies; 4) presence of at least one measurable target lesion based on Response Evaluation Criteria in Solid Tumors, version 1.1 (RECIST v1.1) (13); 5) expected survival of over 3 months. Key exclusion criteria were as follows: 1) concurrent receipt of other anticancer treatments or any previous anticancer treatments prior to furmonertinib administration; 2) tumors mixed with small cell lung cancer (SCLC) components; 3) allergy to furmonertinib or its metabolic product; 4) history of interstitial pulmonary disease (IPD) or any uncontrolled/severe complicating comorbidity. Clinicopathological variables such as age, sex, Eastern Cooperative Oncology Group performance status (PS), smoking status, histology, stage, metastatic organs, EGFR mutation types, concomitant mutations, and programmed death-ligand 1 (PD-L1) expression were retrospectively collected from documented health records. The present report obtained approval from the Ethics Committee of the First Affiliated Hospital of Zhengzhou.

At the start of the study, tests for EGFR mutations were performed using either real-time polymerase chain reaction (PCR) or next-generation sequencing (NGS). For evaluating PD-L1 expression, tumor samples were analyzed using immunohistochemistry (IHC) with the DAKO 22C3 PharmDx antibody (Dako, Carpinteria, CA,

USA). The levels of PD-L1 were determined by the tumor proportion score (TPS), which measures the percentage of positive tumor cells.

Intervention

Patients received furmonertinib 80 mg orally once daily until disease progression or severe or unmanageable toxicities were developed.

Outcome measure

The primary endpoint was PFS, as assessed by the investigators using RECIST 1.1 criteria. Key secondary endpoints included OS, ORR, disease control rate (DCR), and safety data. The ORR encompassed the percentage of patients achieving either a complete response (CR) or a partial response (PR) to the therapy. The DCR was defined as the number of patients who experienced a CR, a PR, or a stable disease (SD). PFS was defined as the duration from the first dose of furmonertinib until progressive disease (PD) or death. OS was calculated as the time from the start of treatment until death from any cause. Patients were considered censored if they were still alive at the time of their last recorded visit.

Safety

Safety data were collected whenever adverse events (AEs) led to modifications in treatment or serious adverse events (SAEs) occurred.

Statistical analysis

Statistical analyses were carried out using SPSS version 21.0 (IBM Corp., Armonk, NY, USA), and all charts were created using GraphPad Prism version 8.0. Descriptive statistics were applied to all variables where appropriate. Continuous variables were presented with the number of patients, median, and range (minimum and maximum values). Categorical variables were shown as frequency counts and percentages. A p-value of less than 0.05 was considered significant. Survival curves were generated using the Kaplan–Meier method, and survival analysis was conducted using a stratified log-rank test. HRs and 95% CIs were calculated using Cox regression analysis.

Results

Patients' clinicopathological characteristics

From October 1, 2021, to July 19, 2023, we performed a retrospective review of 73 patients with advanced EGFR-mutated NSCLC diagnosed at the First Affiliated Hospital of Zhengzhou University. Out of these patients, a total of 32 (43.8%) were found to have brain metastases at the time of initial diagnosis, with an additional two patients presenting leptomeningeal metastases when first diagnosed. The majority of patients (69 out of 73, or 94.5%) had adenocarcinoma, while two patients had squamous cell carcinoma, and two had adenosquamous carcinoma. Notably, 35 patients (47.9%) were male, and 16 patients (21.9%) were current or former smokers (with quitting time less than 15 years). The median age of the group was 61 years (ranging from 30 to 85 years), which is consistent with the typical clinical profile of patients with EGFR-mutated NSCLC. The EGFR exon 19 deletion mutation occurred in 35 patients and the L858R point mutation in 31 patients. Two patients exhibited primary resistance mutations (T790M point mutation in exon 20) to first- or second-generation EGFR TKIs, and compound mutations were observed in five patients, including one with a rare G719A/S768I mutation (as shown in Figure 1). Additionally, co-occurring TP53 mutations were found in seven patients, with one patient also having concurrent EGFR 21L858R, TP53, and RET–IGR fusion mutations. Other details are summarized and presented in Table 1.

Treatment efficacy

In this series, PFS events occurred in 19 out of 73 patients, with a median follow-up period of 9 months (95% CI, 7.0–11.0, data cutoff date of September 24, 2023). Of these patients, 49 achieved a PR, resulting in an ORR of 67.1% (49/73 patients, with a 95% CI ranging from 56.1% to 78.2%). The ORRs for patients with 19DEL or 21 L858R were 67.6% (95% CI, −51.7 to 83.4) and 64.6% (95% CI, 47.8–81.6), respectively (as indicated in Table 2). There were 32 patients who had brain metastases at the time of initial diagnosis. Of these, 26 had measurable and evaluable target lesions within the brain, and the intracranial ORR was 84.6% (95% CI, 69.8–99.5), with all 26 patients experiencing some degree of tumor reduction (Table 3). The estimated median PFS for all patients with EGFR-mutated NSCLC undergoing treatment with furmonertinib was 19.5 months (95% CI, 14.6–24.4 months), as shown in Figure 2A. The median intracranial PFS was 16 months (95% CI, 15.6–16.4 months), which is detailed in Figure 2B. We further explored the

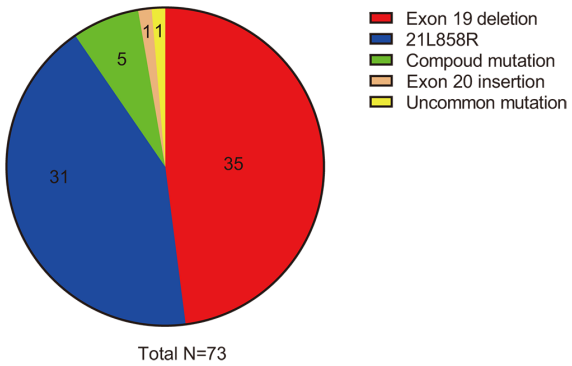

FIGURE 1
EGFR mutation types included in the study. EGFR, epidermal growth factor receptor.

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TABLE 1 Baseline clinicopathological characteristics.

Parameters	N (%)
Total patients	73 (100.0)
Median age (range), years	61 (30–85)
Gender	
Male	35 (47.9)
Female	38 (52.1)
Smoking history	
No	52 (71.2)
Yes	21 (28.9)
Histology	
Adenocarcinoma	69 (94.5)
Squamous	2 (2.7)
Adenosquamous	2 (2.7)
Stage	
IV	73 (100)
ECOG PS	
0	19 (26.0)
1	46 (63.0)
2	8 (11.0)
Mutation status	
19DEL	35 (47.9)
21L858R	31 (42.5)
Rare	2 (2.7)
Compound	5 (6.8)
Metastatic sites	
<3	58 (79.5)
≥3	15 (20.5)
Brain metastases	
Yes	32 (43.8)
No	41 (56.2)
Liver metastases	
Yes	4 (5.5)
No	69 (94.5)
Bone metastases	
Yes	36 (49.3)
No	37 (50.7)
Adrenal metastases	
Yes	6 (8.2%)
No	67 (91.8)

ECOG PS, Eastern Cooperative Oncology Group performance status.

relationship between PFS and several factors: the presence of brain metastases, PD-L1 status (less than 1% *vs.* 1% or greater), EGFR mutation subtype (exon 19 deletion *vs.* 21L858R point mutation), and the presence of TP53 co-mutations. The analyses revealed no statistically significant associations between these factors (as presented in Figures 3A–D). As of the last data update, OS had not been reached due to immature data. In the univariate analysis, gender ($p = 0.04$) and liver metastasis ($p = 0.03$) showed a significant association with progression-free survival (PFS) (Table 4). However, other clinicopathological factors did not show any significant associations with PFS (all $p > 0.05$) (Table 4). In the multivariate Cox regression analysis model, which included all factors significantly associated with PFS from the univariate analysis, no factors were found to be significantly associated with PFS (Table 4).

TABLE 2 Best clinical response of patients receiving furmonertinib.

Best response	All patients (N = 73), n%	Patients with 19DEL mutations (N = 37), n%	Patients with 21L858R mutations (N = 34), n%
CR	0	0	0
PR	49 (67.1)	25 (67.6)	22 (64.7)
SD	21 (28.8)	11 (29.7)	10 (29.4)
PD	3 (4.1)	1 (2.7)	2 (5.9)
ORR	49 (67.1; 95% CI, 56.1–78.2)	25 (67.6; 95% CI, −51.7 to 83.4)	22 (64.7; 95% CI, 47.8–81.6)
DCR	70 (95.9, 95% CI, 91.2–100.6)	36 (97.3; 95% CI, −91.8 to 102.8)	32 (94.1; 95% CI, 85.8–102.5)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ORR, objective response rate; DCR, disease control rate.

Safety profiles

Adverse events were noted in 25 patients who received furmonertinib as a first-line treatment. Diarrhea (24%), anemia (36%), and liver injury (32%) were the most frequently reported adverse events. There were serious adverse events in four patients (16%): one experienced diarrhea, one had thrombocytopenia, another suffered liver injury, and the last had an increase in blood creatinine. There was only one case of grade 1 interstitial lung disease reported, and no patients stopped treatment due to adverse events. Additional adverse events are compiled in Table 5. Overall, the toxicity profile for furmonertinib-treated patients was manageable and largely aligned with that reported in previous studies.

TABLE 3 Best intracranial clinical response for patients with brain metastases receiving furmonertinib.

Best intracranial response	All patients (N = 26), n%
CR	7 (26.9)
PR	15 (57.7)
SD	4 (15.4)
PD	0 (0)
ORR	22 (84.6; 95% CI, 69.8–99.5)
DCR	26 (100, 95% CI, 100–100)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ORR, objective response rate; DCR, disease control rate.

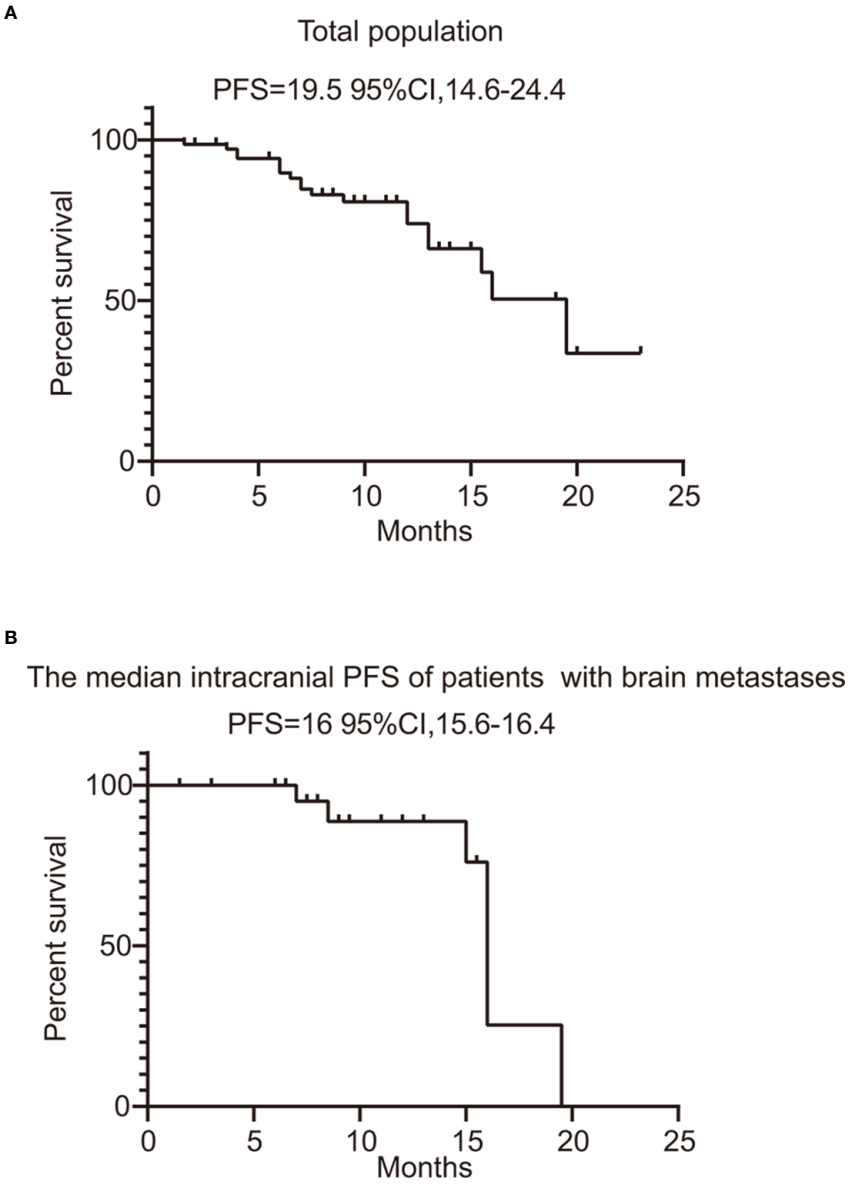

FIGURE 2
PFS of total population and patients with evaluable brain metastases. **(A)** PFS of all the patients treated with furmonertinib. **(B)** the median intracranial PFS of patients with brain metastases treated with furmonertinib. PFS, progression-free survival.

Discussion

As far as we know, this is both the first and largest observational study conducted in China on using furmonertinib as an initial treatment in everyday clinical practice. Our research encompassed a diverse patient group, including those with poor PS, elderly individuals over the age of 75, patients with uncommon and complex EGFR mutations, and those with active brain metastases, including leptomeningeal involvement—populations typically not included in prospective and randomized clinical trials. The findings from our study validate the use of furmonertinib as a viable first-line treatment option for patients with EGFR mutations in real-world conditions, just as it has been shown in controlled clinical trials. Nonetheless, given the small sample size and the retrospective nature of our study, additional research is needed to confirm these results.

Our current study included 35 male patients displaying EGFR mutations, accounting for nearly half of the participants, thereby emphasizing the importance of molecular testing in NSCLC patients regardless of sex. This finding appears to contradict previous research that suggested a lower incidence of EGFR mutations in men (14, 15). Moreover, the study involved 32 patients (43.8%) with brain metastases at the time of their initial diagnosis, which is a higher incidence compared to what has been reported in clinical trials (9). This greater prevalence of brain metastases in a real-world setting could potentially affect the

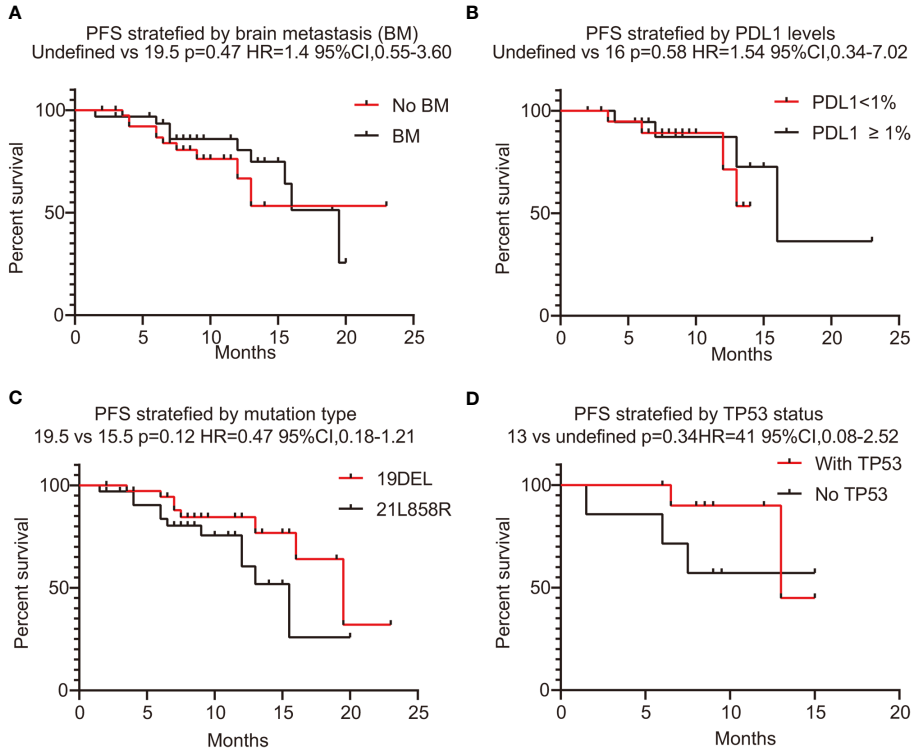

FIGURE 3
PFS stratified with brain metastases, PD-L1 expression level, EGFR mutation status, and co-mutation TP53. **(A)** PFS stratified with brain metastases (yes or no). **(B)** The correlation between PFS and PD-L1 levels (<1% *vs.* ≥1%). **(C)** The correlation between PFS and mutation type (19DEL *vs.* 21L858R). **(D)** The correlation between PFS and co-mutation TP53 (yes *vs.* no). PFS, progression-free survival; PD-L1, programmed death-ligand 1; 19DEL, exon 19 deletion; 21L858R, a substitution of lysine with arginine in exon 21; EGFR, epidermal growth factor receptor.

TABLE 4 Univariate and multivariate Cox regression analyses of PFS.

Parameters	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p-Value	*HR* (95% *CI*)	p-Value
Sex: male *vs.* female	1.26 (0.61–2.28)	0.41		
Age: <65 *vs.* ≥65	2.64 (1.05–6.65)	0.04		0.053
Smoking: yes *vs.* no	0.95 (0.33–2.74)	0.43		
ECOG PS: 0–1 *vs.* ≥2	2.17 (0.72–6.53)	0.16		
Mutation status 19DEL 21L858R Compound Rare		0.40		
Stage IVA *vs.* IVB	0.50 (0.12–2.04)	0.33		
Number of metastatic organs: <3 *vs.* ≥3	1.26 (0.42–3.79)	0.98	2.226 (1.313–3.773)	
Brain metastases: yes *vs.* no	0.71 (0.28–1.83)	0.48		
Liver metastases: yes *vs.* no	2.16 (0.28–16.43)	0.03		0.087
Bone metastases: yes *vs.* no	0.72 (0.30–1.76)	0.48		
Adrenal metastases: yes *vs.* no	2.26 (0.33–15.44)	0.41	1.855 (1.181–2.914)	

PFS, progression-free survival; ECOG PS, Eastern Cooperative Oncology Group performance status.

TABLE 5 Adverse event profiles.

Event	Treated population (25 patients have documented side effects)	
	Any grade	Grades 3, 4, 5
Diarrhea	6/25 (24%)	1/25 (4.0%)
Rash	4/25 (16%)	0
Anemia	9/25 (36.0%)	0
Decreased white blood cell count	4/25 (16%)	0
Neutropenia	4/25 (16.0%)	0
Thrombocytopenia	4/25 (16%)	1/25 (4%)
Interstitial lung disease	1/25 (4.0%)	0
Liver injury (elevated ALT or AST)	8/25 (32.0%)	1/25 (4.0%)
Hypothyroidism	1/25 (4.0%)	0
Hypokalemia	3/25 (12%)	0
Hyperlipidemia	1/25 (4.0%)	0
Increased blood creatinine	1/25 (4.0%)	1/25 (4.0%)

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

survival outcomes observed in this study. An additional noteworthy detail of our report is that all included patients were diagnosed with stage IV cancer, unlike the population in the FURLONG trial. Our study found that the median PFS was 19.5 months, which is less than what was documented in the FURLONG study. Possible explanations for this shorter PFS may include the inclusion of a higher number of male patients, more patients with brain metastases, and a greater number of cases at stage IV.

It is widely recognized that PD-L1 is a key biomarker for predicting responses to ICIs. Some studies have also indicated a correlation between PD-L1 expression and the effectiveness of EGFR-TKIs, though this theory remains the subject of debate (16–20). Quite a few studies have found no significant link between PD-L1 expression levels and the efficacy of EGFR-TKIs (21). In our study, we evaluated PD-L1 expression in 32 patients, finding that the majority were negative (PD-L1 < 1%), aligning with prior research suggesting that individuals with EGFR mutations tend to have low PD-L1 expression levels (22–24). Dong and colleagues compiled data from 15 studies, proposing that PD-L1 expression is inversely related to EGFR mutation status (25). Their investigation into the correlation between mRNA and PD-L1 protein levels in surgical samples from The Cancer Genome Atlas (TCGA) and an internal database (Guangdong Lung Cancer Institute (GLCI)) supported the notion that EGFR-wild type tumors have higher PD-L1 expression compared to EGFR-mutated tumors. Contrarily, there have been reports asserting the opposite (26). Thus, the association between PD-L1 expression and EGFR mutations remains a topic of debate. In our case, the data showed no differences in PFS when categorizing by PD-L1 levels. However, due to the small number of cases included in the study, these findings should be approached with caution.

Previous literature has indicated that the effectiveness of EGFR-TKIs can vary based on the type of EGFR mutation present (7–9). For instance, in the FLAURA China study, individuals with EGFR 19DEL achieved a longer PFS than those with the L858R point mutation in exon 21 (27). Similarly, the AENEAS study showed that patients with the 19DEL mutation had a more favorable response compared to those with the 21L858R mutation, despite achieving comparable benefits to the control group (8). The findings of the FURLONG study echo these observations, suggesting that patients with the 19DEL mutation could be considered a subgroup with a potentially better prognosis compared to those with the 21L858R mutation (9). These results have prompted some experts to propose that patients with 19DEL and 21L858R mutations might benefit from distinct therapeutic approaches. However, in our current research, we observed no significant difference in PFS between patients with 19DEL and 21L858R mutations. The discrepancy with the FURLONG study's results could be due to the limited sample size in our study. Looking to enhance the efficacy of EGFR-TKIs for patients with 21L858R mutations, some researchers have explored the combination of EGFR-TKIs with anti-angiogenesis drugs. Various studies have suggested that combining first-generation EGFR-TKIs with vascular endothelial growth factor (VEGF) inhibitors might extend PFS for patients with the 21L858R mutation to levels similar to those with the 19DEL mutation (28–31). Nevertheless, intriguingly, the addition of VEGF inhibitors does not seem to improve the effectiveness of osimertinib, which is a third-generation EGFR-TKI (32–34). The question of whether first-generation EGFR-TKIs combined with VEGF inhibitors are superior to monotherapy with third-generation EGFR-TKIs for patients with EGFR-sensitive mutations remains open for investigation. Nevertheless, third-generation EGFR-TKIs are currently the preferred treatment option for patients with such sensitive mutations.

In both univariate and multivariate analyses, we observed that PFS was not associated with Eastern Cooperative Oncology Group (ECOG) performance status, the number of metastatic sites, the presence of brain or liver metastases, or other factors. This finding differs from that of previous studies. A possible reason for this discrepancy could be the limited number of cases and the retrospective nature of our study.

Our research has several limitations. First, the follow-up period may not be adequately long, which could introduce bias into our conclusions. Also, due to the limited duration of observation, the OS data were not mature. We plan to provide an update on OS once sufficient events have occurred and an appropriate follow-up duration has been reached. Second, our study was retrospective and included a limited number of cases. Potential concerns should be noted: efficacy evaluations were performed according to RECIST 1.1 by investigators, which could lend toward more objective outcomes. Therefore, further research is necessary.

Conclusions

In conclusion, our study suggests that furmonertinib could be a preferred treatment option as a first-line therapy for patients with

EGFR-sensitive mutations. We observed comparable PFS in the real-world setting relative to that in randomized clinical trials. These findings underscore the potential of furmonertinib as a viable choice in real-world 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 authors.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The studies were conducted in accordance with the local legislation and institutional requirements. Current work was retrospective study and individuals' information will not be shared, hence, the Ethics Committee of the First Affiliated Hospital of Zhengzhou University exempted the informed consent.

Author contributions

NY: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Writing – original draft, Writing – review & editing. SG: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing. SH: Formal analysis, Methodology, Resources, Writing – original draft, Writing – review & editing. HZ: Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. XL: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Efficacy of osimertinib and the role of sequential liquid biopsy in patients diagnosed with NSCLC harboring EGFR and BRAF mutations at baseline: insights from two case reports

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Epidermal Growth Factor Receptor (EGFR) and B-Raf (BRAF) mutations are two of the most important drivers identified in non-small-cell lung cancer (NSCLC). This report highlights two cases of patients diagnosed with metastatic NSCLC bearing concurrent EGFR and BRAF mutations at baseline and treated with osimertinib as first-line treatment. Molecular profiling was conducted in the tissue and plasma at the time of initial diagnosis, and subsequent repeated liquid biopsy examinations were planned after 10 days, 28 days, and at the time of radiological progression in the frame of the prospective translational study REM. These cases suggest that osimertinib may maintain its therapeutic effectiveness even in patients presenting with a baseline BRAF co-mutation. Notably, radiological responses align with liquid biopsy observations: in both instances, follow-up liquid biopsies indicate the clearance of EGFR-mutated circulating tumor DNA (ctDNA).

KEYWORDS

liquid biopsy, EGFR, NSCLC, BRAF, co-mutation, NGS, osimertinib

Introduction

In the last two decades, the introduction of targeted therapies for oncogene-addicted diseases changed the natural history of non-small-cell lung cancer (NSCLC) (1–4). Oncogene addiction is a term used to describe the reliance of certain neoplasms on a single activated oncogenic protein or pathway in order to maintain malignant properties and serves as a rationale for target therapy (5).

Currently, several targeted therapies are approved for patients with oncogene-addicted tumors. EGFR and BRAF are two of the most important driver mutations, accounting for an overall prevalence of 32% and 1%–3% of NSCLC cases, respectively (6, 7).

Following the results of the FLAURA trial, osimertinib, a third generation TKI, became the standard first-line treatment for EGFR mutated metastatic NSCLC (mNSCLC) (1).

Nevertheless, the magnitude of clinical benefit is heterogeneous, and new-combination therapeutic strategies recently demonstrated to be associated with improved progression-free survival (8, 9). While reliable predictive markers are not yet available for clinical practice, the presence of co-mutations, such as p53 and PIK3CA, was found to be associated with limited clinical benefit, albeit little is known about the role of BRAF co-mutations in this particular setting (10). Interestingly, BRAF mutations have been described as a possible mechanism of acquired resistance to EGFR-TKIs (11).

Additionally, even though oncogene driver mutations are generally mutually exclusive (12), the presence of two actionable driver mutations represents a significant clinical challenge in terms of selection of treatment and management.

Here, we present two cases of patients diagnosed with mNSCLC harboring both EGFR and BRAF mutations at baseline. The patients were treated with first-line osimertinib, and tumor molecular profiling was performed in the tissue and plasma at diagnosis and monitored through repeated liquid biopsies at different time points according to the schedule of the REM clinical study. REM is an ongoing multicentric, prospective, observational clinical study that enrolls EGFR-mutated NSCLC patients receiving first-line osimertinib aiming to identify concomitant genetic alterations in plasma at baseline and at progression and to monitor EGFR mutation in plasma to correlate it with the radiological response and the outcome. In this setting, plasma samples were collected before the initiation of treatment and then after 10 days and 28 days of treatment. Three different tests were used to analyze patients' cfDNA: a real-time PCR technique (Cobas EGFR mutation test V2, Roche) and two next-generation sequencing (NGS) tests that included the main EGFR and BRAF mutations (Avenio ctDNA Expanded kit, Roche, and PSS Solid Cancer IVD kit, Sysmex). The study design and informed consent were submitted and approved by the local Ethics Committee.

Case description

Case 1

The first case that we report here is one of an 81-year-old female Caucasian never-smoker patient.

In February 2023, she experienced dyspnea and underwent a CT scan that demonstrated the presence of a left inferior lobe lesion, multiple pleural homolateral nodules, pleural effusion, and a single cerebral lesion, a benign meningioma, that had already been reported in her previous clinical history. Histological diagnosis was obtained by CT-guided percutaneous needle biopsy and was compatible with lung adenocarcinoma. Real-time PCR (RT-PCR) testing in the tissue revealed the presence of both BRAF V600E mutation and EGFR exon 19 deletion (ex19del). No quantitative data of mutation frequency was available, making it impossible to distinguish whether these mutations are clonal or subclonal. Liquid biopsy at baseline confirmed the presence of EGFR ex19del at low levels in cfDNA, with correspondence among the results of three different methods used: Cobas EGFR mutation test V2 ISQ 9.79; Avenio ctDNA Expanded kit VAF 0.34%; Sysmex PSS Solid Cancer IVD kit VAF 0.25% (16 mutant molecules—MM). Conversely, the BRAF V600E mutation was not detected by both NGS tests in plasma.

The patient was in good clinical condition (ECOG PS 0) and had no relevant medical history, apart from controlled hypertension. Considering clinical staging, histological diagnosis, lack of smoking history, and molecular profile, the patient started on osimertinib, and close clinical and radiological monitoring was performed.

Subsequent radiological tumor assessments were performed according to clinical practice, and best radiological response was stable disease according to RECIST 1.1. The treatment was well tolerated, and the only adverse event recorded was a G1 platelet count decrease (according to CTCAE 5.0).

Liquid biopsy testing was performed initially by Cobas EGFR mutation test V2 RT-PCR at different time points, revealing clearance of the EGFR mutation coherent with the clinical response to treatment; indeed, cfDNA samples from T1 to T5 resulted in non-mutated EGFR. Consistently, analysis of serial cfDNA samples by Sysmex PSS Solid Cancer IVD kit detected minimal residual molecular disease at T1 (VAF, 0.046%; 4.22 MM) and T2 (VAF, 0.029%; 2.34 MM) with a kinetics showing gradual reduction in the EGFR mutation during treatment. Indeed, in the following time points (T3–T4–T5), NGS analysis indicated complete clearance of the mutation in plasma. Notably, the BRAF mutation found in tissue biopsy was not detected in any of the cfDNA samples analyzed by Sysmex PSS Solid Cancer IVD kit.

After 12 months, the patient is still on treatment with osimertinib, and she is maintaining a good clinical and radiological response. Further plasma monitoring and re-characterization in the tissue and plasma at the time of progression has been planned.

Case 2

The second case is that of a 77-year-old Caucasian woman.

Following the onset of dyspnea in July 2022, she underwent a CT scan showing bilateral lung nodules and left pleural effusion for which she underwent left thoracentesis with immediate clinical benefit. A CT-scan-guided needle biopsy of one of the lung nodules performed for diagnostic purposes enabled to make the histological diagnosis of lung adenocarcinoma, with no evidence of extra-

thoracic disease. Molecular profiling on the tissue revealed the presence of EGFR L858R and BRAF E501K mutations, without available variant allele fraction information. NGS testing of cfDNA at baseline confirmed the presence of both mutations. Specifically, Avenio ctDNA Expanded kit detected EGFR L858R with VAF = 0.35% and Sysmex PSS Solid Cancer IVD kit detected the same mutation with VAF = 0.15%. Cobas EGFR mutation test V2 RT-PCR analysis was in line with these results, revealing EGFR L858R mutation with ISQ = 5.99. BRAF mutation E501K was confirmed in plasma only by Avenio ctDNA Expanded kit (VAF 0.31%), since this mutation is not covered by Sysmex PSS Solid Cancer IVD kit. The similarity in VAF detected for EGFR and BRAF mutations may suggest the clonal nature of the BRAF mutation.

Considering the good performance status, clinical staging, and molecular characterization of disease, the patient started her first-line systemic treatment with osimertinib.

RT-PCR testing for EGFR was then performed on liquid biopsy at time points T1 and T2, revealing clearance of EGFR mutation right after 10 days since the start of systemic treatment. NGS monitoring by Sysmex PSS Solid Cancer IVD kit was performed only at T2 due to insufficient amount of cfDNA available at T1 and confirmed the clearance of EGFR mutation. BRAF mutation monitoring was not possible because the E501K mutation is not included in the Sysmex PSS Solid Cancer IVD panel.

The first radiological assessment revealed a partial response according to RECIST 1.1 with a 70% reduction in the sum of target lesions. At the time of writing, 17 months after diagnosis, the patient is still on treatment and maintains both radiological response and clinical benefit.

Timeline

The results of longitudinal liquid biopsies and radiological assessment for the patient described in case 1 are reported in Figures 1, 2, while those for case 2 are shown in Figures 3, 4.

Molecular diagnostic assessment

According to the REM protocol, cfDNA samples were collected before starting treatment (T0) and after 10 (T1) and 28 days (T2).

The patient described in case 1 displayed both EGFR and BRAF mutations at tissue biopsy, so she was monitored through longitudinal liquid biopsy at every medical appointment, resulting in a total of six samples (from T0 to T5) being evaluated. The first three blood samples were taken as described, while the additional three samples were collected approximately every 2 months. Regarding case 2, cfDNA samples were collected as planned before and after early time points from osimertinib administration (T0–T1–T2).

We used three methods to test patient's cfDNA: Cobas EGFR mutation test V2 (Roche), Avenio ctDNA Expanded kit (Roche), and PSS Solid Cancer IVD kit (Sysmex).

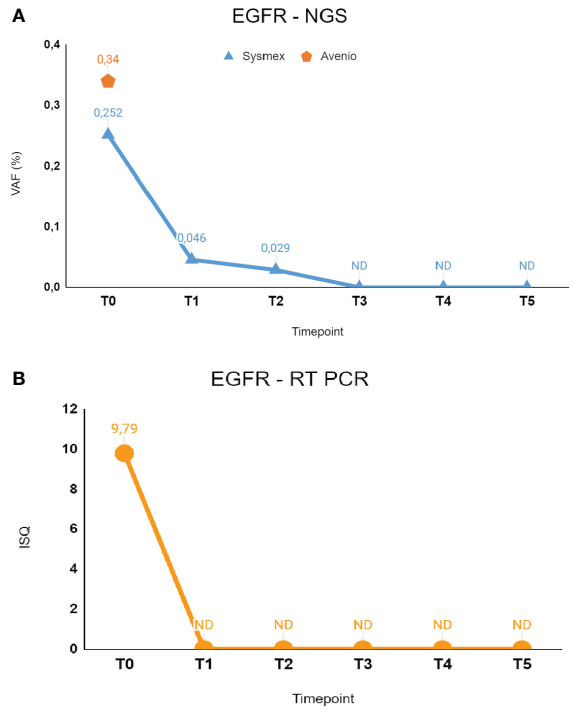

FIGURE 1
The figure shows longitudinal monitoring of EGFR ex19del mutation in the patient described as Case 1. **(A)** Results of NGS analysis: baseline T0 shows VAF values from both Avenio ctDNA Expanded kit and Sysmex PSS Solid Cancer IVD kit analysis. The data reported from T1 to T5 indicate the VAF values detected by Sysmex PSS Solid Cancer IVD kit panel after the start of treatment with osimertinib. **(B)** Monitoring of EGFR mutation by RT-PCR Cobas EGFR Mutation Test V2. Time-points were the same as in **(A)**. T0: 02/09/2023; T1: 02/17/2023; T2: 03/09/2023; T3: 04/06/2023; T4: 06/09/2023; T5: 08/24/2023. VAF, Variant Allele Fraction; ISQ, Semi-Quantitative Index.

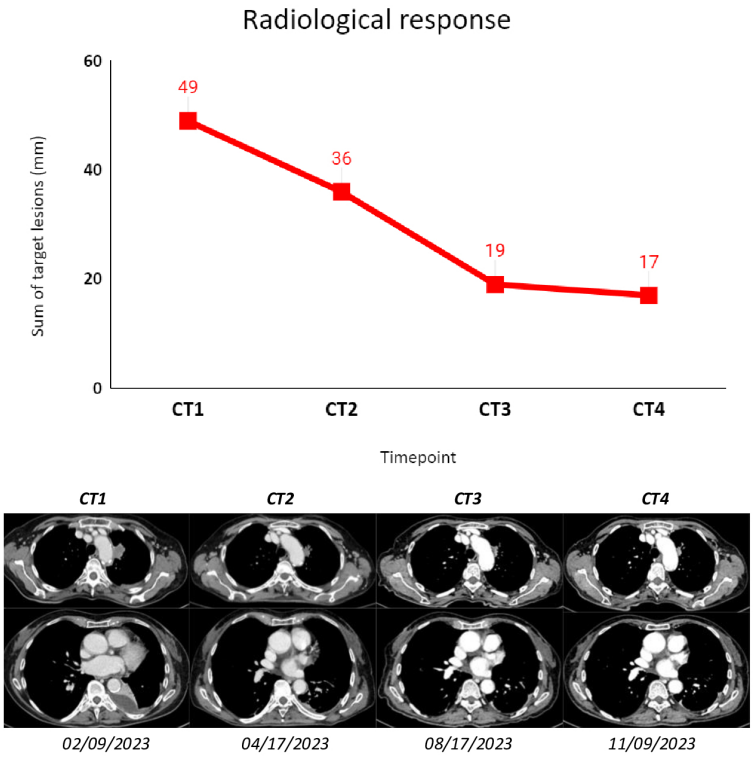

FIGURE 2
The figure shows subsequent radiological assessments (CT scans) that were performed according to clinical practice for the patient described as Case 1. The sum of target lesions was assessed following RECIST 1.1. CT1: 02/09/2023; CT2: 04/17/2023; CT3: 08/17/2023; CT4: 11/09/2023.

The first method is a RT-PCR technique developed to detect EGFR mutations present in exons 18,19, 20, and 21, and it was performed for all samples of plasma collected.

The second and third methods are NGS tests, and both panels include EGFR and BRAF most common mutations as targets. Avenio ctDNA Expanded kit, a relatively large panel of 77 genes, was used only at diagnosis, while the Sysmex PSS Solid Cancer IVD kit, which includes five genes in specific hotspots, guarantees higher sensitivity, and this kind of targeted approach was ideal to monitor the minimal residual disease.

All prepared libraries were sequenced on an Illumina Nextseq 550 instrument with high output kit (300 cycles) in pair-end mode (151×2) for Avenio ctDNA Expanded kit and the mid output kit (150 cycles) in single-end mode (150 cycles) for Sysmex PSS Solid Cancer IVD kit.

Discussion

These two cases suggest that osimertinib might maintain its efficacy even in patients with a BRAF co-mutation at baseline. Since the patients are respectively at their 12th and 17th month on treatment with persistence of clinical benefit and clearance of EGFR mutation in plasma, we suggest that patients carrying BRAF co-mutations might represent a different molecular subset of EGFR mutated patients, when compared with other co-mutations and in particular p53 and KRAS co-mutations (13, 14).

Radiological response was accompanied by liquid biopsy findings: for both cases, subsequent liquid biopsies showed a clearance in the EGFR-mutated cfDNA. Curiously, for the first patient, BRAF V600E was only found in tissue NGS analysis. This could be attributed to the fact that the patient only presented with intrathoracic disease with limited ctDNA shedding. Alternatively, considering that EGFR mutation tested positive in plasma at relatively low levels, we might speculate that the BRAF V600E was subclonal in the tumor of this patient, and therefore, it was missed by cfDNA testing because it was under the detection limit of the NGS assays used. This case clearly illustrates the potential of liquid biopsy in shedding light on tumor heterogeneity and differentiating biological significance of two driver alterations.

Notably, the second patient carried a non-V600 BRAF mutation located in the kinase domain of the protein (15). However, this mutation is currently considered inconclusive because there is conflicting and/or weak data describing the biological significance of the BRAF E501K mutation. *In vitro* studies have demonstrated that this mutation might be inactivating as measured by decreased BRAF kinase activity in a cell line with a second BRAF mutation compared to controls (16). However, another pre-clinical study found increased downstream pathway output compared to wild type (17). Nevertheless, it is still relevant to emphasize that, in this clinical case, this mutation does not seem to have a negative impact on the efficacy of osimertinib.

According to the REM protocol, RT-PCR and NGS cfDNA testing are planned also at radiological progression of disease in order to further investigate possible acquired resistance mechanism. It will be interesting to see whether BRAF mutations will be among

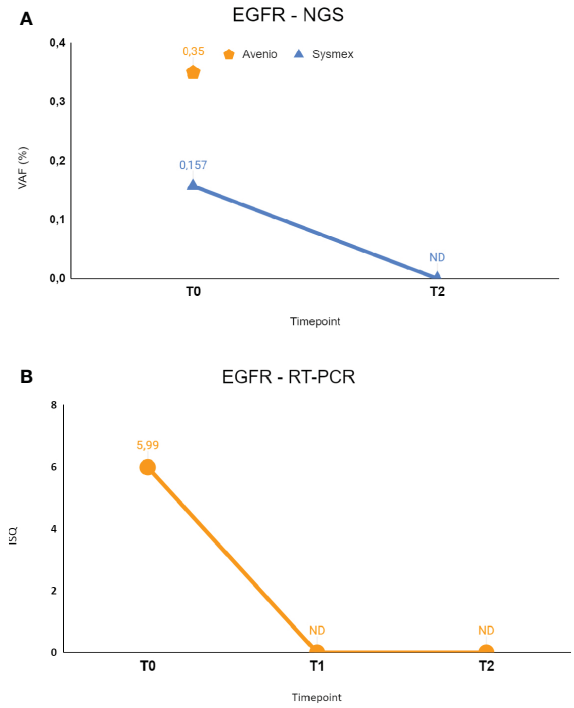

FIGURE 3
The figure shows longitudinal monitoring of EGFR ex19del mutation in the patient described as Case 2. **(A)** Results of NGS analysis: baseline T0 shows VAF values from both Avenio ctDNA Expanded kit and Sysmex PSS Solid Cancer IVD kit analysis. The data reported as T2 indicate the VAF value detected by Sysmex PSS Solid Cancer IVD kit panel after the start of treatment with osimertinib. **(B)** Monitoring of EGFR mutation by RT-PCR Cobas EGFR Mutation Test V2. T0 and T2 were the same as in **(A)**, T1 was the first sample after the start of treatment with osimertinib. T0: 09/06/2022; T1: 09/14/2022; T2: 09/26/2022. VAF, Variant Allele Fraction; ISQ, Semi-Quantitative Index.

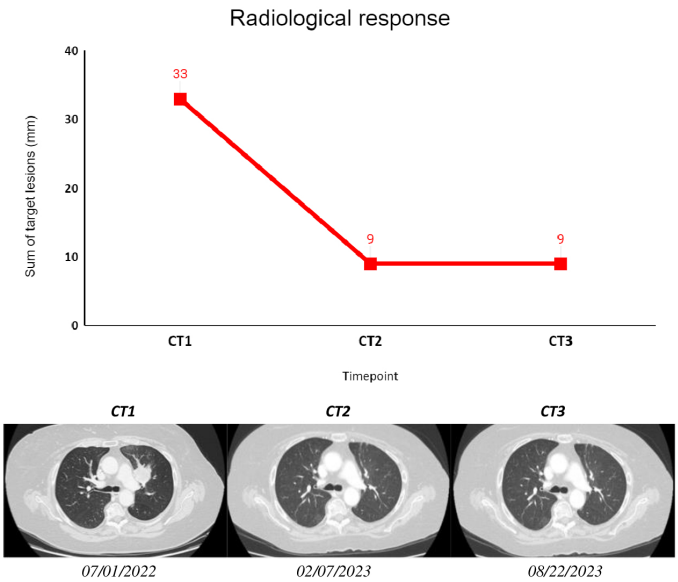

FIGURE 4
The figure shows subsequent radiological assessments (CT scans) that were performed according to clinical practice for the patient described as Case 2. The sum of target lesions was assessed following RECIST 1.1. CT1: 07/01/2022; CT2: 02/07/2023; CT3: 08/22/2023.

these, since combination therapies with anti-EGFR and anti-BRAF TKIs have been described in this setting (18–20).

These two cases demonstrate that liquid biopsy can have an important role in monitoring patients during treatment, showing that molecular response is associated with clinical response and can be evaluated before radiological assessment. In addition, specifically in the context of co-mutations, further data collections are awaited to understand its potential role in unveiling tumor heterogeneity and differential role of concomitant genetic alterations.

In the end, we conclude that a molecular survey of patients' plasma has clinical validity in this setting and can aid to follow EGFR TKI effects also in the rare event of BRAF co-mutations.

Data availability statement

The data presented in the study are deposited in the ENA (European Nucleotide Archive) repository, accession number ERP158121.

Ethics statement

The studies involving humans were approved by ethics committee for clinical trials, Veneto Institute of Oncology IOV – IRCCS, Padua, Italy. The studies were conducted in accordance with the local legislation and institutional requirements. The 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

LCB: Data curation, Investigation, Writing – original draft, Writing – review & editing. AP: Data curation, Investigation, Writing – original draft, Writing – review & editing. ABB: Data curation, Investigation, Writing – review & editing. LC: Writing – review & editing, Investigation. VG: Writing – review & editing, Supervision. LB: Conceptualization, Supervision, Writing – review & editing, Methodology. SI: Conceptualization, Supervision, Writing – review & editing, Methodology.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

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A study of high dose furmonertinib in EGFR exon 20 insertion mutation-positive advanced non-small cell lung cancer

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Background: The epidermal growth factor receptor (EGFR) ex20ins mutation, as a rare subtype of mutation, has gradually attracted attention. Its heterogeneity is high, its prognosis is extremely poor, and the efficacy of existing traditional treatment plans is limited. In this study, we aimed to evaluate efficacy of high dose furmonertinib as a first-line treatment for EGFR ex20ins-positive NSCLC.

Methods: This is a retrospective, multi-center, non-interventional study. From May 2021 to March 2023, 9 NSCLC patients with EGFR ex20ins were enrolled. Efficacy and safety of 160 mg furmonertinib were evaluated. Objective response rate (ORR), disease control rate (DCR), median progression-free survival (PFS) and treatment related adverse events (TRAEs) were assessed.

Results: Of the evaluated patients, six patients experienced partial remission (PR), two patients experienced stable disease (SD) and one patient experienced progress disease (PD). Data indicated 66.7% ORR and 88.9% DCR. The median progression free survival (PFS) was 7.2 months (95% CI: 6.616 - 7.784). Besides, a longgest PFS with 18 months was found in one patient with p.H773_V774insGTNPH mutation. No ≥ level 3 adverse events have been found.

Conclusions: The study proved the potential efficacy of 160mg furmonertinib in patients with advanced NSCLC with EGFR ex20ins. Meanwhile, 160mg furmonertinib had a good safety profile.

KEYWORDS

the epidermal growth factor receptor, exon 20 insertion, furmonertinib, non-small cell lung cancer, first-line treatment

1 Introduction

According to the global cancer statistics in 2020, the incidence of lung cancer ranks second in the world, with 2.2 million new cases per year, accounting for 11.4% of the total number of new tumors, and the death rate of lung cancer is the first cause of cancer death, accounting for 14.3% of the total number of tumor deaths (1). Lung cancer has become one of the most tumors threatening human health, among which non-small cell lung cancer (NSCLC) is the main pathological type of lung cancer, accounting for 80%-85%, and most patients are found to be in advanced stages of the disease. Platinum-based chemotherapy is the main treatment for advanced NSCLC. In recent years, the epidermal growth factor receptor (EGFR) is gradually being recognized. Multiple studies have shown that targeted therapy, represented by EGFR tyrosine kinase inhibitors (EGFR-TKIs), not only significantly prolongs the progression free survival (PFS) of patients with advanced NSCLC, but also improves their quality of life, which has revolutionized the treatment of patients with advanced NSCLC.

The EGFR gene is cmposed of 28 exons, and most of the mutations occur in exons 18-21. Common mutations include exon 19 inframe deletion (ex19del) and exon 21 L858R alterations (ex21L858R), which accounts for 80%-85% of all EGFR mutations (2). These two classical EGFR mutations have good sensitivity to first-generation EGFR-TKIs (erlotinib, gefitinib), second-generation EGFR-TKIs (afatinib, dacotinib) and third-generation EGFR-TKIs (osimertinib) drugs. The remaining EGFR mutations are known as rare mutations. Among rare mutations, EGFR ex20ins mutation is the most common. The incidence of EGFR ex20ins in EGFR mutated NSCLC patients is 4% -12%, while in NSCLC patients, the incidence is 1.8% -2.3% (3).

Before Mobosetinib and Amivantamab were approved for EGFR ex20ins NSCLC by FDA, the main treatment for patients with EGFR ex20ins mutation were traditional EGFR-TKIs, platinum-containing chemotherapy and immunotherapy. However, these treatment options have limited benefits (4–6). Hence, better treatment plans are needed for these patients.

Furmonertinib is an irreversible, selective, third-generation EGFR-TKI. The FURLONG study (NCT03787992) (7) showed that first-line treatment with furmonertinib in Chinese patients with advanced EGFR gene mutation NSCLC resulted in a median PFS of 20.8 months, which was 9.7 months longer than the 11.1 months in the gefitinib group. In terms of safety, the median drug exposure time of patients in the furmonertinib group was longer than that in the gifitinib group (18.3 months vs. 11.2 months), but the incidence of ≥ Level 3 adverse events in the furmonertinib group was lower than that in the gifitinib group (11% vs. 18%). On March 3, 2021, the National Medical Products Administration (NMPA) approved the use of furmonertinibb for second-line treatment in adult patients with locally advanced or metastatic NSCLC carrying EGFR 20 exon T790M (T790M) resistance mutations. Nowadays, the efficacy of furmonertinib as a first-line treatment for EGFR ex20ins-positive NSCLC is currently being studied. Preclinical data and Phase Ib study results published by furmonertinib showed that receiving 240 mg/d of furmonertinib treatment resulted in an ORR of 60% and a DCR of 100%, with no ≥ grade 3 adverse events occurring (8). However, there is still a lack of results about the effect of 160 mg/d of furmonertinib on EGFR ex20ins-positive NSCLC.

In this study, we retrospectively analyzed the efficacy and safety of first-line treatment with 160 mg/d of furmonertinib in patients with advanced NSCLC harboring EGFR ex20ins mutations. We also collected and analyzed the clinical pathological and molecular characteristics of NSCLC patients with EGFR ex20ins mutations.

2 Material and methods

2.1 Study design

This was a retrospective, multi-center, non-interventional study. Patients received furmonertinib 160mg once daily for first-line treatment. We collected the clinical information of patients, including age, sex, pathological type, smoking history, metastatic site, gene status, efficacy assessment, and adverse events (AEs). Our study was approved by the ethics committee of the First Hospital of Changzhou.

2.2 Inclusion criteria

a) Non-small cell lung cancer (NSCLC) diagnosed by histology or cytology at the age of 18 or older. b) Tumor driven gene testing has confirmed the presence of EGFR20 exon insertion mutations in tissue or blood samples. c) Stage IV NSCLC patients who did not undergo any systemic treatment before their first medication. d) Having at least one measurable tumor lesion (according to RECIST 1.1). e) The ECOG score was 0-1, and there was no significant deterioration of the disease within the two weeks prior to screening.

2.3 Efficacy and safety assessments

All patients who received at least one dose of furmonertinib were included in the efficacy and safety assessment. Patients with measurable disease at baseline and had been re-examined were evaluated for efficacy analysis. Radiographic tumor assessments were completed every 4–8 weeks. Response was assessed by the investigators according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1. Adverse events were recorded from the clinical data. The PFS was calculated from the first day of treatment with furmonertinib to disease progression or death or the last follow-up visit. Adverse events were graded according to the NCI Common Terminology Criteria for Adverse Events (CTCAE), version 4.0.

2.4 Statistical analyses

The descriptive statistics for clinical and demographic characteristics were summarized with numbers and percentages of categorical variables. The ORR and DCR were indicated by the rate of responses. The PFS were calculated by Kaplan-Meier

method. All statistical analyses were performed using SPSS software version 26.0 (IBM Corp., NY, USA).

3 Results

3.1 Patient characteristics

Between May 2021 to March 2023, 9 patients were enrolled in this study. The characteristics of 9 patients evaluated in this study are summarized in Table 1. Median age was 59 years (range 49–75), 33.3% (n=3) of patients were female, 66.7% (n=6) of patients were male, and 44.4% (n=4) were never-smokers. The most common sites of metastasis at baseline were bone (55.6%) and lung (44.4%). In addition, the most common progressive lesion is brain metastasis.

3.2 Molecular characterization of EGFR ex20ins mutations

NGS (Next-generation sequencing technology) analysis determined the mutation types of 9 patients. Three patients harbored EGFR p.S768_D770dup, two patients harbored p.P771_P772insT, one patient harbored p.H773_V774insGTNPH, one patient harbored p.P772_H773insT, one patient harbored p.V769_D770insCV and one patient harbored p.P772_H773insPNP mutations (Figure 1). Besides, more than 20 co-mutations detected by NGS and the most common co-mutation is TP53 (66.7%), TERT (33.3%) (Figure 2).

3.3 Efficacy analysis

Of the nine evaluated patients, six patients experienced PR, two patients experienced SD and one patient experienced PD. These data indicated 66.7% ORR and 88.9% DCR. Besides, median PFS time was 7.2 months (95% CI: 6.616 - 7.784) (Figure 3A). PFS of one patients with H773_V774insGTNPH is up to 18 months. In four patients with H773_V774insGTNPH, S768_D770dup, V769_D770insCV and P772_H773insT, lung cancer remained stable for more than 10 months. In two patients with p.P771_P772insT, the tumor was progressing rapidly. Besides, in two patients without co-mutation, they each obtained longer PFS (18 months and 12.7 months) (Figure 3B). These data indicate variable efficacy pattern among patients with EGFR ex20ins positive NSCLC.

3.4 Safety analysis

In general, furmonertinib treatment was well tolerated. The safety profile of this study is summarized in Table 2. Increased γ-glutamyl transpeptidase (GGT) was observed in one patient. Patient's liver function returns to normal after dose reduction to 120 mg once daily. One patient developed diarrhea and one patient developedoral ulcers. In summary, safety profile of furmonertinib 160 mg is well accepted.

TABLE 1 Basic characteristics of the patients included in this study.

	No. of patients	(%)
Total enrolled	9	
Aged (years)		
Median(range)	59(49-75)	
Sex		
Male	6	66.7
Female	3	33.3
ECOG performance status (pre-treatment)		
0	6	66.7
1	3	33.3
Histology		
Adenocarcinoma	8	88.9
Squamous	1	11.1
Disease stage		
IV A	1	11.1
IV B	8	88.9
Site of metastasis		
Bone	5	55.6
Pleura	3	33.3
Lung	4	44.4
adrenal	1	11.2
Central nervous system	2	22.2
smoking history		
Yes	5	55.6
No	4	44.4
EGFR mutation status		
p.H773_V774insGTNPH	1	11.1
p.P772_H773insT	1	11.1
p.S768_D770dup	3	33.3
p.P772_H773insPNP	1	11.1
p.V769_D770insCV	1	11.1
p.P771_P772insT	2	22.2

ECOG, Eastern Cooperative Oncology Group.

4 Discussion

The majority of EGFR ex20ins mutations occur on the C-terminal loop after the C-helix, with a few occurring within the C-helix (9). According to the different positions of insertion mutations, EGFR ex20ins mutations can be divided into C-helix insertion, proximal insertion, and distal insertion. In our study, mutations in eights patients were located in near-loop and just one patients was located in far-loop. It is worth noting that EGFR

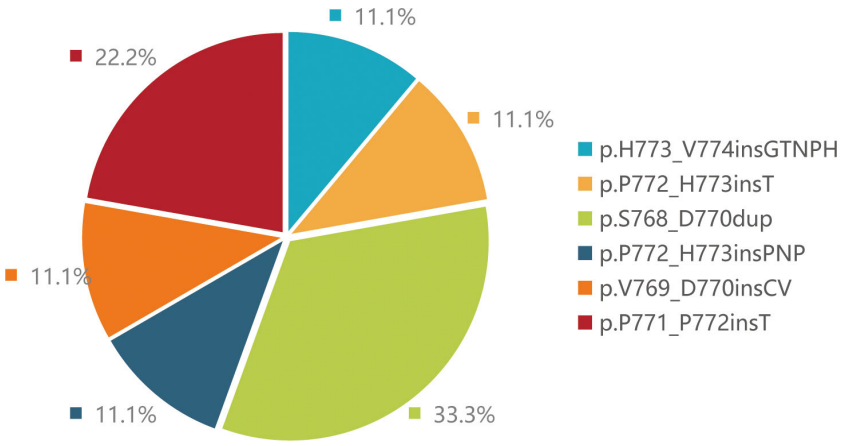

FIGURE 1
Proportion of EGFR ex20ins mutation subtypes identified by next-generation sequencing.

ex20ins has strong molecular heterogeneity. To date, over 100 EGFR ex20ins variant types have been reported (10, 11). Studies shown that the most frequent variant was A767_V769dup (34.9%), S768_D770dup (15.5%) and N771_H773dup (6.2%). However, our study shown that the most frequent variant was S768_D770dup (33.3%) and P771_P772insT (22.2%), which may be related with a smaller sample size of our study. We also found that the most frequent co-mutation were TP53 (66.7%), which was consistent with literature (12, 13). As we know, TP53 is closely related to prognosis of advanced NSCLC with EGFR mutation. A study showed that TP53 was associated with faster resistance in EGFR-mutant NSCLC and mediated acquisition of resistance mutations to EGFR tyrosine kinase inhibitors (14). In our study, we also found that neither of the two patients with the longest PFS had TP53 mutations. However more cases are needed to ensure the impact of co-occurring TP53 mutations.

In the real-world, the first-line treatment of patients with advanced EGFR ex20ins NSCLC mostly use chemotherapy. A retrospective study showed that the ORR and DCR at 6 months of advanced NSCLC patients with EGFR ex20ins treated with first-line platinum based chemotherapy were 19.2% and 41.3% (10). The median PFS was 6.4 months. The first generation TKI drugs (gefitinib, erlotinib) are a reversible ATP competitive inhibitor that can selectively inhibit the ex19del and ex21L858R mutant EGFR, but have almost no effect on the ex20ins mutation. Previous studies have shown that the median PFS of advanced NSCLC patients with EGFR ex20ins mutations receiving first-generation EGFR-TKIs treatment was only 1.9 months (15). As the third-generation orally highly efficient and irreversible EGFR-TKIs, the therapeutic effect of osimitinib on EGFR ex20ins is quite controversial in various studies. A small sample study reported that six advanced EGFR ex20ins NSCLC patients treated with osimitinib with an objective response rate (ORR) of 100%, mPFS of 6. 2 months and a good safety profile (16). A single arm phase II study was conducted on NSCLC patients with EGFR ex20ins mutation, in which the dose of osimitinib was increased to 160 mg. The results showed that the ORR of 20

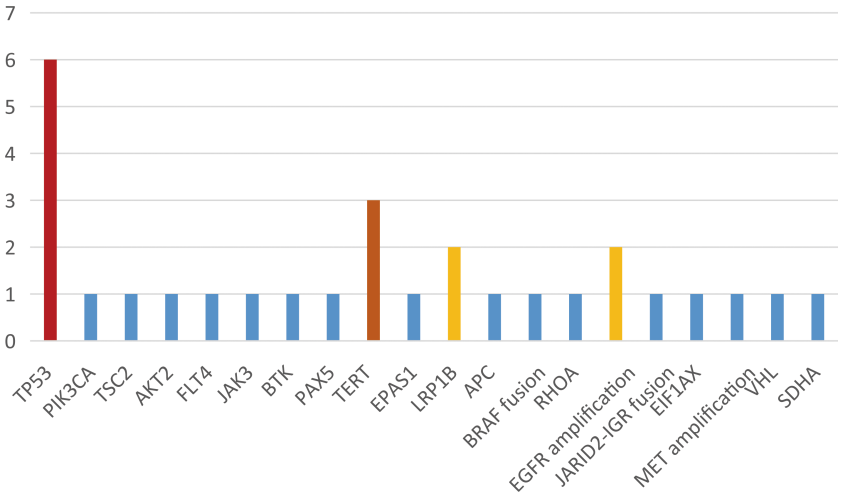

FIGURE 2
Distribution of co-mutation identified by next-generation sequencing.

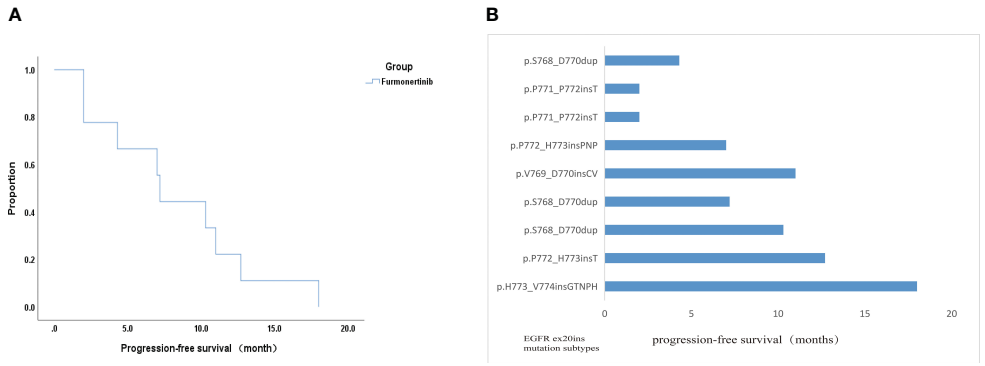

FIGURE 3
Efficacy analysis data of this study. **(A)** Kaplan-Meier curve displaying overall survival. **(B)** PFS of all patients with the EGFR ex20ins mutations in this study.

patients was 25%, with a median PFS of 9.7 months and a median DOR was 5.7 months (17). This result indicates that high-dose osetinib can be used as a treatment option for patients with good tolerance. However, there are unreasonable aspects to this research data, such as the median DOR is much smaller than the median PFS. Considering that the survival curve of small sample studies often presents a stepped shape, it indicates that the estimation of median PFS may not be stable. Besides, VAN VEGGEL et al. (18) found that osimitinib had limited effect on EGFR ex20ins NSCLC. After increasing the dose of osimitinib, they found that the ORR was 6.5% and median PFS was only 2.3 months, which did not show a better treatment effect. In September 2021, the FDA accelerated the approval of mobocertinib for the treatment of locally advanced or metastatic NSCLC patients with EGFR ex20ins mutations confirmed by FDA approved testing methods during or after platinum chemotherapy. The data from a phase I/II multicenter study (NCT02716116) showed that among advanced EGFR ex20ins mutant NSCLC patients who had previously received containing platinum chemotherapy, the ORR receiving mobocertinib (160 mg/d) was 43%, the median DOR was 13.9 months, the DCR was 86%, and the median PFS was 7.3 months (19). Another Phase I/II study published at the 2022 European Society for Medical Oncology (ESMO) conference showed a confirmed ORR of 28.1%, DCR of 78%, median PFS of 7.3 months, and median OS of 20.2 months (20). However, it lacks more data on first-line treatment.

Furmonertinib is one of the third generation EGFR-TKIs drug. The preclinical studies showed that the half-maximal inhibitory concentration (IC50) of furmonertinib for EGFR ex20ins type was 5-10 times lower than that of EGFR wild, which indicates furmonertinib has shown encouraging anti-tumor activity in EGFR 20ins (8). The FAVOUR Ib phase study targeting EGFR ex20ins mutant patients showed that the ORR of first-line treatment with furmonertinib 240 mg was 69%, the DCR was 96.6%, and the median PFS was 10.7 months (21). Another study targeting EGFR ex20ins mutant NSCLC patients with ≥ 2 lines showed that the overall ORR and DCR of 15 patients receiving furmonertinib treatment were 53.5%, 100%, and the 3-month PFS rate was 100% (22). Moreover, both of the above studies have shown good safety and tolerability of furmonertinib, and no ≥ level 3 adverse events have been found. However, they did not explore the efficacy of the furmonertinib 160mg as a first-line treatment on EGFR ex20ins mutant patients. In our study, we found that the ORR of first-line treatment with furmonertinib 160 mg was 66.7%, the DCR was 88.9% and the median PFS was 7.2 months (95% CI: 6.616 - 7.784)months. Our results showed the first-line treatment efficacy of furmonertinib was superior to traditional chemotherapy and classic EGFR-TKI drugs, such as gefitinib, erlotinib and osimitinib. Moreover, compared with furmonertinib 240 mg daily, our study showed that furmonertinib 160 mg daily also could produce good therapeutic effects on EGFR ex20ins mutant patients.

As we known, EGFR ex20ins has strong molecular heterogeneity and different insertion sites result in different drug efficacy. John V Heymach et al. found that poziotinib sensitivity was highly dependent on the insertion location, with near-loop insertions (amino acids A767 to P772) being more sensitive than far-loop insertions (23). In our study, we also have an interesting discovery that the PFS of one patient with p.H773_V774insGTNPH is up to 18 months. We noticed that the patient only had an EGFR ex20ins mutation and there is no concomitant mutation, which may be one of the reasons why patients have long PFS. Moreover, we noticed that the patients with N771_H772insT are more likely to develop central nervous system metastases and have poor response to furmonertinib. So far, no literature has reported this result. This result also requires a larger sample size to confirm. Hence, we can pay more attentions on the therapeutic effect of furmonertinib on different insertion location of EGFR ex20ins.

In summary, this study has demonstrated that furmonertinib had good anti-tumor activity and tolerance in NSCLC patients with EGFR ex20ins mutation. In first-line treatment, it is superior to

TABLE 2 Treatment-related adverse events.

TRAEs,n (%)	Grades 1–2	Grades 3–5
Diarrhea	1	0
Oral ulcer	1	0
Liver function damage	1	0

traditional targeted drugs and platinum containing chemotherapy regimens. Therefore, furmonertinib may be as a first-line treatment option for patients with advanced EGFR ex20ins NSCLC. In addition, the efficacy of furmonertinib may associated with different EGFR ex20ins variant types.

5 Conclusions

The study proved the potential efficacy of 160mg furmonertinib in patients with advanced NSCLC with EGFR ex20ins. Meanwhile, 160mg furmonertinib had a good safety profile.

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 humans were approved by the ethics committee of the First Hospital of Changzhou. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because this is a retrospective, multi-center, non-interventional study.

Author contributions

SH: Data curation, Writing – original draft. HM: Data curation, Writing – original draft. QH: Writing – original draft. MD: Writing – review & editing, Data curation. HD: Methodology, Writing – review & editing. CL: Methodology, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Activity of afatinib in patients with NSCLC harboring novel uncommon *EGFR* mutations with or without co-mutations: a case report

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Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) represent first-line standard of care in unresectable *EGFR* mutation-positive (*EGFR*m+) non-small cell lung cancer (NSCLC). However, 10–20% of patients with *EGFR*m+ NSCLC have uncommon *EGFR* variants, defined as mutations other than L858R substitutions or exon 19 deletions. NSCLC harboring uncommon *EGFR* mutations may demonstrate lower sensitivity to targeted agents than NSCLC with L858R or exon 19 deletion mutations. Prospective clinical trial data in patients with NSCLC uncommon *EGFR* mutations are lacking. Afatinib is a second-generation TKI and the only Food and Drug Administration-approved drug for some of the more prevalent uncommon *EGFR* mutations. We present a series of seven case reports describing clinical outcomes in afatinib-treated patients with NSCLC harboring a diverse range of extremely rare mutations with or without co-mutations affecting other genes. *EGFR* alterations included compound mutations, P-loop αC-helix compressing mutations, and novel substitution mutations. We also present a case with NSCLC harboring a novel *EGFR*::*CCDC6* gene fusion. Overall, the patients responded well to afatinib, including radiologic partial responses in six patients during treatment. Responses were durable for three patients. The cases presented are in line with a growing body of clinical and preclinical evidence that indicating that NSCLC with various uncommon *EGFR* mutations, with or without co-mutations, may be sensitive to afatinib.

KEYWORDS

EGFR, non-small cell lung cancer (NSCLC), afatinib, uncommon mutation, tyrosine kinase inhibitor

Introduction

Activating epidermal growth factor receptor (*EGFR)* mutations occur in 14–38% of non-small cell lung cancer (NSCLC) (1). EGFR tyrosine kinase inhibitors (TKIs) represent first-line standard of care in unresectable *EGFR* mutation-positive (*EGFR*m+) disease (2). Most *EGFR*m+ NSCLC is driven by the so-called classical or common *EGFR* mutations: L858R or exon 19 deletions (Del19) (3). Approximately 10–20% of *EGFR*m+ NSCLC cases harbor uncommon *EGFR* mutations, defined as activating *EGFR* mutations other than L858R and Del19 (3–5). Different variants demonstrate varying sensitivity to EGFR TKIs, and uncommon *EGFR* mutations show lower sensitivity to many targeted agents than classical *EGFR* mutations; therefore, precise characterization of uncommon *EGFR* mutations is important to optimize treatment strategies (4).

The most prevalent uncommon *EGFR* variants in NSCLC are S768I, L861Q, and G719X, for which the preferred first-line treatments in advanced disease are afatinib or osimertinib (6–9). Afatinib is the only U.S. Food and Drug Administration-approved drug against S768I, L861Q, and G719X *EGFR* mutations with demonstrated efficacy in prospective clinical studies (10). Little prospective data exist for uncommon mutations; however, retrospective studies (7, 11–15) and databases (11, 16–18) provide some insight. Novel mutations continue to be identified that have no available clinical data to guide treatment decisions.

A recent preclinical investigation defined a structure-based classification system that permitted prediction of sensitivity to different generations of EGFR TKIs (first generation: erlotinib, gefitinib; second generation: afatinib, dacomitinib; third generation: osimertinib) (4). "Classical-like" mutations (e.g. L858R; Del19; S720P; L861Q/R) were predicted to be sensitive to all generations of EGFR TKI; "T790M-like" mutations (e.g. T790M; certain T790M-containing compound mutations) were predicted to be sensitive to third-generation EGFR TKIs; exon 20 insertions (e.g. S768dupSVD; A767dupASV) were predicted to be sensitive to exon 20 insertion-targeted compounds and second-generation EGFR TKIs; and P-loop αC-helix compressing (PACC) mutations (e.g. G719X; S768I; delE709_T710insD, and other uncommon *EGFR* mutations were predicted to be particularly sensitive to second-generation EGFR TKIs (4). PACC mutations occur across exons 18–21 and alter the orientation of the P-loop or αC-helix of EGFR, affecting interactions with certain TKIs. Second-generation TKIs do not interact with the P-loop of EGFR and are therefore predicted to have greater activity against PACC mutations than other generations of EGFR TKI (4). Some retrospective data support this prediction (4).

Treatment decisions can be very challenging in patients with NSCLC with multiple *EGFR* mutations (compound mutation) or uncommon *EGFR* mutations co-occurring with other gene alterations in the tumor. Treatment might be dependent on which mutation has the higher allele frequency (19) or which other cancer-related genes have co-occurring mutations (20, 21). For example, *TP53*, the most commonly mutated gene in NSCLC, co-occurs in ~65% of cases of *EGFR*m+ NSCLC, and has been associated with poor prognosis and primary/acquired resistance to EGFR TKIs (22–27).

This case series describes outcomes of patients with NSCLC harboring uncommon *EGFR* mutations who received afatinib. Cases were collected during routine clinical treatment across six centers in Germany between 2017 and 2023.

Case descriptions

Patients with an *EGFR* PACC mutation as part of a compound mutation

Case 1: G719A/L833F

After presenting with a cough, a 58-year-old female with a history of paroxysmal atrial fibrillation and hemangioma was diagnosed with lung adenocarcinoma (stage IB) in May 2017, and underwent resection of the left upper lobe and lymphadenectomy. In August 2017, a single symptomatic metastasis to the third lumbar spine vertebrae with infiltration of the major psoas was detected. The patient received radiotherapy and began treatment with denosumab (120 mg every 4 weeks; Figure 1A). However, in September 2017, a new abdominal lesion next to the left lobe of the liver was reported. The patient refused biopsy to confirm diagnosis of distant metastases. Hybrid capture next-generation sequencing (NGS) of the initially resected tumor tissue identified a novel compound *EGFR* mutation, comprising two substitution mutations on exons 18 (G719A, a PACC mutation) and 21 (L833F, a classical-like mutation). A *TP53* point inactivating mutation (p.T140 frame shift [non-activating mutation]) was also detected.

The patient began treatment with first-line afatinib, 40 mg once per day (QD), in September 2017. In November 2017, following grade 3 diarrhea, grade 2–3 stomatitis, and rhagades of the fingers, the dose of afatinib was reduced to 30 mg QD.

The patient achieved complete remission of the abdominal lesion, with the response lasting 28 months. Metastases were detected in the left adrenal gland in January 2020. In February 2020, the patient underwent adrenalectomy (R1) followed by radiotherapy and continued afatinib treatment. Disease was stable until June 2020.

In August 2020, disease progression was observed in the area of the former adrenal gland. Urinary retention was treated with a double J-tumor stent and the patient experienced urosepsis (*Proteus mirabilis*, two events) and nephroptosis. Following local progression and subsequent left nephrectomy in October 2020, afatinib therapy was terminated in December 2020, and the patient received second-line therapy with carboplatin/paclitaxel, atezolizumab, and bevacizumab. The total duration of afatinib treatment was 35 months.

Case 2: G719A/L861R

A 71-year-old male with a history of polymyalgia rheumatica consulted his general practitioner with concerns relating to a family history of cancer. In May 2021, elevated serum tumor markers were

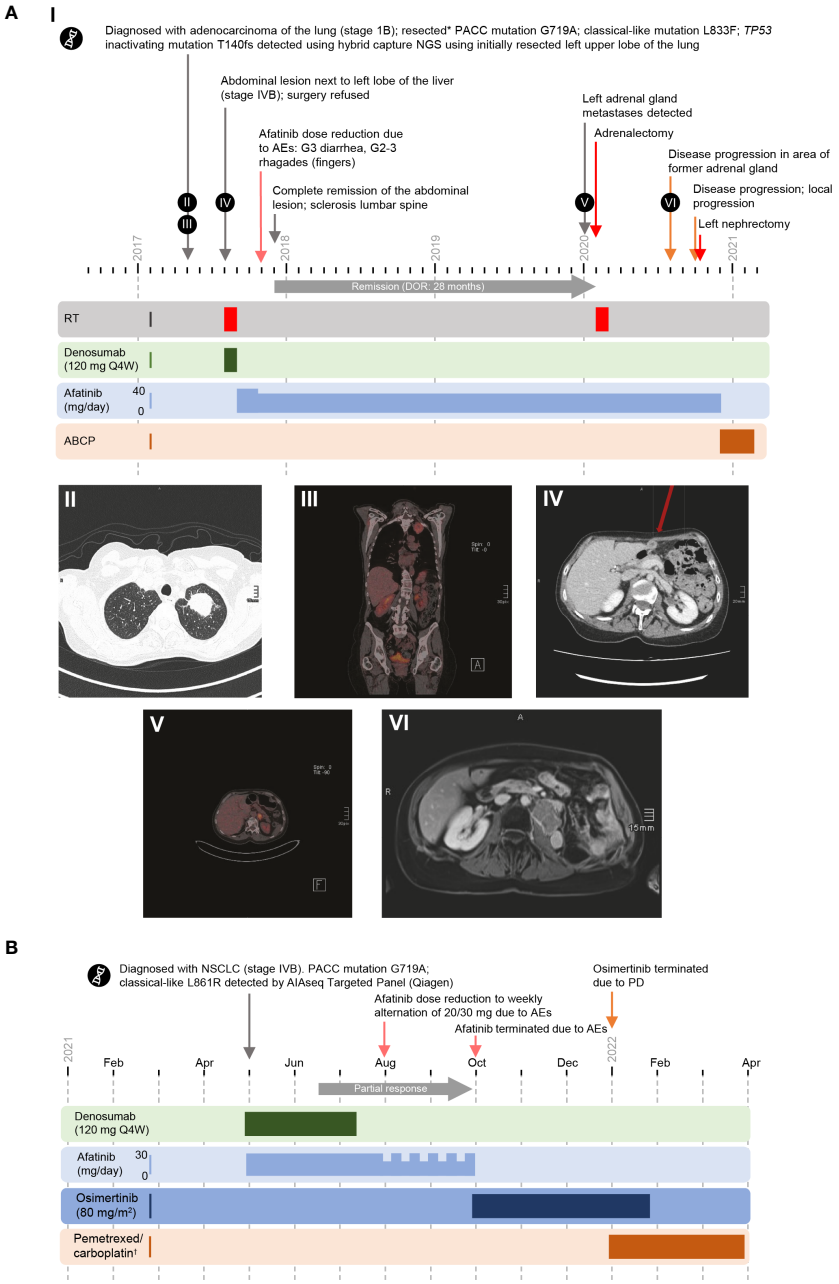

FIGURE 1
Cases 1 and 2 (PACC/classical compound *EGFR* mutation). **(A)** I Timeline of Case 1. II, III. May 2017. Tumor staging pT2a pN0 M0, St. IIA UICC 8. IV. August 2017. A single symptomatic metastasis and infiltration of the major psoas was detected. V. January 2020. Adrenal metastases detected. VI. August 2020. Disease progression was observed in the area of the former adrenal gland. **(B)** Timeline of Case 2. *Single symptomatic metastasis and infiltration of the major psoas. †488.12 mg carboplatin AUC 80%; 796 mg pemetrexed 80%. ABCP, atezolizumab plus bevacizumab plus carboplatin plus paclitaxel; AE, adverse event; DOR, duration of response; G, grade; NGS, next generation sequencing; NSCLC, non-small cell lung cancer; PACC, P-loop and αC-helix compressing; PD, progressive disease; Q4W, every 4 weeks; RT, radiotherapy; UICC, Union for International Cancer Control.

reported, and the patient was subsequently diagnosed with NSCLC (stage IVB; programmed death-ligand 1 [PD-L1]: 1%; pulmonary and bone metastases) (Figure 1B). NGS (QIAseq Custom Lung Panel, Qiagen) identified a novel compound mutation comprising substitution mutations on exon 18 (G719A, PACC) and exon 21 (L861R, classical-like).

The patient began treatment in May 2021, with first-line afatinib (30 mg QD) plus denosumab (120 mg every 4 weeks). Following presentation with exanthem (June/July 2021, treated with topical corticosteroid) and diarrhea (August 2021, treated with loperamide), the dose of afatinib was reduced to weekly alternation of 20/30 mg.

Partial responses (PRs) were reported in June, July, and August 2021. After approximately 5 months on treatment, stable disease (SD) was reported. However, afatinib was terminated in October 2021 owing to intolerable adverse events (AEs), and osimertinib

(80 mg QD) was initiated. Progressive disease (PD) was reported in January 2022, which resulted in discontinuation of osimertinib and initiation of pemetrexed and carboplatin treatment.

Patients with PACC exon 18 deletion insertion mutations

Case 3: delE709_T710insN

A 64-year-old male presented with persistent cough. A computerized tomography (CT) scan revealed pulmonary nodules on both sides of the lung, and following a biopsy by bronchoscopy the patient was diagnosed with thyroid transcription factor-1 (TTF1)-positive adenocarcinoma (stage IA) in May 2010, with resection in the same month (Figure 2A). Relevant comorbidities included arterial hypertension, treated with candesartan (32 mg). In June 2019, aged 73 years, he was diagnosed with bilateral pulmonary metastases (TTF1-positive adenocarcinoma), following biopsy by bronchoscopy. NGS detected an uncommon *EGFR* exon 18 deletion insertion mutation (delE709_T710insN), classified as PACC (4).

The patient began first-line afatinib (30 mg QD) in October 2019. Following emergence of grade 3 diarrhea, afatinib was paused and the patient was treated with loperamide and hydration until symptoms had resolved. Following grade 1 paronychia, the dose of afatinib was reduced to 20 mg QD. No further AEs occurred.

The patient achieved a best response of PR after 3 months of treatment with afatinib. The patient reported good quality of life with no clinical symptoms of disease. Afatinib was continued for 27 months and was discontinued in January 2022 at the patient's request. In May 2023, progression of the lung metastases was observed following a CT scan. Afatinib was resumed and PR was observed in July 2023. As of September 2023, the patient remains symptom free.

Case 4: delE709_T710insD

A 64-year-old female was diagnosed with NSCLC (stage IVB) in November 2017 during workup of a painful pathologic fracture in the 8th thoracic vertebra (Figure 2B). Radiologic imaging revealed a central tumor of the left lower lung lobe, as well as additional bone and brain metastases. Relevant comorbidities included arterial hypertension, chronic bronchitis, osteoporosis, and gastritis. NGS identified delE709_T710insD with a co-occurring *CD274/PD-L1* mutation (P146R).

The patient received whole-brain radiotherapy from November to December 2017, with palliative radiotherapy (thoracic spine, lumbar, 20 Gy, 5 Gy/fraction) in November 2017. The patient began first-line erlotinib (150 mg QD) plus intravenous zoledronic acid (4 mg every 3 weeks) in December 2017. Following PD in May 2018, treatment was discontinued in June. The patient received second-line afatinib (40 mg QD) starting in June 2018, and achieved a PR in

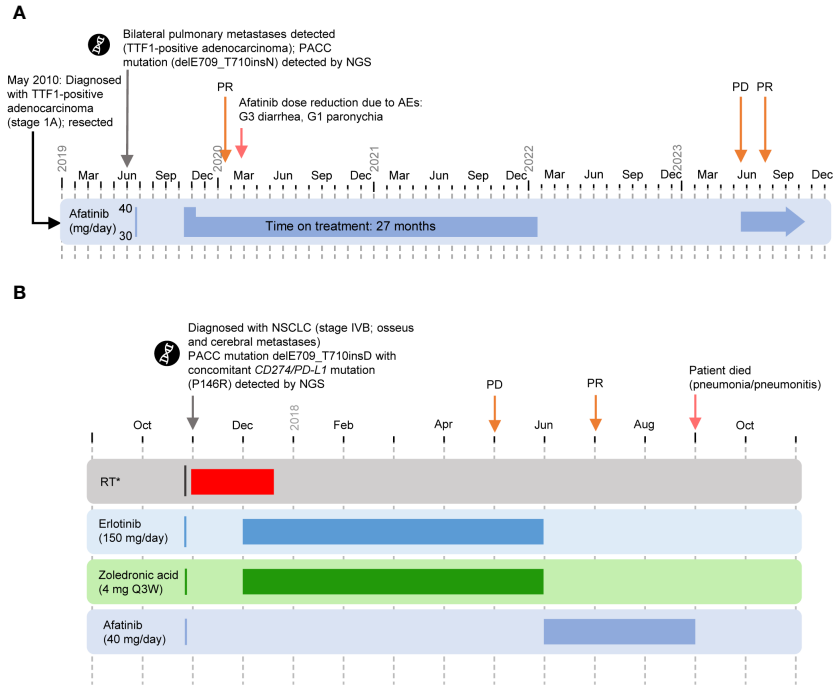

FIGURE 2
Cases 3 and 4. (PACC exon 18 deletion insertion mutation) **(A)** Timeline of Case 3. Afatinib was discontinued in January 2022 at the patient's request. In May 2023, progression of the lung metastases was observed following a CT scan. Afatinib was resumed and PR was observed in July 2023. As of September 2023, the patient remains symptom free. **(B)** Timeline of Case 4. *Nov 2017: palliative RT (thoracic spine, lumbar, Os pubis left [5 Gy/Fraction]. WBRT: Nov–Dec 2017. Zoledronic acid: Dec 2017–Jun 2018. First-line treatment with erlotinib: Dec 2017–Jun 2018. Second-line treatment with afatinib: Jun–Sep 2018. AE, adverse event; G, grade; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; PACC, P-loop and αC-helix compressing; PD, progressive disease; PR, partial response; Q3W, every 3 weeks; RT, radiotherapy; TTF-1, thyroid transcription factor-1; WBRT, whole brain radiotherapy.

July 2018. Subsequently, the patient experienced pneumonitis probably related to preceding radiotherapy of the thoracic spine, leading to death in September 2018 after approximately 3 months on treatment.

Rare substitution mutations

Case 5: H988R substitution

A 74-year-old male presenting with weight loss was diagnosed with NSCLC (stage IVA, with pulmonary and pleural metastases) in February 2018 (Figure 3A). Relevant comorbidities included arterial hypertension, non-erosive reflux disease, chronic hepatitis C infection, hiatal hernia, and other gastrointestinal conditions. NGS testing confirmed a rare *EGFR* exon 25 mutation, H988R, with co-occurring *TP53* and *CDKN2A* mutations.

First-line treatment with afatinib (40 mg QD) started April 2018 and a PR was reported in August 2018. In January 2020, afatinib treatment was paused for 2 weeks because of diarrhea, rash, and neutropenia, and then restarted at a dose of 30 mg QD. The dose of afatinib was further reduced to 20 mg QD in February 2020. PR was maintained until at least February 2021 (date of last imaging). The patient deteriorated clinically without tumor progression and died in July 2021. Total duration on afatinib was 39 months; no other treatment was reported.

Case 6: Q982K substitution

In September 2018, an asymptomatic 65-year-old male with a history of arterial hypertension, latent diabetes mellitus, and degenerative spinal syndrome, was diagnosed with NSCLC (stage IIIA/IV) following magnetic resonance imaging examination of the cervical spine (Figure 3B); suspicious enlargement of the left adrenal gland was also observed. Positron emission tomography-CT standardized uptake values were 11–15 for the primary lung tumor and mediastinal lymph nodes, and four for the left adrenal gland. After discussion with the interdisciplinary tumor board, it was agreed to treat the patient according to stage III disease management practice and continue to monitor the left adrenal gland with serial imaging. Molecular testing confirmed a novel point mutation in *EGFR* exon 24 (Q982K) with co-occurring *TP53*, *CDKN2A*, and *PDGFRA* mutations.

Cisplatin (80 mg/m^2 day 1 every 3 weeks) plus vinorelbine (25 mg/m^2 day 1 and day 8 every 3 weeks), was initiated in October 2018. An episode of tinnitus prompted a switch from cisplatin to carboplatin (AUC 5) from cycle 2. Sequential radiotherapy (60 Gy) began in January 2019. Best overall response to chemoradiotherapy was SD in March 2019.

Cerebellar metastases were detected in April 2019, and were resected in the same month, followed by whole-brain radiotherapy in May 2019. The patient received second-line afatinib (40 mg QD) starting in May 2019. Despite a stable thoracic tumor, new liver

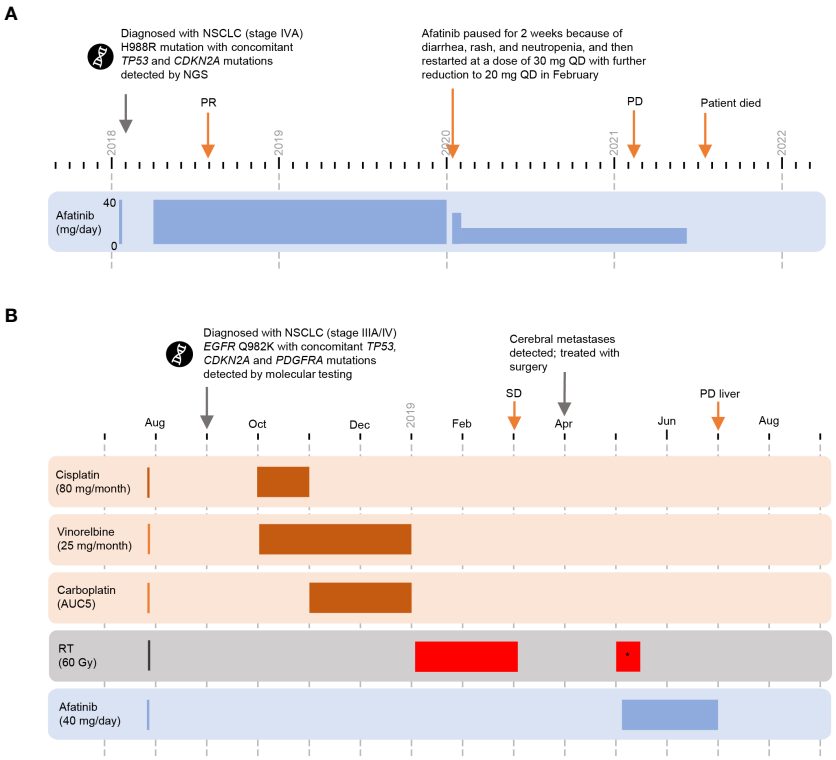

FIGURE 3
Cases 5 and 6 (novel substitution mutations) **(A)** Timeline of Case 5. **(B)** Timeline of Case 6. *whole brain radiotherapy. *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *EGFR*, epidermal growth factor receptor; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; PD, progressive disease; PR, partial response; QD, each day; RT, radiotherapy; SD, stable disease; *TP53*, tumor protein 53.

lesions were detected in July 2019 and afatinib treatment was terminated. The total duration of afatinib treatment was 2 months.

EGFR fusion

Case 7: *EGFR::CCDC6* fusion

A 56-year-old female ex-smoker (until 2005, 20 pack-years) with a history of bronchial asthma who presented with pleural effusion affecting the left thorax was diagnosed with stage IVB (UICC) adenocarcinoma NSCLC (primary lesion left lower lobe) in November 2022 (TTF-1-positive, *CK7* positive, Ki-67 score: 70%; PD-L1 Tumor Proportion Score 0%, PD-L1 immune cells 0%; cT3 pN2 cM1a [pleural, pulmonary, osseus, and cervical lymph nodes]). First-line treatment (carboplatin/pemetrexed/pembrolizumab ± vibostolimab/denusomab) as part of the KeyVibe-007 trial (EUDRA-CT: 2021-004564-94) began in January 2023 (Figure 4). At restaging in May 2023, multiple new bilateral pulmonary metastases were detected and participation in the trial ended.

Molecular testing (Archer FusionPlex Lung Panel) in January 2023 detected an *EGFR* exon 24::*CCDC6* (coiled-coil domain containing 6) exon 2 fusion. Treatment with second-line afatinib 30 mg QD began in June 2023. Regression of the primary lesion and complete resolution of pulmonary metastases were observed after 4 weeks. Treatment and response are ongoing.

Discussion

This report describes outcomes with afatinib in NSCLC with a diverse range of extremely rare *EGFR* alterations found in routine clinical practice (Supplementary Figure 1). Five patients harbored rare aberrations that, to the best of our knowledge, have not been previously described in literature. Four patients had known PACC mutations either in isolation or as part of a compound *EGFR* mutation. Two patients had PACC mutations with co-occurring mutations in *TP53* or *PDGFRA*. One patient had an *EGFR* gene fusion, a rare type of driver event. Overall, these patients responded well to afatinib (Supplementary Table 1), consistent with preclinical modelling (4) and previous studies of afatinib treatment in patients with uncommon mutations (11, 28).

Cases 1 and 2 involved compound mutations comprising PACC and classical-like mutations which both responded to afatinib. Case 1 had a durable response to afatinib despite the presence of a co-occurring *TP53* mutation plus a novel compound *EGFR* mutation, that included substitution mutations on exons 18 (G719A; a PACC mutation) and 21 (L833F; a classical-like mutation). Cases 3 and 4 exhibited PACC exon 18 deletion insertion mutations, and both patients had a clinical response to afatinib treatment, including a long (>2 years) response reported for Case 3. Case 4 also harbored a concomitant *CD274/PD-L1* mutation, which we believe has not previously been described. Accumulating evidence indicates that delE709_T710insD is sensitive to afatinib and does not appear to be affected by the concomitant mutation. Cases 5 and 6 had *EGFR* substitution mutations in exon 25 (H988R) and exon 24 (Q982K), respectively; situated in the cytoplasmic region C-terminal domain, beyond the tyrosine kinase domain. Mutations here may destabilize receptor conformation, potentially causing upregulation of kinase activity and irregular downstream signaling (29). Both had co-occurring mutations in other genes, which are known to be prognostic biomarkers (23, 30–32). In Cases 5 and 6, prolonged survival was observed with first-line afatinib. In Case 7, the patient with the fusion, a dramatic response was observed in response to second-line afatinib. It is currently unknown how these rare mutations, and the *EGFR*::*CCDC6* fusion, align with the structure-based classification system (4), highlighting the difficulty associated with making treatment decisions for patients with novel mutations.

The selection of optimal treatment for patients with rare or compound *EGFR* mutations is often complex. Previous case reports describe compound mutations comprising substitution mutations classified as PACC and classical-like that respond to TKIs (33, 34). We found only one other report of a L833F-containing mutation, in which a patient with an L833F/L861Q mutation also achieved durable PR in response to first-line afatinib (progression-free survival [PFS]: 10 months) and clinical benefit to later-line osimertinib (34). A previous review briefly mentions the patient described in Case 5 with the H988R substitution (35). The review also mentions an additional patient with an H988R mutation who did not respond to afatinib treatment (35). While the recent structure-based classification system (4) has provided helpful information regarding predicted sensitivities of uncommon mutations, the sensitivities of rare compound mutations and the influence of co-occurring mutations remain difficult to predict in the absence of prospective clinical trials. Treatment decisions for these patients requires careful consideration.

Previous case reports of *EGFR* PACC insertion deletion mutations in NSCLC indicate sensitivity to afatinib and other EGFR TKIs (gefitinib, erlotinib) (36–38), including a 23-month PFS response with afatinib (37). Case 4 achieved a PR with afatinib after PD on erlotinib, which is also consistent with a previous case study where clinical benefit with afatinib following prior erlotinib treatment was reported (39). We have identified 16 reports of patients with a delE709_T710insD mutation who received EGFR TKIs (16), and consistent with the preclinical modelling (4), delE709_T710insD-mutated NSCLC appears to be more sensitive to afatinib than first-generation TKIs. In a review of 14 cases, PFS was significantly improved with afatinib compared with first-generation TKIs (median 7.0 *vs.* 3.1 months; $p = 0.005$) and all patients receiving afatinib achieved a PR (36).

Although *EGFR* gene fusions are rare, clinical responses in *EGFR* fusion-driven tumors have been reported with EGFR TKIs (40, 41). The *EGFR*::*CCDC6* fusion is novel, to our knowledge; however *CCDC6*-tyrosine kinase fusions (for example with *ALK*, *ROS1*, or *RET*), are recognized–and druggable–driver events in lung cancer (42). The durable response in this patient reinforces the importance of testing for fusion driver events, as this important class of somatic alteration can underly disease sensitive to targeted agents.

The interplay between *EGFR* alterations and co-occurring mutations in different genes represents a new frontier for NSCLC clinical research. In our case series, three patients had co-mutations

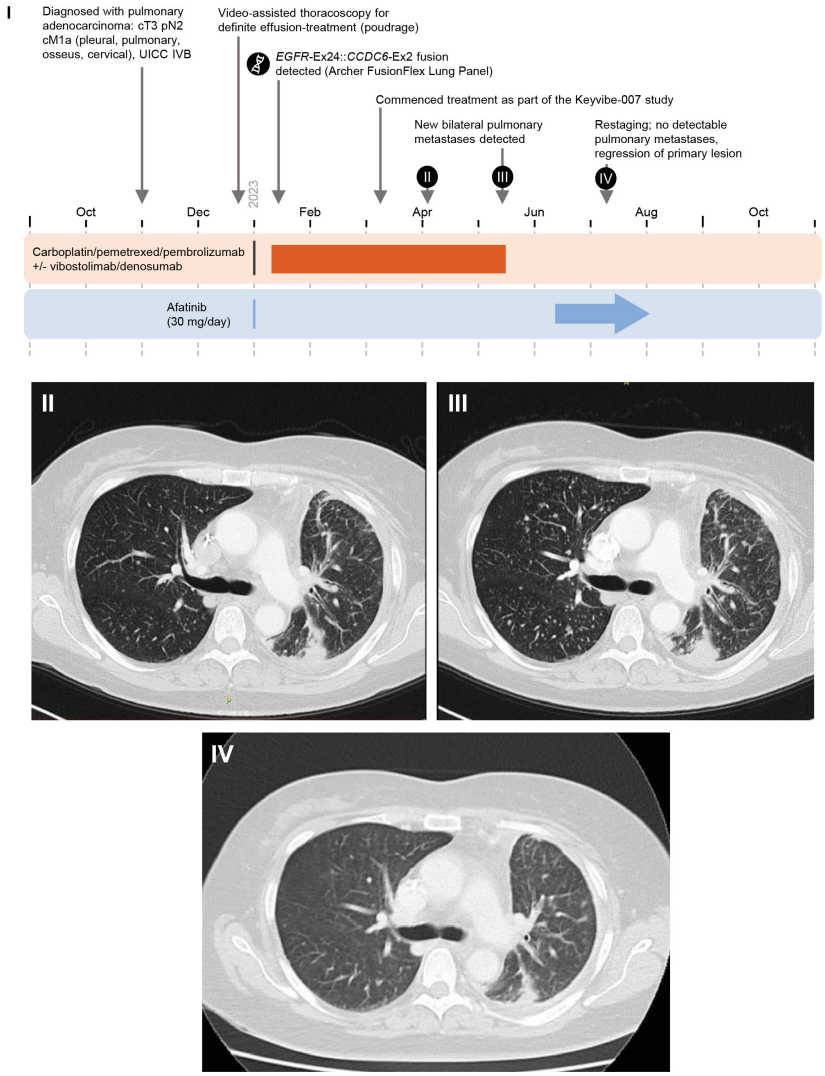

FIGURE 4
Case 7 (novel *EGFR::CCDC6* fusion) I. Timeline of Case 1. II. April 2023. III. May 2023, New bilateral pulmonary metastases detected. IV. July 2023. Restaging: no detectable pulmonary metastases, regression of primary lesion. Ex, Exon; UICC, Union for International Cancer Control.

in *TP53*, two had co-mutations in *CDKN2A*, and one had overexpression of PD-L1, plus a co-mutation affecting, *CD274/PD-L1*. These alterations occur commonly in patients with *EGFR*m+ NSCLC and have been associated with poor prognosis and resistance to TKIs (23, 30–32). However, in our case series, co-mutations did not prevent patients gaining benefit from afatinib treatment. Two patients with co-occurring *TP53* mutations exhibited prolonged time on treatment (35 and 39 months), durable response to afatinib was observed in one of the two patients with *CDKN2A* mutations (both patients with substitution mutations), and a PR was observed in the patient with a *CD274/PD-L1* mutation. Decisions about the initial treatment of NSCLC with uncommon *EGFR* mutations have key importance for the subsequent course and should be made carefully based on published evidence about TKI efficacy, as this can vary widely according to the specific mutation (43), and real-world data indicate that approximately 30–35% of patients do not receive treatment after the first line (44).

Reports of NSCLC with TKI-sensitive uncommon *EGFR* mutations may also prompt further clinical trial research in this setting; however, if clinical practice is to evolve, obstacles in terms of mutation testing must also be overcome. Current guidelines recommend that broad molecular profiling should be carried out for all patients diagnosed with NSCLC, which generally means NGS-based testing (9, 45). Globally, rates of molecular profiling in lung cancer patients are below 50% with a wide regional variation (46); financial constraints, quality and standardization of testing, access to testing, awareness, and turnaround times have all been cited as barriers to testing. Furthermore, some commonly used testing panels may miss uncommon mutations or those occurring outside of Exons 19, 20, and 21 (47, 48). Advances in testing strategies and methodologies have the potential to improve molecular profiling in NSCLC; these include the adoption of the structure-based classification system into testing panels and the use of liquid biopsy as a rapid, non-invasive means of assaying genomic profiles (49). Liquid biopsy may be particularly useful for

monitoring temporal changes in mutation and biomarker status and is already in use to detect resistance to EGFR TKIs (50).

Integration of classification systems and real-world evidence may support future treatment decisions in patients with uncommon *EGFR*m+ NSCLC. Better understanding of the impact of co-occurring mutations is required. Patients with uncommon *EGFR* mutations are now being included in several ongoing randomized clinical trials assessing the efficacy and safety of EGFR TKIs in NSCLC (51–54). In a recent analysis of the phase III ACHILLES trial in treatment-naive patients with uncommon or compound *EGFR* mutations, PFS with afatinib (10.6 months) was significantly longer than with platinum-based chemotherapy (5.7 months; hazard ratio: 0.422, $p = 0.0007$), supporting the use of first-line EGFR TKIs in this setting (55).

Conclusion

These cases corroborate the clinical and preclinical evidence that certain uncommon *EGFR* mutations are sensitive to afatinib. This series illustrates the importance of further study in this area and the need for publicly available mutation databases to support prescribing decisions in the absence of prospective clinical trial data for patients with rare mutations.

Data availability statement

The datasets generated and analyzed during the study are available from author AS on reasonable request. Requests to access these datasets should be directed to AS: https://anja.stammberger@boehringer-ingelheim.com.

Ethics statement

Ethical approval was not required for the studies involving humans because Manuscript details a collection of case studies from patients treated in routine practice, not results for a formal study. The studies were conducted in accordance with the local legislation and institutional requirements. The 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. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

PC: Data curation, Formal analysis, Writing – review & editing. FH: Data curation, Formal analysis, Writing – review & editing. PH: Data curation, Formal analysis, Writing – review & editing. MF: Data curation, Formal analysis, Writing – review & editing. MT: Data curation, Formal analysis, Writing – review & editing. H-GK: Data curation, Formal analysis, Writing – review & editing. AA: Data curation, Formal analysis, Writing – review & editing. AS: Data curation, Formal analysis, Writing – review & editing. EL: Data curation, Formal analysis, Writing – review & editing.

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Conflict of interest

AS is a current employee of Boehringer Ingelheim. PC reports research funding from Amgen, AstraZeneca, Boehringer Ingelheim, Novartis, Roche, Takeda, and advisory board/lecture fees from AstraZeneca, Boehringer Ingelheim, Chugai, Daiichi Sankyo, Gilead, Janssen, Novartis, Pfizer, Roche, Takeda, and Thermo Fisher.

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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.2024.1347742/full#supplementary-material

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Weighted gene co-expression network analysis and whole genome sequencing identify potential lung cancer biomarkers

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Background: Tuberculosis (TB) leads to an increased risk of lung cancer (LC). However, the carcinogenetic mechanism of TB remains unclear. We constructed gene co-expression networks and carried out whole-exome sequencing (WES) to identify key modules, hub genes, and the most recurrently mutated genes involved in the pathogenesis of TB-associated LC.

Methods: The data used in this study were obtained from the Gene Expression Omnibus (GEO) and WES. First, we screened LC-related genes in GSE43458 and TB-related genes in GSE83456 by weighted gene co-expression network analysis (WGCNA). Subsequently, we screened differentially expressed genes related to LC and TB in GSE42834. We also performed WES of 15 patients (TB, n = 5; LC, n = 5; TB+LC, n = 5), constructed mutational profiles, and identified differences in the profiles of the three groups for further investigation.

Results: We identified 278 hub genes associated with tumorigenesis of pulmonary TB. Moreover, WES identified 112 somatic mutations in 25 genes in the 15 patients. Finally, four common genes (EGFR, HSPA2, CECR2, and LAMA3) were confirmed in a Venn diagram of the 278 hub genes and the mutated genes from WES. KEGG analysis revealed various pathway changes. The PI3K–AKT signaling pathway was the most enriched pathway, and all four genes are included in this pathway. Thus, these four genes and the PI3K–AKT signaling pathway may play important roles in LC.

Conclusion: Several potential genes and pathways related to TB-associated LC were identified, including EGFR and three target genes not found in previous studies. These genes are related to cell proliferation, colony formation, migration, and invasion, and provide a direction for future research into the mechanisms of LC co-occurring with TB. The PI3K–AKT signaling pathway was also identified as a potential key pathway involved in LC development.

KEYWORDS

lung cancer, tuberculosis, co-expression, whole exome sequencing, EGFR

Introduction

Lung cancer (LC) is a leading cause of cancer-related deaths and a critical barrier to increasing life expectancy worldwide (1). Tuberculosis (TB) is one of the major deadly infectious diseases and remains a global public health threat (2). TB increases the risk of LC and affects the prognosis of LC patients. The incidence of lung cancers is approximately 11-fold higher in the cohort of patients with TB compared with non-tuberculosis subjects (26.3 versus 2.41 per 10,000 person-years) (3, 4). Several prospective and retrospective studies have suggested that TB is associated with an increased risk of lung cancer (5–7).

Early diagnosis bias and late treatment strategies for TB might be factors responsible for the high co-occurrence of TB and LC (8–11). TB diagnosis is performed by QuantiFERON-TB Gold In-Tube tests as a gold standard (5, 12). Pulmonary comorbidities can considerably obscure LC symptoms and delay diagnosis, or may preclude a comprehensive diagnostic examination with adequate illness staging. The risk of LC should be assessed before starting treatment for TB, with the aim of preventing LC development. Therefore, there is an urgent need to explore signature genes closely associated with the development of LC to allow early diagnosis of the development of LC in TB patients.

Co-expression networks are useful to describe pairwise relationships between gene transcripts (13). Here, we used weighted gene co-expression network analysis (WGCNA) to calculate associations between gene significance (GS) and module membership (MM), analyze the correlation between modules to construct a weighted gene co-expression network, and merge differentially expressed genes (DEGs) with key module genes for functional analysis. By constructing the protein–protein interaction (PPI) network, we detected certain hub genes as key factors regulating the occurrence of LC.

For further research, 15 patients (TB, n = 5; LC, n = 5 (3 adenocarcinoma, 2 non-small cell lung cancer); TB+LC, n = 5) were recruited and whole-exome sequencing (WES) was performed on the primary fresh tissues. Mutational profiles of the 15 patients were constructed based on the sequencing data, and differences in the mutational profiles between the three groups of patients were investigated further. The combination of the WGCNA and the identified DEGs revealed four target hub genes (EGFR, HSPA2, CECR2, and LAMA3) that may be potential biomarkers for LC diagnosis and treatment.

Abbreviations: LC, lung cancer; TB, tuberculosis; WGCNA, weighted correlation network analysis; WES, Whole-Exome Sequencing; GEO, Gene Expression Omnibus; DEGs, differentially expressed genes; PPI, protein–protein interaction; MM, module membership; GS, gene significance; TOM, Topological overlap matrix; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function.

Material and methods

Data information

The GSE43458, GSE83456, and GSE42834 datasets were obtained from NCBI Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/). GSE43458 consists of 80 lung cancer samples and 30 control samples run on an Affymetrix Human Gene 1.0 ST Array [HuGene-1_0-st]. GSE83456 contains 45 pulmonary tuberculosis samples and 61 control samples run on an Illumina HumanHT-12 V4.0 Expression BeadChip. GSE42834 contains 20 controls, eight patients with lung cancer, and 19 pulmonary tuberculosis patients, also run on an Illumina HumanHT-12 V4.0 Expression BeadChip. The R packages affy and annotate were used to process the raw data, make an expression matrix, and match probes to their gene symbol. The R package Limma was used to screen the DEGs based on the GSE42834 data. All DEGs were screened with q-value < 0.001 and |log2FC| > 0.5 as thresholds. The common differential genes in these results were selected for functional analysis.

Patient samples

This study was performed according to the Declaration of Helsinki (2013) of the World Medical Association. The study protocol was approved by the Ethics Committees of The Xinjiang Uygur Autonomous Region Chest Hospital (approval number 2021BAT011). Fresh primary tissues were collected from 15 pathologically confirmed patients undergoing surgery for lung cancer, pulmonary tuberculosis, or lung cancer combined with pulmonary tuberculosis at The Eighth Affiliated Hospital of XinJiang Medical University (Urumqi, China). Histological diagnosis of tumors was performed and confirmed by two pathologists. Samples were immediately frozen in liquid nitrogen and stored at −80°C until further analysis. The clinicopathological features of the 15 patients are presented in Supplementary Table S5.

WGCNA construction

Based on the expression and clinical pathological data of the GSE43458 and GSE83456 datasets, the genes showing the top 60% variance were selected for weighted gene co-expression network analysis (WGCNA). This was used to calculate the correlation coefficients between genes for clustering and to construct a co-expression weighted network. The hierarchical clustering function was used to classify genes with similar expression profiles into modules based on the topological overlap matrix (TOM) dissimilarity with a minimum size of 30 for the gene dendrogram. The blue modules were significantly associated with TB, and the turquoise and yellow modules were significantly associated with LC. The dissimilarity of module eigengenes (MEs) was calculated to choose a cut-off to merge some modules. Lastly, the network of eigengenes was also visualized.

Identification of clinically significant modules

To determine the appropriate modules, we computed the association between MEs and clinical traits. The log10 transformation of the P-value (GS = lgP) in the linear regression between gene expression and clinical data was then used to establish gene significance (GS). The average GS for every gene in a module was also defined as the module membership (MM). The module associated with a clinical feature was the one with the highest absolute MM ranking out of all the modules that were chosen.

Protein–protein interaction network analysis

The protein–protein interaction (PPI) network was constructed using the Search Tool for the Retrieval of Interacting Genes (STRING) database (https://string-db.org), which covered almost all functional interactions between the expressed proteins, and interactions with a combined score greater than 0.4 were considered statistically significant. The results of this investigation were shown using Cytoscape software (version 3.7.0).

Enrichment analysis

The R packages clusterProfiler and org.Hs.eg.db were used for Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the hub genes for all data.

DNA extraction and quality control

Genomic DNA (gDNA) was extracted from the recently frozen tissues using the QIAmp DNA Tissue Kit (TIANGEN, Beijing, China) following the manufacturer's instructions. The quantity and purity of the gDNA were assessed using a Qubit® 2.0 fluorometer (Thermon Fisher Scientific. Waltham, MA, USA) and a NanoDrop 2000 (Thermo Fisher Scientific, Inc.). The fragmentation status was evaluated using the Agilent 2200 TapeStation system using the Genomic DNA ScreenTape assay (Agilent Technologies, Santa Clara, CA, USA) to produce a DNA integrity number. An additional quality control step to determine the DNA integrity was performed using a multiplex PCR approach.

Library preparation, hybridization capture, and amplification

A total of 300 ng of each gDNA sample, based on the Qubit quantification, was mechanically fragmented (duty factor, 10%; peak incident power, 175 W; cycles per burst, 200; treatment time, 240 s; bath temperature, 2–8°C) on an M220 focused ultrasonicator (Covaris, Inc.). The target DNA fragment size was 350 bp. Subsequently, 200 ng of sheared gDNA was used to perform end repair, A-tailing, and adapter ligation with a library preparation kit (Agilent Technologies, Inc.) according to the manufacturer's protocol. Subsequently, the libraries were captured using Agilent SureSelect XT custom 0.5–2.9M probes (Agilent Technologies, Inc.) and amplified. The captured libraries were sequenced on an Illumina NovaSeq 6000 PE150 (Illumina Inc.) for 2 × 150 paired end reads, using Cycle Sequencing v3 Reagents (Illumina).

Bioinformatics analysis

Clean data were obtained after filtering out the adapters and reads with a proportion of N > 10%, with N being the unidentified bases in the sequencing process, using fastp (fastp 0.20.0). Low-quality bases (Phred score < 15) were excised from the 3′ ends of reads. Only sequenced samples with >80% of data with a quality score ≥ Q30 (95% of base call accuracy) were used in the analysis. Reads with length < 50 bp after excision were removed. The clean data were mapped to the human reference genome (University of California Santa Cruz ID: hg38) using the Burrows–Wheeler Alignment algorithm (BWA 0.7.17). The alignment in SAM format was converted to BAM files using SAMtools (SAMtools 1.9.0). Next, the genome analysis toolkit (GATK; v4.0.2.1) was used for sorting, duplicate marking, and base recalibration. The final BAM files were analyzed using QualiMap v.2.2.1 to provide a global overview of the data, including mapped reads, mean mapping quality, and mean coverage. The variants (single nucleotide variants (SNV) and insertion–deletion mutations (InDels)) were called for unpaired tumor sequences with 40 pooled blood samples (from healthy individuals) using the GATK mutect2 tumor-only mode (parameter: af-alleles-not-in-resource, 0.00025%), and germline mutations and contaminations were filtered out using GATK FilterMutectCalls (parameter: max-germline-posterior, 0.995). Somatic variants were annotated using the ANNOVAR software tool.

The following filter conditions were used to identify candidate somatic alterations: i) all variations with COSMIC evidence (http://cancer.sanger.ac.uk/cosmic) were retained; and ii) SNV acquisition conditions: (1) tumor samples require at least 10× coverage; (2) at least 5× coverage supports mutant alleles in tumor DNA; (3) mutant allele frequency (AF) ≥ 0.05; and (4) genomic locations with mutant allele frequencies greater than 0.1% in the Thousand Genomes Project and Exome Aggregation Consortium (ExAC) were filtered out (AF ≥ 0.001).

Statistical analysis

The mutational landscape across the cohort was created using the maftools package in R software (R 4.3.0, R Core Team; https://www.R-Project.org). A cut-off value of an adjusted P-value (p.adjust) < 0.05 was used to identify significantly enriched terms.

Results

Weighted co-expression network construction and identification of key modules

WGCNA analysis was performed for both GSE43458 and GSE83456. To construct a scale-free topology network, soft threshold powers (β) of 12 in GSE43458 (scale-free R2 = 0.80) (Figure 1A) and 12 in GSE83456 (scale-free R2 = 0.80) (Figure 1B) were estimated. For GSE43458, the hierarchical clustering tree revealed that 17 co-expression modules were clustered (Figure 1C), and the orange module was negatively correlated with the LC proportion (Cor = 0.9, P = 1×10−22) (Figure 1E). For GSE83456, dynamic hybrid cutting clustered 20 co-expression models (Figure 1D), with the black module having the strongest positive correlation with the TB proportion (Cor = 0.83, P = 1×10−14), and the salmon module showing the strongest negative correlation (Cor = 0.85, P = 6×10−16) (Figure 1F). In the orange module, scatter plots showed strong negative correlations between MM and GS for LC (Cor = 0.92, P = 1×10−200) (Figure 1G); strong positive correlations were also observed between MM and GS for TB in the black module

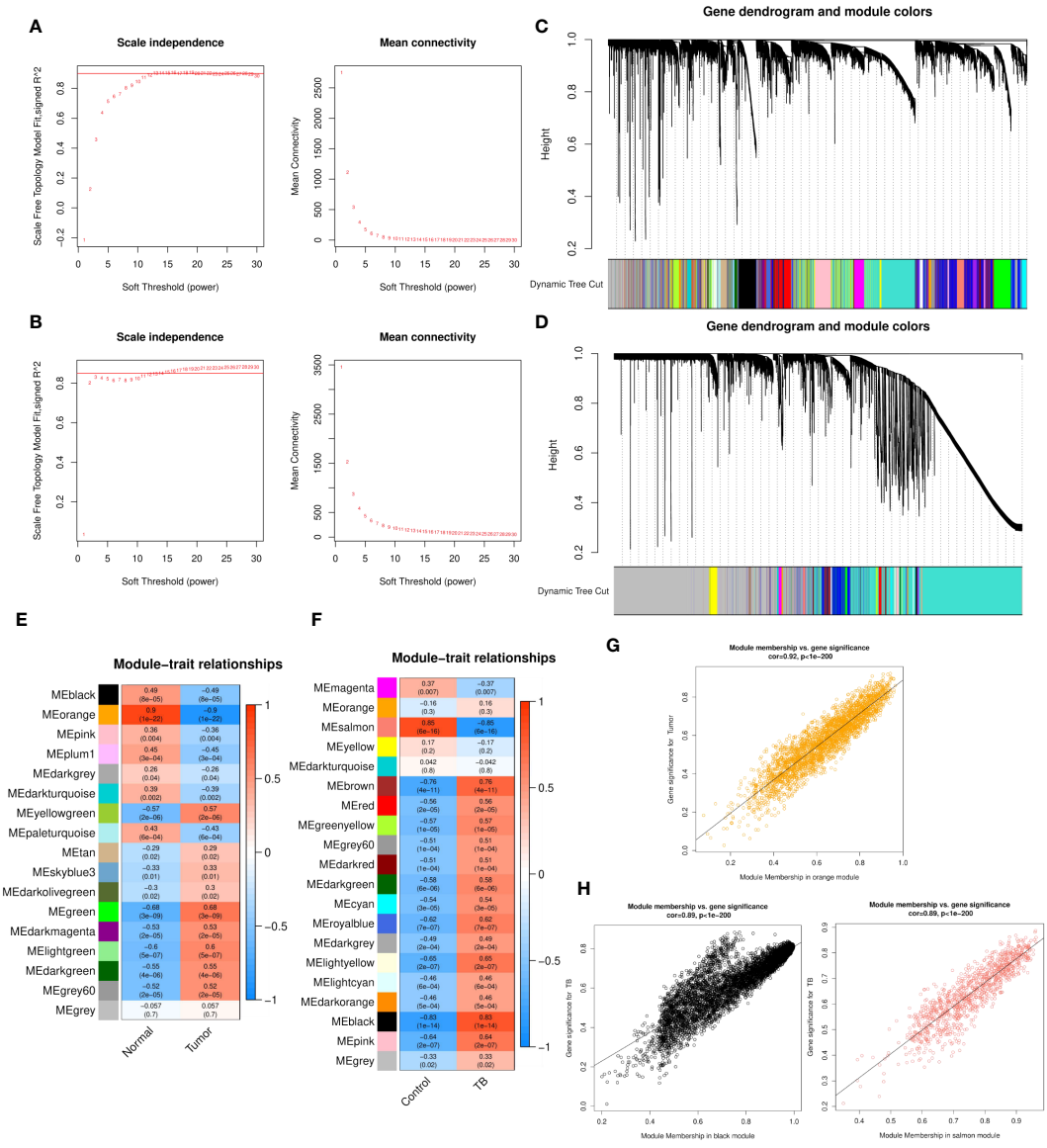

FIGURE 1
(A, B) Soft threshold powers (β) of 12 and 12 were selected based on the scale-free topology criterion for GSE43458 **(A)** and GSE83456 **(B)**, respectively. **(C, D)** Clustering dendrograms showing the clustering of genes with similar expression patterns into co-expression modules in GSE43458 **(C)** and GSE83456 **(D)**. The gray module indicates genes that were not assigned to any module. **(E, F)** Module–trait relationships revealing the correlations between each gene module eigengene and phenotype in GSE43458 **(E)** and GSE83456 **(F)**. **(G, H)** Scatter plots of the MM and GS of each gene in the orange module of GSE43458, showing a negative correlation with the LC proportion **(G)**, and the black and salmon modules of GSE83456 showing a positive and negative correlation, respectively, with the TB proportion **(H)**. Horizontal axis, correlation between gene and co-expression module; vertical axis, correlation between gene and phenotype. LC, lung cancer; TB, tuberculosis; MM, module membership; GS, gene significance.

(Cor = 0.89, P = 1×10−200), as well as large negative correlations between MM and GS in the salmon module (Cor = 0.89, P = 1×10−200) (Figure 1H). Hence, these three modules were selected for in-depth investigation. A total of 5157 and 1042 genes were incorporated in the black and salmon modules, respectively, while the orange module contained 2743 genes.

Differentially expressed genes of GSE42834

To identify differential genes in LC, TB, and TB patients with LC, we analyzed the DEGs in three modules using another dataset, GSE42834 from the GEO database. We set the cut-off as |log2FC| > 0.5 and q-value < 0.001 to screen DEGs from GSE42834. Figure 2A shows a volcano plot of the DEGs. We overlapped the DEGs and the genes in the three modules (LC vs control, LC vs TB, and TB vs control) by Venn diagram and found that 3606 common genes were present in all three modules (Figure 2B). Figures 2C, D demonstrate the GO and KEGG analyses of these 3606 genes. Extracellular matrix was the most enriched cellular component (CC) term, G protein-coupled receptor activity was the most enriched molecular function (MF) term, and neuroactive ligand–receptor interaction was the most enriched KEGG pathway.

Functional analyses of hub genes

To assess the function of the hub genes, we extracted the common genes derived from WGCNA and DEGs. As shown in

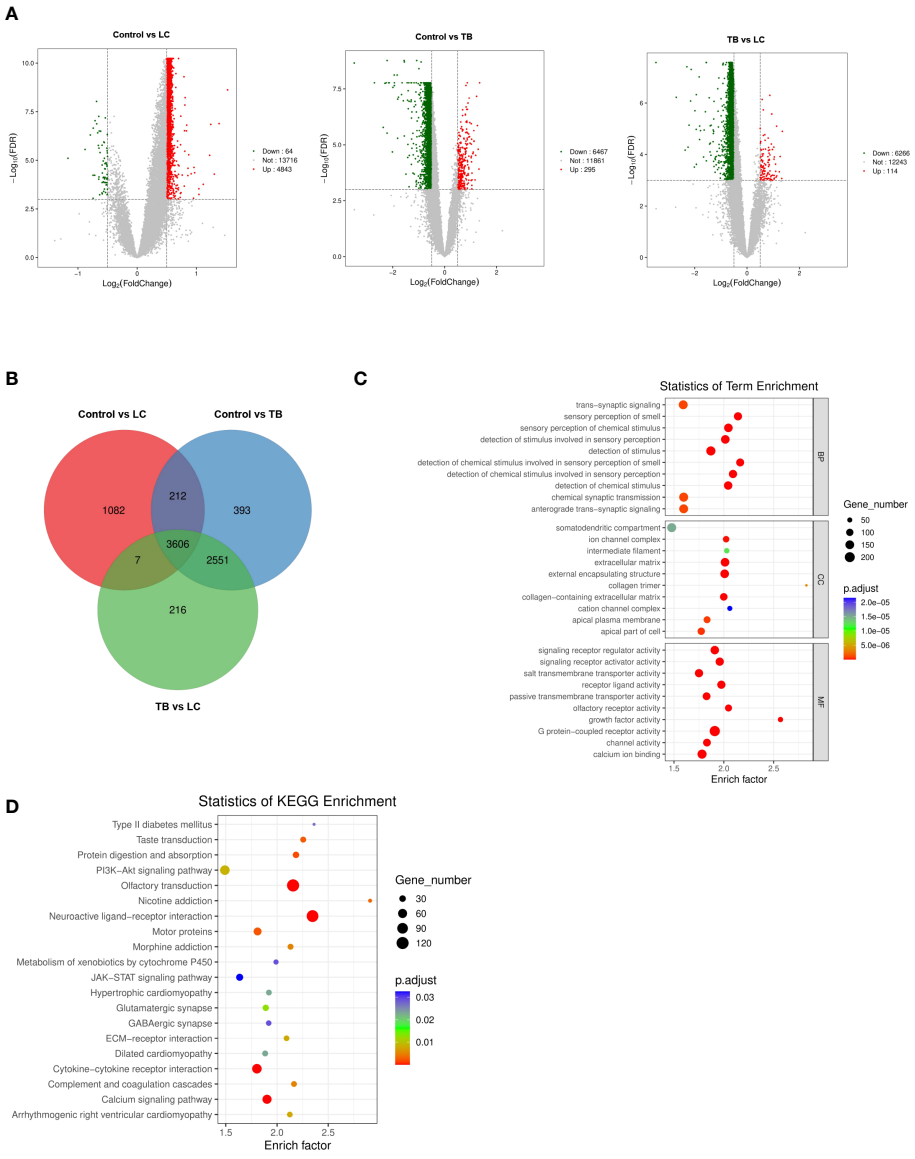

FIGURE 2
(A) Volcano plot visualizing DEGs in GSE42834 (20 control, 8 lung cancer, and 19 pulmonary tuberculosis samples). **(B)** Identification of common genes between the DEGs in control, lung cancer, and pulmonary tuberculosis by overlap. **(C, D)** GO analyses of the enriched BP, CC, and MF terms **(C)** and KEGG pathway analysis **(D)** of the 3606 genes. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function.

Figure 3A, 278 common genes at the intersection of the three hub gene sets were detected and visualized via a Venn diagram. Subsequently, we performed GO and KEGG analyses on these 278 genes. Cell–substrate adhesion was the major enriched biological process (BP) term, and collagen-containing extracellular matrix and growth factor activity were the major enriched CC and MF terms, respectively (Figure 3B). The RAS and PI3K–AKT signaling pathways were the main enriched KEGG pathways (Figure 3C). The PPI network was constructed with 278 genes. The EGFR pathway is an oncogenic pathway in human non-small cell lung cancer (NSCLC), which also affects the levels of some pathway-related binding proteins or downstream activities (14, 15). Pathway analyses further revealed that the levels of some EGFR-related genes were altered, suggesting that EGFR might serve as a regulatory signal node in the development of TB-associated lung cancer (Figure 3D).

Recurrently mutated genes in TB and LC with and without TB

To further investigate the role of EGFR in lung cancer and pulmonary TB, we performed exon sequencing on samples from lung cancer, lung cancer associated with pulmonary tuberculosis, and pulmonary tuberculosis patients. Supplementary Figure S1 presents a summary of the MAF files (Supplementary Table S6) for the 15 patients. Totally, 2094 meaningful variations in 497 genes were identified. Totally, 2094 meaningful variations in 497 genes were identified. A waterfall plot depicts 25 of the genes containing indel mutations (Figure 4). For the SNVs, T > C was the most frequent SNV class. The median number of variants identified in the 15 samples was 5 (range, 1–24).

Figure 4 presents the mutational profile of the 15 patients with LC and pulmonary TB, including 25 mutated genes, organized by the TB, LC, and TB+LC groups. The mutated gene with the highest frequency was *ADCK5* (67%). *EGFR*, one of the most frequently mutated genes in lung cancer, had a mutation rate of 20%, similar to the previously reported *EGFR* mutation rate of 5%–30% in LC (16). The other recurrently altered genes were mucin-3A (*MUC3A*; 60%), keratin-associated protein 5–7 (*KRTAP5–7*; 53%), killer cell immunoglobulin-like receptor 2DL4 (*KIR2DL4*; 33%), zonadhesin (*ZAN*; 27%), fatty acid desaturase 6 (*FADS6*; 27%), cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*; 27%), and ataxin-3 (*ATXN3*; 20%). Supplementary Figure S2 illustrates the frequency of mutations and the resulting protein structure.

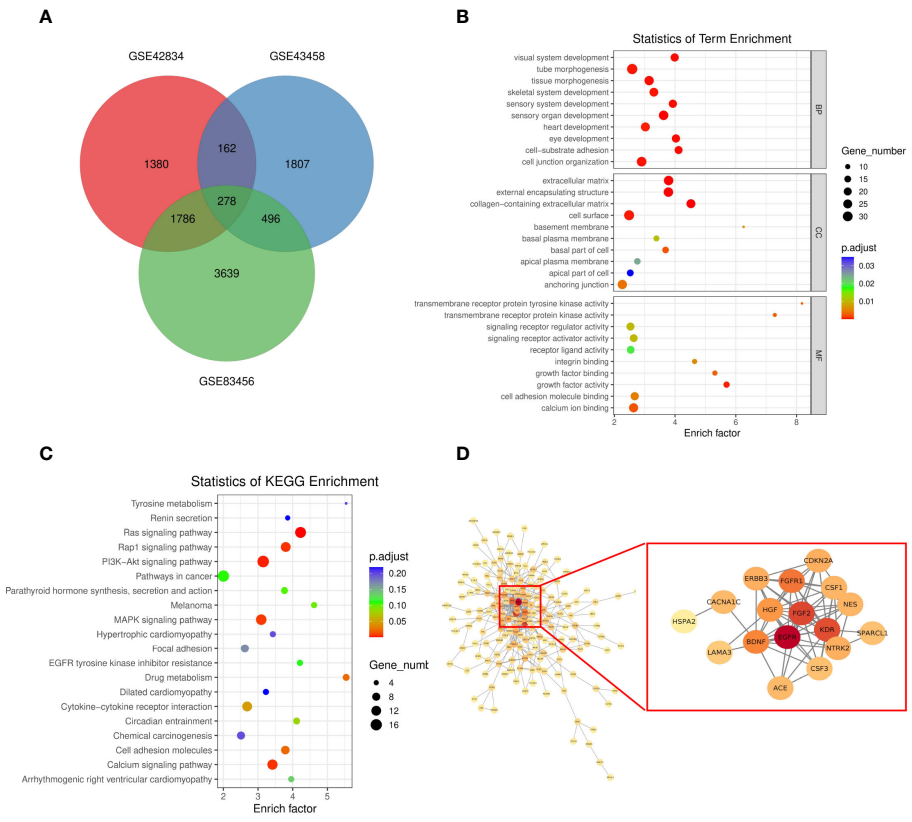

FIGURE 3
(A) Venn diagram showing the intersection between the orange (GSE43458), black (GSE83456), and salmon (GSE83456) module genes and the GSE42834 DEGs. **(B, C)** GO analyses of the enriched BP, CC, and MF terms **(B)** and KEGG pathway analysis **(C)** of the 278 genes. **(D)** Three-dimensional network of the 278 genes. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function.

KEGG signaling pathway enrichment analysis of all somatically mutated genes

To further investigate the biological functions of the mutated genes, KEGG signaling pathway enrichment analyses were performed. Supplementary Table S7 shows enriched signaling pathways associated with the mutated genes. The results of the KEGG signaling pathway analysis are presented in Figure 5, showing that the genes are involved in the PI3K–AKT signaling pathway and non-small cell lung cancer, which is consistent with our previous analysis.

Correlation analysis between key genes and EGFR

EGFR had the highest degree in the aforementioned PPI, with a mutation rate of 20% in lung cancer. This implies that EGFR is involved in the progression of lung cancer. Thus, we investigated the relationship between EGFR and other genes. The Venn diagram in Figure 6A depicts the genes common to both the 278 hub genes and all mutated genes, including *EGFR*, *HSPA2*, *CECR2*, and *LAMA3* (Figure 6A). To investigate the effects of *EGFR* expression on *HSPA2*, *CECR2*, and *LAMA3*, we performed the CIBERSORT algorithm on 15 tumor samples to calculate the expression of *EGFR* and the three key genes in each sample. As shown in Figures 6B–D, *CECR2*, *LAMA3*, and *HSPA2* were positively correlated with *EGFR* expression.

Discussion

Lung cancer is the most dangerous of the common malignant tumors in China, causing the most cancer deaths each year (17). Tuberculosis is an infectious illness of the lungs caused by *Mycobacterium tuberculosis*, and tuberculosis of the lungs raises the risk of a patient getting lung cancer by causing inflammatory irritation that leads to persistent irritation of the lungs (18, 19).

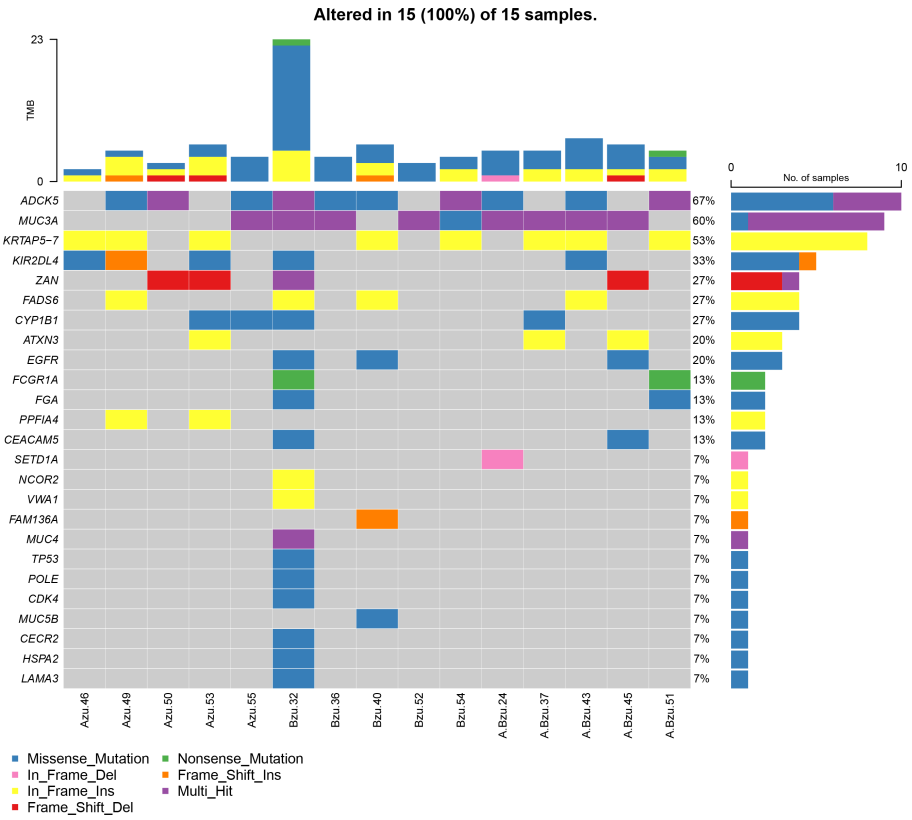

FIGURE 4
The mutational landscape of 15 patients with LC, TB, and LC+TB was determined using whole-exome sequencing. Azu, TB; Bzu, LC; A.Bzu, TB+LC; *ADCK5*, AarF domain containing kinase 5; *MUC3A*, mucin-3A; *KRTAP5–7*, keratin-associated protein 5–7; *KIR2DL4*, killer cell immunoglobulin-like receptor 2DL4; *ZAN*, zonadhesin; *FADS6*, fatty acid desaturase 6; *CYP1B1*, cytochrome P450 family 1 subfamily B member 1; *ATXN3*, ataxin-3; *EGFR*, epidermal growth factor receptor; *FCGR1A*, high affinity immunoglobulin gamma Fc receptor I; *FGA*, fibrinogen alpha chain; *PPFIA4*, liprin-alpha-4; *CEACAM5*, carcinoembryonic antigen-related cell adhesion molecule 5; *SETD1A*, histone-lysine N-methyltransferase SETD1A; *NCOR2*, nuclear receptor corepressor 2; *VWA1*, von Willebrand factor A domain-containing protein 1; *FAM136A*, family with sequence similarity 136, member A gene; *MUC4*, mucin-4; *TP53*, tumor protein p53; *MUC5B*, mucin-5B; *CECR2*, cat eye syndrome chromosome region candidate 2; *HSPA2*, heat shock protein A2; *LAMA3*, laminin subunit alpha 3.

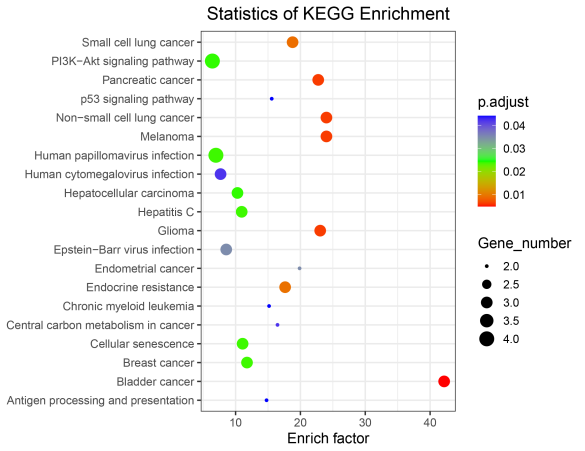

FIGURE 5
Kyoto Encyclopedia of Genes and Genomes signaling pathway enrichment analysis of all somatically mutated genes.

Younger patients show a significantly higher association between TB and lung cancer (20). Several studies have shown that LC in TB patients have lower survival rates than non-TB patients (21, 22). A study of 6934 patients among patients with primary cancer and TB infection demonstrated that TB is a risk for facilitating primary cancer to metastasize to the lung (23). Delayed diagnosis and treatment of TB increases the chance of patient complications and mortality and enhances TB transmission in the population (24). Therefore, it is of practical significance to explore the mechanism of the association between TB and lung cancer development and provide new targets for clinical examination and future targeted therapy of lung cancer patients.

In this study, WGCNA was performed by extracting co-expression networks of grouped genes from a large expression dataset. Among the 37 modules, we found that the orange, black, and salmon modules were most significantly related to LC or TB. We analyzed the GSE42834 dataset, which includes LC, TB, and control groups, to find 3606 DEGs. The confluence of these differential genes with the genes from the three WGCNA modules resulted in 278 genes for which we determined the PPI network, showing that *EGFR* and related genes are highly correlated with TB and LC. KEGG pathway analysis revealed that the hub genes were primarily enriched in pathways related to growth, survival, and metabolism of cancer cells, such as the RAS and PI3K–AKT signaling pathways. The PI3K–AKT signaling pathway is dysregulated in almost all cancers due to gene amplification (25). Studies suggest that in patients with an *EGFR* mutation, the AKT/mTOR pathway is constitutively activated in 67% of cases (26, 27). RAS signaling is a major nexus controlling essential cell parameters, including proliferation, survival, and migration, utilizing downstream effectors such as the PI3K–AKT signaling pathway (28, 29).

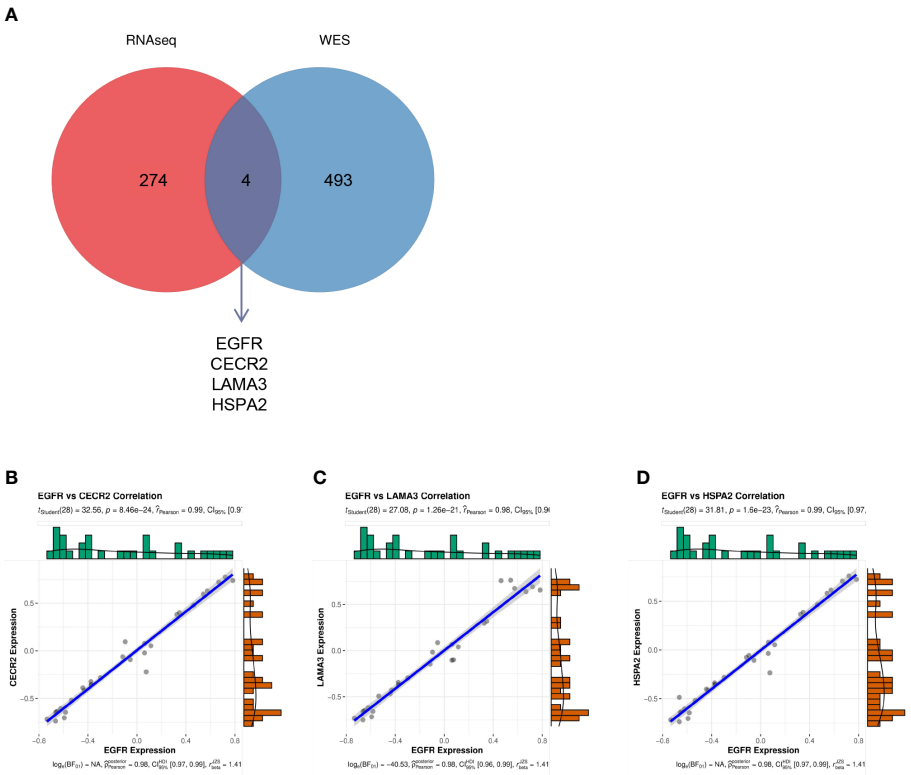

FIGURE 6
(A) The intersection between the 278 hub genes and the mutated genes shown in a Venn diagram. **(B–D)** The correlation between *EGFR* and *CECR2* **(B)**, *LAMA3* **(C)**, and *HSPA2* **(D)**.

Next, we examined the somatic mutation patterns of 15 individuals to acquire a better understanding of the progression from TB to LC. In addition to somatic alterations in previously known LC-associated genes, such as *EGFR*, *ADCK5*, *MUC3A*, and *KIR2DL4* (16, 30–33), we identified mutations in new genes, such as *CECR2*, *LAMA3*, *FADS6*, *CYP1B1*, and *ATXN3*. Interestingly, we found that four (*EGFR*, *CECR2*, *LAMA3*, and *HSPA2*) of the 278 genes obtained in the three GEO datasets were mutated in all 15 patients. *EGFR* had a mutation rate of 20%, similar to the previously reported *EGFR* mutation rate (5%–30%) in LC (16). The epidermal growth factor receptor (*EGFR*) gene encodes signaling proteins crucial for cell proliferation and survival, and *EGFR* mutations are major driver mutations occurring in lung adenocarcinomas (16, 34, 35). The incidence of LC *EGFR* mutations was found to be higher in East Asian countries, as was the prevalence of TB infection (2, 34). Studies have examined the expression of *CECR2* in breast cancer and found that it regulates the tumor immune microenvironment to promote breast cancer metastasis (29). However, there have been no reports to date implicating *CECR2* in LC. Reducing the methylation of the *LAMA3* promoter inhibits the proliferation, invasion, migration, and drug resistance of lung adenocarcinoma cells (36). The data reported show that *HSPA2* does not promote a malignant NSCLC phenotype. HSPA2-deficient keratinocytes show accelerated differentiation in a reconstituted human epidermis model (37–39). These four genes may be key proteins that predict the development of LC in TB. Additionally, the altered genes were discovered to be highly enriched in the PI3K–AKT signaling pathway, consistent with other previous studies (40–42). Nevertheless, further research is required to fully explore their roles in TB and LC.

We acknowledge that there were some limitations and shortcomings of this study. First, this study was mainly focused on data mining and data analysis, which are based on methodology. Clinical information available in public databases is limited, and contaminated tissues and biases in sequencing may lead to biased results in WGCNA. In addition, after obtaining the hub gene, the association with the tumor microenvironment should also be analyzed and further verified by experiments (43, 44). Second, the single method of whole-exon sequencing used, and the minimal sample size, may have an impact on the accuracy of the results. Our future research will include large sample sizes, analyzed by different methods.

Conclusion

We applied WGCNA and WES to explore the development of LC, and determined a mutational profile of 15 patients by WES. This identified four genes (*EGFR*, *CECR2*, *LAMA3*, and *HSPA2*) that play an important role in LC tumorigenesis. Furthermore, the present study confirms *EGFR* mutations in LC and verifies the enrichment of gene alterations in the PI3K–AKT signaling pathway in a small cohort of Chinese patients with LC. These results may shed light on opportunities for diagnosis and personalized treatment of TB with LC.

Data availability statement

The datasets used and/or analyzed during the current study are available from the GEO database (ID: GSE43458, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43458; GSE83456, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE83456; GSE42834, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42834. The WES datasets used and/or analyzed during the current study are available from the BioProject database (BioProject ID: PRJNA1080061; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1080061).

Ethics statement

The studies involving humans were approved by Ethics Committees of The Xinjiang Uygur Autonomous Region Chest Hospital (approval number 2021BAT011). The studies were conducted in accordance with the local legislation and institutional requirements. The 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

MA: Conceptualization, Writing – original draft. ZL: Investigation, Resources, Visualization, Writing – review & editing. BA: Data curation, Methodology, Project administration, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1355527/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Summary and visualization of maf files using the maftools package in R software. **(A)** Variant classification. **(B)** Variant type. **(C)** SNV class. **(D)** The count of variants per sample. **(E)** Variant classification summary. **(F)** Top 30 mutated genes. The colors of variant classification in subfigure D, E and F are in accordance with subfigure A. Del, deletion; Ins, insertion; SNP, single nucleotide polymorphism; SNV, single nucleotide variant.

SUPPLEMENTARY FIGURE S2
Proportion of all mutated genes.

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Case report: Sustained remission after combined sintilimab, anti-VEGF therapy, and chemotherapy in a patient with non-small cell lung cancer harboring acquired *EGFR* 19Del/T790M/*cis*-C797S mutation resistance

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Third-generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are highly effective against tumors harboring the T790M mutation. However, patients treated with these inhibitors ultimately develop resistance, and the most common mechanism is the emergence of the *EGFR* C797S mutation. Few treatment regimens have been reported for this condition. In this report, we present a successful combination treatment with the programmed cell death 1 (PD-1) inhibitor sintilimab, anti-vascular endothelial growth factor (VEGF) therapy, and chemotherapy with pemetrexed and cisplatin in a patient with non-small cell lung cancer (NSCLC) who developed acquired resistance with *EGFR* 19 exon deletion (19Del)/T790M/*cis*-C797S mutation following progression with ametinib therapy. This regimen was well tolerated, and the patient has remained progression-free for 15 months. Our case provides clinical evidence that the combination of PD-1 inhibitor, anti-VEGF therapy, and chemotherapy may be an efficacious therapeutic strategy for NSCLC patients with acquired *EGFR* 19Del/T790M/*cis*-C797S mutation resistance following progression with EGFR TKI therapy.

KEYWORDS

non-small cell lung cancer, growth factor receptor, tyrosine kinase inhibitors, programmed cell death 1 inhibitor, anti-vascular endothelial growth factor therapy

Introduction

Despite initial response to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), most patients with non-small cell lung cancer (NSCLC) harboring *EGFR* activating mutations inevitably develop resistance after approximately one year (1, 2). The *EGFR* T790M mutation is the most common mechanism of resistance to first- and second-generation EGFR TKIs, and third-generation EGFR TKIs, such as osimertinib and ametinib, selectively target the T790M mutation. However, patients treated with third-generation EGFR TKIs ultimately encounter secondary resistance. Although the mechanisms of resistance vary, the most common is the emergence of the *EGFR* C797S mutation (3), with reported frequencies up to 24% (4–6). According to the allelic relationship with T790M, C797S is defined as *cis*-C797S or *trans*-C797S (7). Tumors harboring T790M/*trans*-C797S are sensitive to combined first- and third-generation EGFR TKIs (7, 8). However T790M/*cis*-C797S, the more frequently mutation, is resistant to first-, second-, and third-generation EGFR TKIs (3, 9). Currently, there is no standard therapeutic regimen for NSCLCs harboring the T790M/*cis*-C797S *EGFR* mutation. Platinum-based chemotherapy with or without bevacizumab is one of the recommended regiments (10), however, the survival is poor. Here, we report a successful case of combination therapy with PD-1 inhibitor (sintilimab), anti-VEGF therapy, and chemotherapy in a patient with NSCLC who developed acquired *EGFR* 19 exon deletion (19Del)/T790M/*cis*-C797S mutation resistance following progression on EGFR TKI therapy.

Case report

A 61-year-old man, a former smoker with no relevant family or genetic history, underwent computed tomography (CT) of the chest in November 2018, due to a cough. The CT scan revealed a nodule in the right upper lung near the mediastinum, suggesting a neoplastic lesion (Figure 1). One month later, he was diagnosed with Stage IVA (T4N2M1a) lung adenocarcinoma with brain metastasis in the left occipital lobe. Genomic profiling of pleural effusion cell pellets using next-generation sequencing (NGS) identified an *EGFR* 19 exon delete (19Del; c.2235_2249del p.Glu746_Ala750del). Consequently, he was treated with icotinib (125 mg tid), achieving a partial response (PR).

In June 2019, magnetic resonance imaging (MRI) revealed bone metastases at the L3 lumbar and S2 and S3 sacral vertebrae. He received intensity-modulated radiotherapy using RAPID-Arc, delivering 55 Gy in 22 fractions to gross target volume (GTV) and 40 Gy in 22 fractions to clinical target volume (CTV). Since bone-related examinations were not performed at the initial diagnosis, baseline images were unavailable. NGS analysis of a blood sample did not detect an *EGFR* mutation, and CT scans showed reduced lung lesions, indicating effectiveness of icotinib. Consequently, icotinib treatment was continued.

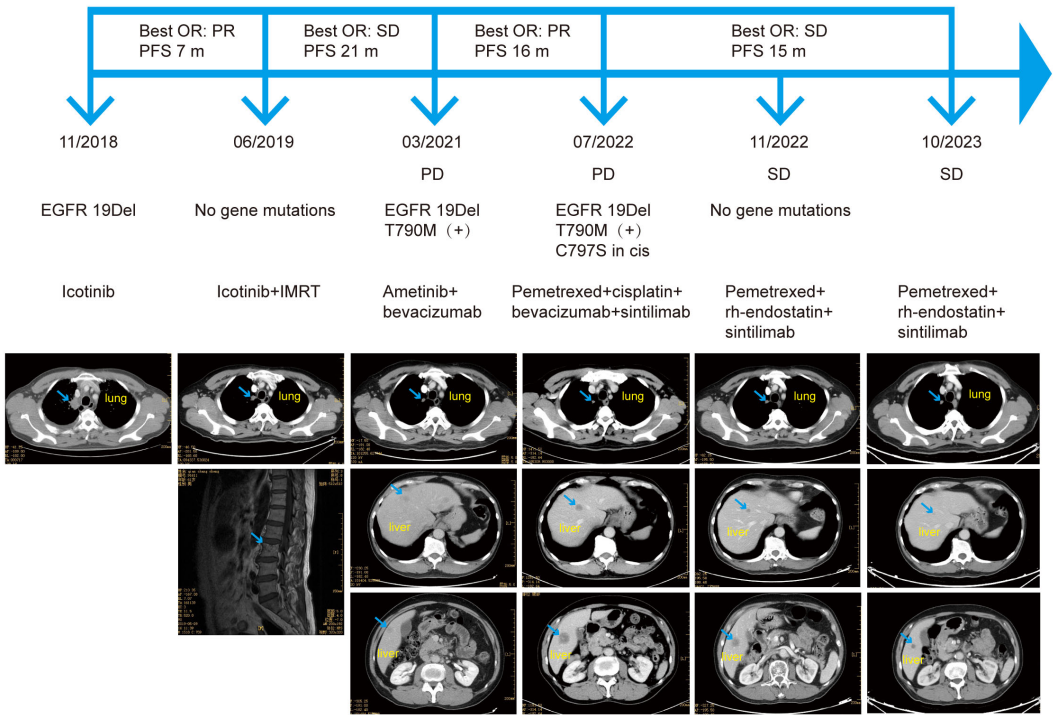

FIGURE 1
The patient's course, treatment, next-generation sequencing results, and imaging results. OR, objective response; PFS, progression-free survival; PR, partial response; SD, stable disease; IMRT, intensity-modulated radiotherapy; *EGFR*, epidermal growth factor receptor; 19Del, exon 19 deletion; PD, progressive disease.

The patient maintained stable disease (SD) for 21 months, until CT scans revealed new lesions in both lungs and the liver. NGS analysis of a blood sample identified an *EGFR* T790M mutation (c.2369C>Tp.Thr790Met) along with the *EGFR* 19Del (c.2235_2249del p.Glu746_Ala750del). Subsequently, he commenced treatment with ametinib (110 mg, qd) combined with bevacizumab (400 mg q3w), achieving a PR. However, disease progression was observed in July 2022 with enlarged liver metastases and an increased number of liver lesions. NGS analysis of a blood sample revealed a novel *EGFR cis*-C797S mutation (c.2389T>Ap.Cys797Ser) in addition to the existing *EGFR* 19Del and T790M mutations.

The ORIENT-31 trial, a prospective, double-blind, phase 3 clinical trial, evaluated the efficacy and safety of sintilimab with or without bevacizumab biosimilar IBI305 plus pemetrexed and cisplatin, compared with pemetrexed and cisplatin alone, for patients with locally advanced or metastatic *EGFR*-mutated NSCLC who had disease progression after receiving EGFR TKI therapy (11). Based on the preliminary results from this trial, we initiated a treatment regimen of pemetrexed and cisplatin combined with bevacizumab and sintilimab (200 mg q3w) in July 2022 for our patient, who had an Eastern Cooperative Oncology Group performance status (ECOG PS) score of 1. After six courses of this regimen, he transitioned to maintenance therapy with pemetrexed, bevacizumab and sintilimab.

A CT scan in November 2022 showed that the primary lung lesion and multiple lung metastases were mostly unchanged, although the liver lesions had shrunk, indicating an objective response (OR) of SD. NGS analysis of a blood sample did not identify the *EGFR* 19Del, T790M, or *cis*-C797S mutations, and no other mutations were detected. Due to the patient's worsening economic situation, bevacizumab was replaced with the lower cost recombinant human endostatin (30 mg, d1–7, q3w). As of October 2023, the patient continued to respond to the treatment regimen of pemetrexed combined with recombinant human endostatin and sintilimab, with a progression-free survival (PFS) exceeding 15 months. The only treatment-related side effect was grade 2 diarrhea, according to the Common Terminology Criteria for Adverse Events (CTCAE) Version 5.0, which occurred after four courses, and was alleviated with symptomatic treatment. A colonoscopy in November 2022 indicated no abnormalities.

Discussion

Due to the molecular heterogeneity of NSCLC, the resistance mechanisms to third-generation TKIs are complicated and not fully understood. Acquired resistance to EGFR TKIs can be broadly categorized into *EGFR*-dependent (on-target) and *EGFR*-independent (off-target) (12, 13). Relevant therapeutic options have been found to prolong clinical benefits. For instance, the combination of the ALK inhibitor brigatinib with cetuximab may be effective for patients with acquired *EGFR* T790M/*cis*-C797S-mediated resistance to osimertinib (14, 15). Fourth-generation EGFR TKIs, such as EAI045, JBJ-04–125-02, and BLU-945, can overcome both the T790M and C797S mutations (16). Additionally, the phase III MARIPOSA-2 study demonstrated that PFS was significantly longer for the combination of amivantamabe-lazertinibe and chemotherapy compared to chemotherapy alone in patients with *EGFR*-mutated advanced NSCLC who had progressed on or after osimertinib (median of 8.3 versus 4.2 months, respectively) (17). Furthermore, the antibody-drug conjugate (ADC) patritumab deruxtecan (HER3-DXd) showed clinically meaningful efficacy in the phase II HERTHENA-Lung01 study, and a phase III HERTHENA-Lung02 trial is ongoing (ClinicalTrials.gov identifier: NCT05338970) (18).

In our case, the patient acquired an *EGFR cis*-C797S mutation after treatment with a third-generation TKI. However, fourth-generation EGFR TKIs are not readily accessible to Chinese patients in clinical practice, and the cost of brigatinib and cetuximab is high, increasing the financial burden on patients. Therefore, economical, accessible and effective therapeutic regimens are needed to manage those NSCLC Chinese patients who acquire an *EGFR cis*-C797S mutation.

A few randomized phase 3 trials have shown that combining PD-1 or programmed cell death ligand 1 (PD-L1) inhibitors with VEGF inhibitors and chemotherapy enhances antitumor activity and provides a PFS benefit for patients with advanced *EGFR*-mutated NSCLC who progressed after receiving EGFR TKI therapy. A subgroup analysis of the IMpower150 trial showed that treatment with the PD-L1 inhibitor atezolizumab, bevacizumab, and chemotherapy (carboplatin and paclitaxel) improved survival outcomes in NSCLC patients who developed *EGFR* mutations after TKI treatment (19, 20). Additionally, the ORIENT-31 trial demonstrated that treatment with the PD-1 inhibitor sintilimab, bevacizumab biosimilar IBI305, and standard chemotherapy (pemetrexed and cisplatin) significantly improved PFS compared to chemotherapy alone (median 7.2 months *vs* 4.3 months; hazard ratio 0.51; p<0.0001) for NSCLC patients who had progressed after EGFR TKI therapy (11). However, the trial included patients with multiple *EGFR* mutations, including exon 19Del, exon 21 L858R, and others, not exclusively those with acquired *EGFR cis*-C797S mutations. As of October 2023, the last follow-up time, our patient is still responding to the combination of a PD-1 inhibitor, anti-VEGF therapy and chemotherapy, with a progression-free survival (PFS) of over 15 months, exceeding the median PFS of 7.2 months reported in the ORIENT-31 trial.

To date, the mechanism of this treatment regimen remains unclear. Due to low response rates to immune checkpoint inhibitors (ICIs) in patients with *EGFR*-mutant NSCLC (21), this population has typically been excluded from first-line treatment with immunotherapy. Nevertheless, recent translational studies have shown that ICIs are more effective in patients with PD-L1 higher expression in tumor cells, a higher tumor mutation burden, or a higher density of tumor-infiltrating lymphocytes following EGFR TKI treatment (22–24). Moreover, multiple clinical studies have

indicated that the efficacy of ICIs may be enhanced when combined with VEGF inhibitors (25–27). Anti-angiogenic therapy induces normalization of tumor vasculature, promoting T cell infiltration into the tumor and creating a tumor immune microenvironment favorable for ICI therapy (28, 29). Additionally, VEGF expression can be promoted by *EGFR* signaling, potentially increasing the sensitivity of tumors harboring *EGFR* mutations to anti-VEGF therapy (30, 31).

In our case, several limitations should be considered. Repeated tissue biopsies are necessary to identify histological changes in complex cancers and to elucidate resistance mechanisms if the combination treatment of a PD-1 inhibitor, anti-VEGF therapy, and chemotherapy fails. After three months of combination therapy, no gene mutations were detected, yet the patient continued to respond to treatment. The underlying mechanism warrants further investigation. Despite these limitations, the patient has acquired survival benefits and the three-drug regimen has been well tolerated. The only side effect was grade 2 diarrhea, which was alleviated with symptomatic treatment. Our case may shed lights on overcoming *EGFR* 19Del/T790M/*cis*-C797S mutation resistance.

Conclusion

The combination treatment with the PD-1 inhibitor sintilimab, anti-VEGF therapy, and chemotherapy demonstrated a significant improvement in PFS in a NSCLC patient who developed acquired resistance due to *EGFR* 19Del/T790M/*cis*-C797S mutation after progression on EGFR TKI therapy. This therapeutic regimen may be efficacious and offers an optimal strategy for managing these patients.

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 humans were approved by The Clinical Research Ethics Committee of the Sixth Affiliated Hospital of South China University of Technology. The studies were conducted in accordance with the local legislation and institutional requirements. The 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

WH: Project administration, Software, Writing – original draft. LT: Investigation, Writing – review & editing. WY: Methodology, Writing – review & editing. YY: Writing – review & editing, Project administration. YL: Project administration, Writing – review & editing. WT: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1298389/full#supplementary-material

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EGFR kinase domain duplication in lung adenocarcinoma with systemic and intracranial response to a double-dose of furmonertinib: a case report and literature review

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Background: EGFR kinase domain duplication (EGFR-KDD) is an infrequent oncogenic driver mutation in lung adenocarcinoma. It may be a potential target benefit from EGFR-tyrosine kinase inhibitors (TKIs) treatment.

Case presentation: A 66-year-old Chinese male was diagnosed with lung adenocarcinoma in stage IVb with brain metastases. Next-generation sequencing revealed EGFR-KDD mutation. The patient received furmonertinib 160mg daily for anti-cancer treatment and obtained therapeutic efficacy with partial response (PR). Progression-free survival (PFS) duration from monotherapy was 16 months. With slow progressions, combined radiotherapy and anti-vascular targeted therapy also brought a continuous decrease in the tumors. The patient has an overall survival (OS) duration of more than 22 months and still benefits from double-dose furmonertinib.

Conclusions: This report provided direct evidence for the treatment of EGFR-KDD to use furmonertinib. A Large-scale study is needed to confirm this preliminary finding.

KEYWORDS

EGFR-KDD, furmonertinib, brain metastasis, lung adenocarcinoma, targeted therapy

Introduction

Global cancer statistics demonstrate that lung cancer remains the leading cause of cancer-related deaths worldwide (1). Adenocarcinoma is the most common pathological type of lung cancer, accounting for 35-40% of all cases. Patients with oncogenic mutations of the epidermal growth factor receptor (EGFR) tyrosine kinase domain have been identified as a significant subgroup of non-small cell lung cancer (NSCLC). Compared with standard chemotherapy, EGFR tyrosine kinase inhibitors (TKIs) generally obtain better tumor control in patients with EGFR-mutated lung adenocarcinoma (2, 3). The most common EGFR-activating mutations include in-frame deletions in exon 19 (Ex19del) and point mutations in exon 21 (Eex21 L858R), which are generally sensitive to EGFR-TKIs (4). EGFR kinase domain duplication (EGFR-KDD) represents a rare form of EGFR mutation, with an incidence of 0.24% in EGFR mutation of lung cancer (5). Predominantly, EGFR-KDD is caused by in-frame tandem duplication of EGFR exons 18–25. Aberrant duplication forms an intramolecular dimer and constitutively activates EGFR signaling. Some studies also reported duplications spanning exons 17 to 25 or exons 14 to 26 (6). Several reports have demonstrated significant anti-tumor responses to EGFR TKI treatment for lung adenocarcinoma patients with EGFR-KDD alterations, including gefitinib, erlotinib, afatinib, and osimertinib (7–9). However, there is currently no standardized approach for treating EGFR-KDD alterations. Here, we present a case report of a patient with harboring EGFR-KDD alteration and brain metastases. The patient is treated with furmonertinib and obtains an optimal cancer response duration. This is the first case report of furmonertinib use in EGFR-KDD alterations.

Case report

A 66-year-old Chinese male presented with lower limbs weakness and was admitted to a local hospital. Cerebral computed tomography (CT) scan revealed a right occipital lobe mass. Further positron emission tomography-CT (PET-CT) imaging revealed that F-18 fluorodeoxyglucose hypermetabolic speckled in a large lesion in the right lung, suggesting of peripheral lung cancer with brain metastasis. In December, 2021, the patient transferred to our hospital. He was previously diagnosed with hypertension, and has no family history of malignancy. The Eastern Cooperative Oncology Group (ECOG) score of the patients was 1. Physical examination showed sightly attenuated breath sounds in the right lung. A chest CT scan revealed a 10 x 8 cm mass in the upper right lung and enlarged lymph nodes in the mediastinum (Figure 1A). Magnetic resonance imaging (MRI) demonstrated metastatic masses in the right parietal and occipital lobes measuring 2 x 1.8 cm and 3.2 x 3.1 cm (Figure 1B), respectively, as well as metastases in the second and third sacral vertebra. CT-guided biopsy of the right lung mass was performed and showed adenocarcinoma on pathology (Figure 1C). According to the 8th edition of AJCC staging, the patient was classified as stage IVb (cT4N2M1c). Next-generation sequencing (NGS) revealed an EGFR-KDD mutation involving exons 18-25 (frequency 18%), accompanied by TP53 D281F indel (frequency 9%). The patient received furmonertinib at a double dose of 160 mg for targeted therapy in January 2022, in order to increase drug concentration in the brain tissue. Following treatment, the patient's bilateral lower limb weakness gradually ameliorated, and he regained the ability to walk independently. One month later, a chest CT scan revealed that the right lung tumor had shrunk from 10 cm to 5.7 cm. Subsequent regular follow-up examinations revealed that the best therapeutic efficacy was partial response (PR). The right lung tumor shrunk to 4.2 x 2.6 cm (in September, 2022), and the brain lesions also significantly decreased in size. Images of CT and MRI scans were showed in Figures 1A, B. Importantly, the patient showed good tolerance to the double dose of furmonertinib without diarrhea, liver function injury, interstitial lung disease or other adverse reactions. There was no reduction or discontinuation of furmonertinib due to any adverse events regarding to double dose.

In December 2022, a chest CT scan and cerebral MRI showed slow progression of the right lung tumor and intracranial metastases, but did not meet the criteria for diagnosing progressive disease (PD). The patient refused the suggestion of biopsy to clarify secondary mutations and continued to receive furmonertinib at a daily dose of 160mg. In January 2023, the patient experienced bilateral lower limb weakness again and received stereotactic body radiation therapy (SBRT) for the brain metastases. In February 2023, a follow-up CT scan revealed further enlargement of the right lung tumor, and the patient underwent intensity-modulated radiation therapy (IMRT) for the lesion in the right lung and the surrounding lymph drainage area.

In May 2023, the patient suffered from lower limbs weakness again. A cerebral MRI showed an increase in intracranial lesions with peritumoral edema, while a chest CT scan showed that right lung tumor continued to shrink, maintaining a PR evaluation. Following multidisciplinary discussions, the recommended treatment was surgical resection of the intracranial metastases. However, the patient refused surgery. In order to further enhance intracranial drug concentrations and reverse tumor resistance, we added bevacizumab at a dose of 7.5mg/kg every three weeks as anti-vascular targeted therapy, starting from May 17, 2023. Furmonertinib has been continuously administered up to now. The lower limbs weakness improved, and multiple reassessments indicated a PR treatment evaluation. Since the diagnosis, the patient has achieved a progression-free survival (PFS) duration of 16 months with first-line monotherapy of furmonertinib, and currently continues to benefit from furmonertinib, with an overall survival (OS) duration exceeding 22 months. The patient has not suffered from significant adverse drug reactions during the treatment. The flowchart of treatment is showed in Figure 1D.

Discussion

Exon 19 deletion and exon 21 L858R mutation are the two most common EGFR mutations, accounting for 75% of all EGFR

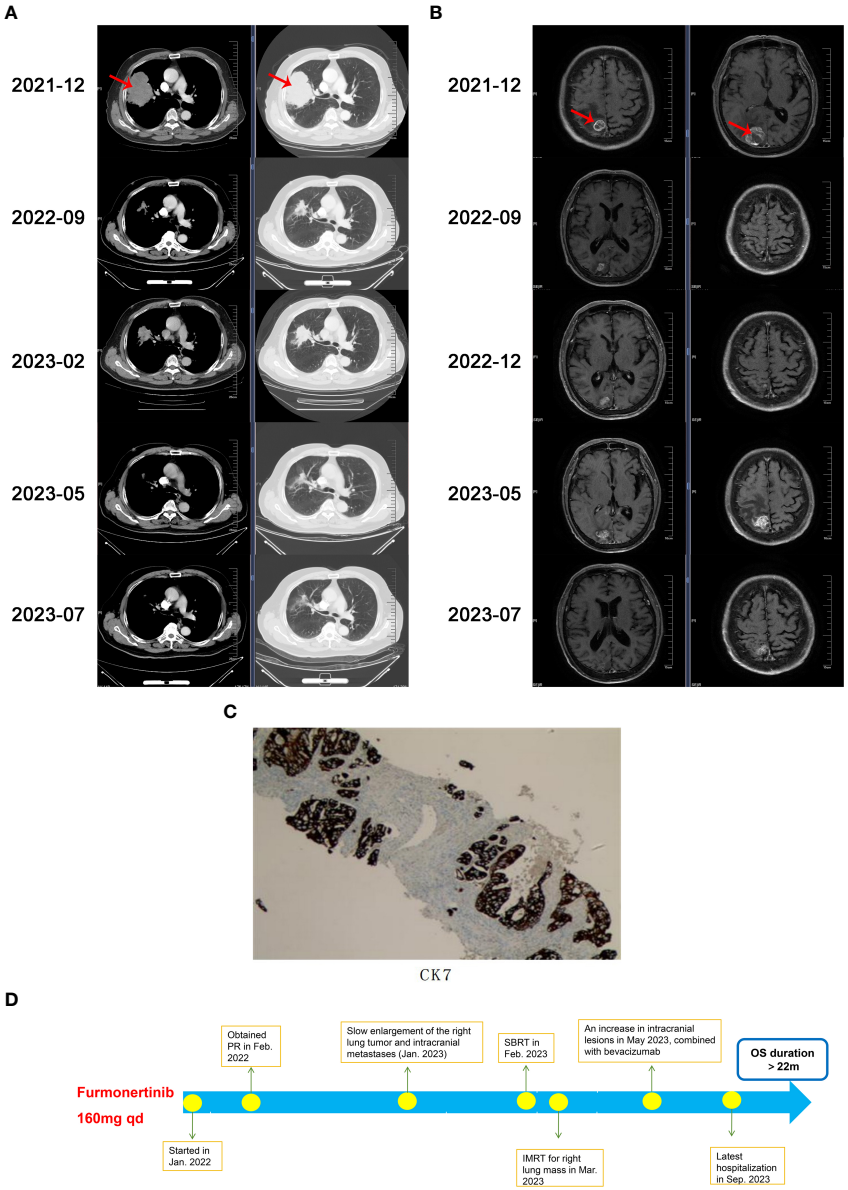

FIGURE 1
(A) Chest computed tomography (CT)scans during the treatment, **(B)** Cerebral magnetic resonance imaging (MRI)scans during the treatment, **(C)** Immunohistochemical image of CK7, **(D)** The timeline demonstrating the history of treatment for the patient. PR, partial response; SBRT, stereotactic body radiation therapy; IMRT, intensity-modulated radiation therapy; OS, overall survival.

mutations and representing the sensitive mutations for EGFR-TKI treatment (10). EGFR-KDD is a rare mutation. Wang et al. (5) reviewed 10759 cases of lung cancer and found that EGFR-KDD accounted for only about 0.24% of EGFR mutations. As a driver gene, EGFR-KDD mutations are commonly observed in exons 18-25, with exon 17-25 and exon 14-26 being less common. Vitro studies have shown that repetitive mutation in EGFR exons 18-25 kinase domain lead to constitutive activation of the EGFR kinase, resulting in over-activation of downstream signaling pathways, promoting cell proliferation and tumor development. Additionally, clinical pathological studies reported that EGFR-KDD alteration may increase sensitivity to EGFR-TKIs (8, 11). However, there is currently no consensus on the treatment of EGFR-KDD. Previous publications about EGFR-KDD treatments are mostly case reports, with EGFR-TKI being the choice for most first-line treatments, although the treatment is not as effective as EGFR sensitive mutations. Yang et al. (12) reported a case of an EGFR-KDD mutant patient who received chemotherapy with unsatisfactory results. Table 1 summarizes the published cases of EGFR-KDD mutation treating with first-line TKIs. The longest reported PFS is 12 months, although patients still benefit from targeted therapy at the time of publication. The only patient with brain metastasis experienced disease progression after only 2 months of treatment with osimertinib.

We are the first to report a case of EGFR-KDD mutated lung adenocarcinoma with concurrent brain metastasis treated with

TABLE 1 Clinical characteristics and outcomes of patients with NSCLC EGFR-KDD mutation treating with first-line TKIs in previous studies.

No.	Publication	Year of publication	Age	Gender/ Ethnicity	Diagnosis/ Stage	EGFR-TKI Treatment	Concurrent mutations	Response to TKI	PFS (months)
1	Zhu et al. (13)	2018	63	Female/ Chinese	LUAD/IV	Icotinib	NA	SD	11
2	Chen et al. (14)	2020	59	Male/ Chinese	LUAD/IV	Afatinib	TP53 R282W CTNNB1 S37Y	SD	10
3	Zhao et al. (11)	2021	61	Male/ Chinese	LUAD/IIIB	Afatinib	None	PR	12
4	Kim et al. (15) *	2021	50	Male/ African-American	LUAD/IVb	Osimertinib	None	PR	2
5	Wang et al. (16)	2019	74	Female/ Chinese	LUAD/IIb	Afatinib	None	SD	6
6	Zhang et al. (9)	2021	#1:44	#1:Male/ Chinese	#1:LUAD/ IVa	#1: Afatinib→ Osimertinib	#1: CDK6 TP53	#1: PR SD	#1: 7(1st line) NR(2nd line)
			#2:61	#2: Female/ Chinese	#2:LUAD/ IVb	#2:icotinib	#2:PTEN	#2:NR	#2:8
7	Wang et al. (5)	2019	#1:61	#1: Male/ Chinese	#1: LUAD/IV	#1: Erlotinib→ Osimertinib	#1: TP53	#1: PD	#1: 2(1st line) 2 (2nd line)
			#2: 60	#2: Male /Chinese	#2: LUAD/IV	#2: Gefitinib→ afatinib→ osimertinib	#2: ERBB2	#2: PR	#2: 5(1st line), 2(2nd line), 4(3rd line)
			#3: 67	#3: Female/ Chinese	#3: LUAD/IV	#3: Gefitinib	#3: NA	#3: SD	#3: 11
			#4: 43	#4: Male/ Chinese	#4: LUAD/IV	#4: Icotinib + patinib	#4: TP53, PIK3CA	#4: PR	#4: 4
			#5: 52	#5: Female/ Chinese	#5: LUAD/IV	#5: Gefitinib→ erlotinib	#5: NA	#5: PD	#5: 3(1st line) 5(2nd line)

* accompanied with brain metastasis; #: a report with multiple patients
NSCLC, non-small cell lung cancer; EGFR-KDD, epidermal growth factor receptor kinase domain duplication; TKI, tyrosine kinase inhibitor; PFS, progression-free survival; LUAD, lung adenocarcinoma; NA, not available; SD stable disease; PR, partial response; NR, not reach; PD, progressive disease

furmonertinib. Furmonertinib (AST2818) is a highly brain-penetrant, third generation EGFR tyrosine kinase inhibitor (TKI) developed by Shanghai Allist Pharmaceuticals Co., Ltd, Shanghai, China. It has been approved for the first-line treatment of patients with 19Del or L858R mutations and treatment of patients with T790M mutations whose disease has progressed on or after EGFR TKI therapy (17, 18). Compared with osimertinib, it has atrifluoroethoxy pyridine-based molecule structure that binds to a hollow hydrophobic pocket in the binding region composed of amino acids M793 and L792, enhancing its binding activity and kinase selectivity for EGFR. Additionally, this modification improved the metabolic profile of furmonertinib and inhibited the formation of non-selective metabolites. Furthermore, furmonertinib and its metabolites exhibit low affinity for wild-type EGFR-encoded proteins, which minimizes inhibition of wild-type EGFR-encoded proteins and reduces related side effects, thus improving its safety. Meng et al. revealed that furmonertinib is mainly distributed in the lung after administration (19). Therefore, furmonertinib improve treatment efficacy of lung cancer. In the FAVOUR (NCT04858958, CTR20201697) phase Ib study, furmonertinib 240 mg once daily and 160 mg once daily both showed promising efficacy and a predictable and manageable safety profile in patients with NSCLC harboring EGFR Exon20ins mutations (20). Furmonertinib might play a role in the treatment of rare EGFR mutation. A pooled, *post-hoc* analysis of two phase 2 studies (NCT03127449 [phase 2a study of furmonertinib], NCT03452592 [phase 2b study of furmonertinib]) demonstrated that better response and longer median central nervous system-PFS (CNS-PFS) were observed in

patients treated by furmonertinib 160 mg orally once daily to furmonertinib 80mg (21). Regarding the safety of medication use, FUTONG study indicated that furmonertinib and gefitinib have similar rates of drug-related adverse reactions at standard doses. However, for grade 3 or higher adverse reactions, the incidence in the furmonertinib group (18%) was lower than that in the gefitinib group (11%) (18). The most frequent severe adverse reactions with furmonertinib are QTc prolongation and diarrhea. Furthermore, a retrospective single-arm study presented at the 2023 World Conference on Lung Cancer (WCLC) demonstrated that out of 20 patients with advanced non-small cell lung cancer (NSCLC) harboring EGFR mutations and brain metastases treated with a first-line double dose of furmonertinib, only one patient experienced grade 3 treatment-related adverse events (TRAEs) (22). Three patients had their dosage reduced, and one patient interrupted treatment due to TRAEs, but no patients discontinued the study medication. These findings suggest that a double dose of furmetinib also exhibits a favorable tolerability profile. In this case, the patient has not experienced significant adverse reactions during treatment, demonstrating great safety of furmonertinib. Furmonertinib may emerge as a new option for treating EGFR-KDD mutation.

TP53 is a critical tumor suppressor gene and has the highest mutation rate among malignancies. In NSCLC patients, 50%-65% of EGFR mutation-positive cases also harbor TP53 mutations. Clinical research has consistently shown that TP53 mutations impact the effectiveness of EGFR-TKIs. A meta-analysis including 24 studies with 2,227 patients with EGFR-mutated NSCLC found that patients with TP53-EGFR co-mutations had significantly shorter PFS and overall survival (OS) duration compared to those with wild-type TP53 (23). Subgroup analysis indicated that mutations in exons 5-8 were associated with a poorer prognosis. Further research has found that in advanced EGFR-mutated NSCLC patients, the presence of TP53 exon 4 or 6 mutations leads to an even worse outcome (24). Although the effect of specific TP53 mutation subtypes on prognosis is not fully agreed upon, the co-occurrence of TP53 and EGFR mutations generally decreases the efficacy of EGFR-TKIs in NSCLC patients. Some studies have attempted to explore the effects of combination therapy. The RELAY study (25), a phase III trial, investigating the efficacy of ramucirumab plus erlotinib versus placebo plus erlotinib in EGFR-positive NSCLC patients, showed that patients with TP53 co-mutations at baseline benefited more from combination therapy in terms of PFS, regardless of exon 19del or exon 21 L858R mutations. The ACTIVE study (26) suggested that a combination of gefitinib and apatinib favored PFS for patients with TP53 mutations compared with gefitinib alone, particularly for those with exon 8 mutations. Additionally, a retrospective study (27) demonstrated that the combination of EGFR-TKI with chemotherapy could provide more survival benefits than EGFR-TKI monotherapy for NSCLC patients with the TP53-EGFR co-mutation. Despite the potential benefits, combination therapy also increases the rate of adverse reactions, which may be challenging for patients to tolerate. In our case, the patient had a TP53 exon 8 D281F mutation, which could be a negative prognostic factor. However, the patient still achieved satisfactory tumor control with monotherapy of furmonertinib.

Previous studies demonstrated that continuing the original TKI treatment in combination with consolidative local therapy benefits patients with central progression or oligoprogression after targeted therapy (28). Furthermore, multiple studies suggested that the combination of targeted therapy and anti-angiogenesis treatment significantly improves PFS (29, 30). The approach of anti-angiogenesis plus targeted therapy is a crucial component of chemotherapy-free treatment strategies. The combined therapy simultaneously blocks the EGFR/VEGF pathway and downregulates signaling pathways at multiple sites, exhibiting synergistic anti-tumor activity and delaying the occurrence of TKI resistance. Additionally, the vessel normalization effect of anti-angiogenic agents alleviates the impact of the blood-brain barrier, improving drug transport in the central nervous system and increasing intracranial drug concentrations. In this case, the patient experienced slow enlargement of intracranial and right lung lesions after 12 months of furmonertinib use. After receiving local radiotherapy, the tumor continued to shrink. In May 2023, the tumor progressed again, and the patient declined further local interventions. We added bevacizumab to the furmonertinib and the patient obtained persistent tumor control. As of now, the patient continues to benefit from the combination treatment approach, with an OS duration exceeding 22 months.

In summary, we report the first case of furmonertinib using in advanced non-small cell lung cancer (stage IVb) with an EGFR-KDD mutation. The patient achieved a sustained anti-tumor response in primary tumor and central nervous system metastases with a double-dose of furmonertinib. Compared to first-generation and second-generation EGFR TKIs, furmonertinib has better penetration across the blood-brain barrier and demonstrates efficacy in treating central nervous system metastases in non-small cell lung cancer, showing a favorable response rate. In comparison to other third-generation EGFR TKIs such as osimertinib, furmonertinib has fewer adverse reactions and higher safety, which bring better patient compliance. This case report supports the use of furmonertinib in advanced NSCLC patients with EGFR-KDD and central nervous system metastasis. Large scale study is needed to confirm this preliminary finding.

Data availability statement

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

Ethics statement

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

HL: Formal analysis, Methodology, Software, Writing – original draft. ZY: Data curation, Formal analysis, Methodology, Writing – original draft. ZL: Formal analysis, Methodology, Writing – original draft. JC: Conceptualization, Project administration, Supervision, Writing – review & editing. HW: Conceptualization, Investigation, Supervision, Writing – review & editing. YL: Supervision, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Efficacy of epidermal growth factor receptor-tyrosine kinase inhibitor for lung adenosquamous cell carcinoma harboring EGFR mutation: a retrospective study and pooled analysis

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Objectives: To explore the efficacy of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) on lung adenosquamous cell carcinoma (ASC) with EGFR mutation.

Methods: Efficacy of EGFR-TKIs in the treatment of advanced or recurrent lung ASC with EGFR mutations was assessed retrospectively in 44 patients. Pooled analysis of 74 patients using EGFR-TKIs, including 30 patients selected from 11 publications, was conducted.

Results: In our retrospective research, patients treated with EGFR-TKI in ASC with EGFR mutations had objective response rate (ORR) of 54.5%, disease control rate (DCR) of 79.5%, median progression free survival (mPFS) of 8.8 months, and median overall survival (mOS) of 19.43 months, respectively. A pooled analysis reveals ORR, DCR, mPFS, and mOS are, respectively, 63.4%, 85.9%, 10.00 months, and 21.37 months for ASC patients. In patients with deletions in exon 19 and exon 21 L858R mutations, mPFS (11.0 versus 10.0 months, P=0.771) and mOS (23.67 versus 20.33 months, P=0.973) were similar. Erlotinib or gefitinib-treated patients had an overall survival trend that was superior to that of icotinib-treated patients.

Conclusions: ASC harboring EGFR mutations can be treated with EGFR-TKI in a similar manner to Adenocarcinoma (ADC) harboring EGFR mutations. There is still a need for further investigation to identify the separate roles of ASC's two components in treating EGFR.

KEYWORDS

tyrosine kinase inhibitor, adenosquamous lung carcinoma, EGFR, mutation, lung cancer

Introduction

Lung adenosquamous cell carcinoma (ASC)has a low incidence of about 0.4%-4%, making it one of the rarest types of lung cancer (1). ASC is characterized by the presence of both glandular and squamous components, each constituting at least 10% of the tumor (2). This dual histology contributes to the aggressive nature of ASC and poses significant therapeutic challenges. The prognosis for ASC patients is generally poorer compared to those with pure adenocarcinoma or squamous cell carcinoma, reflecting its more aggressive biological behavior and limited treatment options. Although immunotherapy improves survival of ASC patient, compared to squamous cell carcinoma and adenocarcinoma, ASC patients have a worse prognosis (3–5).

In patients with non-small cell lung cancer (NSCLC) harboring epidermal growth factor receptor (EGFR) mutations, treatment with EGFR tyrosine kinase inhibitors (EGFR-TKIs) are now norm. Third-generation EGFR-TKIs, such as osimertinib, have become the current standard of care, particularly for patients with EGFR T790M resistance mutations (6, 7). EGFR mutations are predominantly found in adenocarcinoma, but they can also be detected in 54.8% of ASC patients (8). Despite the proven efficacy of EGFR-TKIs in treating EGFR-mutant NSCLC, the evidence for their effectiveness in ASC is limited due to the rarity of the condition and the consequent scarcity of comprehensive studies (9). Current treatment guidelines and clinical trials predominantly focus on adenocarcinoma, leaving a gap in tailored therapeutic strategies for ASC patients with EGFR mutations.

In this context, our study aims to explore the efficacy of EGFR-TKIs in patients with EGFR-mutant ASC through a retrospective analysis and pooled data from published studies. We seek to provide insights into the clinical outcomes and potential benefits of EGFR-TKIs therapy in this unique patient population, thereby addressing a critical gap in the management of ASC.

Patients and methods

Patients

Between January 2009 and April 2022, we collected clinical data on ASC patients treated with EGFR-TKI at West China Hospital. All patients underwent bronchofiberscope or percutaneous lung biopsies, followed by immunohistochemistry (IHC) for pathological confirmation. These retrospective analyses were carried out with informed consent from each patient.

We searched PUBMED for all publications describing the use of EGFR-TKI in advanced or recurrent EGFR mutant ASC patients for further research into its efficacy. There were three subject headings used during the search period of 2005 to 2022: lung cancer, mutation, and EGFR. Journals and publications were not limited by the strategy, but abstracts of conferences were not accepted. An evaluation of EGFR-TKIs used to treat advanced or recurrent ASC patients harboring EGFR mutations was included in this study. The choice was limited to researches published in the English journal. EGFR-TKI therapy was offered to patients who met all three criteria: (1) advanced or recurrent ASC, (2) EGFR mutation, and (3) acceptance of EGFR-TKI therapy (erlotinib 150mg/day, gefitinib 250mg/day, icotinib 125mg tid or dacomitinib 45mg/day). Data, such as EGFR mutation type, EGFR-TKI line, and treatment with EGFR-TKI, were collected as baseline factors. The authors were asked for data that wasn't included in the article.

Test method for EGFR mutation

In the retrospective data, tissues that were embalmed or freshly harvested were used to extract DNA. The mutations in EGFR were identified using a quantitative PCR analysis using the Amplification Refractory Mutation System.A EGFR mutation was detected using the protocol represented in each study, according to the published data.

Clinical assessments

The Response Evaluation Criteria in Solid Tumors were used to evaluate the efficacy of the EGFR-TKI targeted therapy. There were four types of responses: progressive disease (PD), stable disease (SD), partial response (PR), and complete response (CR). The objective response rate (ORR) is determined by dividing the percentage of patients who were CR or PR by all patients. CR, PR, and SD patients were divided by total patients to determine the disease control rate (DCR). A prognosis of progression free survival (PFS) was calculated from the beginning of treatment to the onset of PD. We also calculated overall survival (OS) from the moment treatment began until death. It was on July 22, 2022, that the last follow-up visit was carried out. In statistical analysis, patients who did not progress or were alive were censored on July 22, 2022.

Statistical methods

Qualitative variables were illustrated as the way of absolute and percentage amounts, while continuous variables were illustrated as medians with ranges. In order to conduct the survival analysis, Kaplan-Meier methods were used. An univariate analysis of log-rank tests was performed in order to determine which prognostic factors affect PFS and OS. A multivariate analysis was conducted by using Cox regression. The significance of P values is determined by using 0.05. Analyses were conducted using SPSS version 22.0.

Results

Patient characteristics

EGFR-TKI treatment was administered to 44 ASC patients with EGFR mutations at the two cancer centers for the purposes of assessing efficacy. Of the 44 ASC patients, there were 22 females and 22 males. Age range was 34-82 years (median 60.5 years). There were 17 patients with a history of smoking. Among the patients, 20

had a mutation in exon 19 (19-DEL), 21 had a mutation in exon 21 (L858R), while 3 had a rare sensitive mutation (G719X, L861Q). As a first-lines treatment, 27 patients were treated with EGFR-TKI, and 17 patients were treated in a second or more line of treatment. There were 11 patients treated with erlotinib, 21 patients treated with gefitinib, 11 patients treated with icotinib, and 1 patient treated with dacomitinib (Table 1).

Efficacy of EGFR-TKI

ASCs with EGFR mutations responded to EGFR-TKI with 2 CRs, 22 PRs, 11 SDs, and 9 PDs. The ORR was 54.5% and the DCR was 79.5% for the 44 patients (Table 2). Ten patients had not yet progressed, while 21 patients were still alive on July 22, 2022. Figure 1 shows the mPFS was 8.8 months (95% CI 3.38-14.22), and Figure 2 shows the mOS of 19.43 months (95% CI 15.42-23.45).

TABLE 1 Clinical characteristics of ASC patients with EGFR mutation.

Characteristics	Study data (n=44)	Published data(n=30)	Total data (n=74)
Age (years)	60.5(34-82)	57(30-76)	58.5(30-82)
Age	44	21	65
<60	22(33.8%)	12(18.5%)	34(52.3%)
≥60	22(33.8%)	9(13.9%)	31(47.7%)
Gender	44	30	74
Male	22(29.7%)	11(14.9%)	33(44.6%)
Female	22(29.7%)	19(25.7%)	41(55.4%)
Smoking status	44	28	72
Smoker (current/former)	17(23.6%)	5(7.0%)	22(30.6%)
Non-smoker (never)	27(37.5%)	23(31.9%)	50(69.4%)
EGFR mutation type	44	30	74
19 Del	20(27.0%)	20(27.0%)	40(54.0%)
21 L858R	21(28.4%)	8(10.8%)	29(39.2%)
G719X, L861Q	3(4.0%)	1(1.4%)	4(5.4%)
21L858R and T790M	0(0.%)	1(1.4%)	1(1.4%)
Lines of EGFR-TKI	44	22	66
1ST line	27(40.9%)	10(15.2%)	37(56.1%)
2nd+ line	17(25.8%)	12(18.1%)	29(43.9%)
EGFR-TKI treatment	44	23	67
Erlotinib	11(16.4%)	14(20.9%)	25(37.3%)
Gefitinib	21(31.4%)	9(13.4%)	30(44.8%)
Icotinib	11(16.4)	0(0%)	11(16.4%)
Dacomitinib	1(1.5%)	0(0%)	1(1.5%)

ASC, adenosquamous cell carcinoma; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

TABLE 2 Best response to EGFR-TKI in ASC patients.

Best response	Study data (n=44)	Published data(n=30)	Total data (n=74)
Complete response (CR)	2	1	3
Partial response (PR)	22	20	42
Stable disease (SD)	11	5	16
Progressive disease (PD)	9	1	10
Objective response rate (ORR)	54.5% (24/44)	77.8% (21/27)	63.4% (45/71)
Disease control rate (DCR)	79.5% (35/44)	96.3% (26/27)	85.9% (61/71)

ASC, adenosquamous cell carcinoma; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

Pooled analysis

A total of 30 patients who met the inclusion criteria from eleven research studies were included in this study (10–20). The 11 researches consisted of 8 retrospective studies and 3 case reports. A total of eight of these researches were conducted in East Asian countries. Thirteen patients were from western countries. Definitive data of age, gender, smoking status, EGFR mutation type, lines of EGFR-TKI and EGFR-TKI treatment could be extracted in 21 (70.0%), 30 (100.0%), 28 (93.3%), 30 (100.0%), 22 (73.3%) and 23 (76.7%) of the 30 patients, respectively (Tables 1, 3, 4).

Finally, we pooled data from 74 patients. Ages ranged from 30 to 82 years (median 58.5). Gender and smoking history were: male

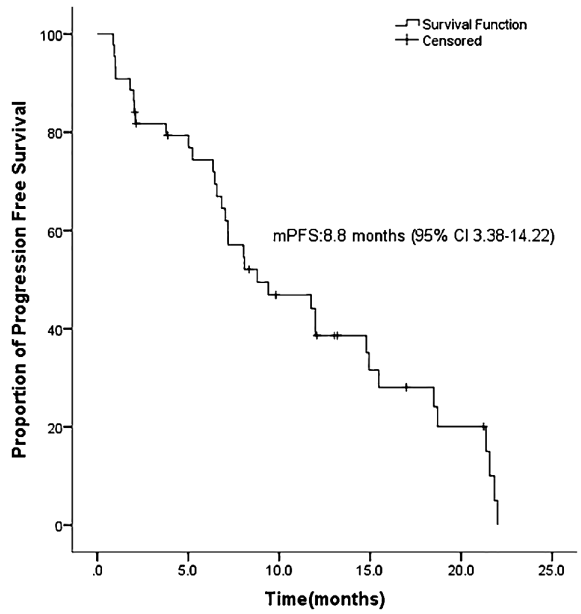

FIGURE 1
Progression free survival (PFS) of ASC in our bicenter research.

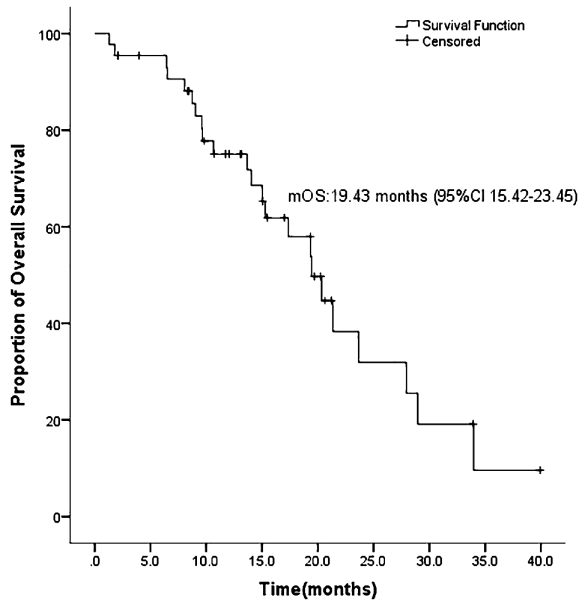

FIGURE 2
Overall survival (OS) of ASC in our bicenter research.

(33/74, 44.6%), female (41/74, 55.4%); never-smoker (50/72, 69.4%), smoker (22/72, 30.6%); There were 40 patients (40/74, 54.0%) with exon 19 deletion, 29 patients (29/74, 39.2%) with L858R mutation and 4 patients (4/74, 5.4%) with rare sensitive mutation (G719X, L861Q). One patient (1.4%) who had both a resistant mutation (T790M) and sensitive mutation (L858R) was excluded in the analysis of PFS and OS. Twenty-five (25/67, 37.3%) patients received erlotinib, 30 patients (30/67, 44.8%) received gefitinib, 11 patients (11/67, 16.4%) received icotinib and 1 patients (1/67, 1.5%) received dacomitinib. In 37 cases (37/66, 56.1%), EGFR-TKI was used as the first line of treatment while in 29 cases (29/66, 43.9%), second or more lines of treatment with EGFR-TKI were used. (Table 1).

There are 27 patients whose tumor responses were identified from published research. In total, 71 patients were evaluated for response. There were three patients with CR, 42 patients with PR, 16 patients with SD, and 10 patients with PD. It had an ORR of 63.4% (45/71) and DCR of 85.9% (61/71) (Table 2).

19 patients with PFS were identified in published research. In total, 63 patients were analyzed for PFS. All patients had a mPFS of 10.00 months (95% CI 6.73-13.27). Exon 19 deletion patients had a mPFS of 11.00 months (95% CI 6.70-15.30), while exon 21 L858R mutation patients had a mPFS of 10.00 months (95% CI 5.89-14.11) (P=0.771). Compared to rare sensitive mutations (G719X, L861Q) patients, exon 19 deletion patients or exon 21 L858R mutation patients had a longer mPFS (11.00 months vs. 2.10 months, *P*=0.005; 10.00 months vs. 2.10 months, *P*=0.019). Univariate analysis did not show significant correlations between the data sources, age, gender, smoking status, EGFR-TKI lines, and EGFR-TKI treatment and PFS. Multivariate analysis revealed no significant correlation between clinical features and PFS (Table 5).

The data of OS was extracted in 18 patients from the published researches. The pooled analysis of OS included 62 patients. The mOS was 21.37 months (95% CI 16.01-26.73). Exon 19 deletion patients had a mOS of 23.67 months, while exon 21 L858R mutation patients had a mOS of 20.33 months (P=0.973). In univariate analysis, erlotinib treatment led to a longer OS compared with icotinib treatment (25.00 months vs. 15.01 months, P=0.061); In univariate analysis, a mOS of 23.67 months was seen in patients treated with gefitinib compared with 15.01 months in patients treated with icotinib (P=0.009); Univariate analyses showed no significant correlation between the data sources, age, gender, smoking status, and lines of EGFR-TKIs and OS. In multivariate analysis, no clinical features were found to be correlated significantly with OS (Table 6).

TABLE 3 The 11 published reports which we could extract the data of recurrent or advanced ASC patients who had EGFR mutation and were treated with EGFR-TKI.

Author	Year published	Study design	Country of origin	NO. ASC patients Harboring EGFR mutation
Tokumo et al. (10)	2005	Retrospective trial	Japan	1
Ichihara et al. (11)	2007	Retrospective trial	Japan	1
Xu et al. (12)	2009	Retrospective trial	China	1
Paik et al. (13)	2012	Retrospective trial	America	9
Iwanaga et al. (14)	2012	CASE REPORT	Japan	1
Cho et al. (15)	2012	Retrospective trial	Korea	3
Baik et al. (16)	2013	CASE REPORT	America	2
Inoue et al. (17)	2013	Prospective trial	Japan	2
Powrozek et al. (18)	2014	Prospective trial	Poland	2
Tamura et al. (19)	2015	CASE REPORT	Japan	1
Song et al. (20)	2013	Prospective trial	China	7

ASC, adenosquamous cell carcinoma; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

TABLE 4 Individual patient data of the ASC patients with EGFR mutations extracted from the 11 studies that evaluated the efficacy of EGFR-TKI for ASC patients with EGFR mutations.

Author	No.	mutation	Age (y)	Sex	PS	Smoking	Line	TKI	Response	PFS (m)	OS (m)
Tokumo et al. (10)	1	L858R	77	F	1	–	2	G	SD	–	–
Ichihara et al. (11)	2	L858R+T790M	–	F	–	–	–	G	SD	1.6	8.7
Xu et al. (12)	3	L858R	–	M	–	Y	≧2	G	PR	5.3+	5.3+
Paik et al. (13)	4	19-del	61	M	–	N	2	E	PR	12.1	27.5
	5	19-del	71	F	–	N	1	E	–	19.6	32.9+
	6	19-del	58	F	–	N	2	E	SD	23.6	32.2+
	7	19-del	45	F	–	N	2	E	–	–	15.9
	8	19-del	46	M	–	N	1	E	PR	5+	6.6+
	9	19-del	73	M	–	Y	3	E	–	–	29.8
	10	19-del	76	M	–	N	1	E	PR	5.3	5.3
	11	L858R	30	F	–	N	2	E	PR	8.4	10.9+
	12	19-del	50	M	–	N	1	E	PR	9.2+	9.6+
Iwanaga et al. (14)	13	19-del	56	F	–	Y	2	G	CR	36.0	–
Cho et al. (15)	14	19-del	48	F	–	N	2	G	PR	4.53	16.93
	15	19-del	43	F	–	N	2	E	PR	8.23	24.03
	16	19-del	51	F	–	N	2	E	PR	13.53	25.0
Baik et al. (16)	17	L858R	53	F	–	N	1	E	PR	9.0	19.0
	18	19-del	61	F	–	N	1	E	PR	4+	–
Inoue et al. (17)	19	19-del	67	F	0	N	1	G	SD	2.97+	36.0
	20	L858R	60	F	0	Y	1	G	PR	10.0	32.8+
Powrezek et al. (18)	21	19-del	58	M	2	N	1	G	PR	11.0	16.0
	22	L861Q+G719X	51	M	1	N	1	E	PR	6+	8+
Tamura et al. (19)	23	19-del	66	F	1	N	2	G	PR	9.0	–
Song et al. (20)	24-30	19del(4)/ L858R(3)	–	F(4)/ M(3)	–	Y(1)/N(6)	–	E/G	PR(5)/SD(1)/ PD(1)	m8.7	–

ASC, adenosquamous cell carcinoma; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; CR, complete response; PR, partial response; SD, stabledisease; PFS, Progression Free Survival; OS, Overall Survival; G, Gefitinib; E, Erlotinib.

Discussion

Literature studies concerning EGFR-TKI sensitivity in ASC harboring EGFR mutation are limited due to the low incidence of ASC in lung cancer. As a result, there is not enough evidence to support the efficacy of EGFR-TKI in the treatment of ASC. In our pooled analysis, there was an ORR of 63.4% and DCR of 85.9% in ASC patients treated with EGFR-TKI, and a mPFS of 10.00 months and a mOS of 21.37 months in these patients. Hence, ASC containing mutant EGFR are effectively treated with EGFR-TKI. Meanwhile, EGFR mutation can be detected in 54.8% of ASC patients which demonstrated that the mutation rate is parallel to ADC (8, 21). As EGFR mutations are highly prevalent in ASC patients and EGFR-TKIs are highly effective, we recommend routine EGFR mutation testing for all ASC patients. To our knowledge, our study represents one of the largest studies of EGFR-TKI efficacy in lung ASC patients harboring mutations in EGFR. We believe this data deserves clinical reference.

ADC has been successfully treated with EGFR-TKI in previous clinical studies, with ORRs of 70-85% and mPFS of 8-13 months (7, 22). Previous research has indicated that ASC patients with EGFR mutations achieve mPFS of 9.3 months when treated with first-generation EGFR-TKI (9). As a result of our study, lung ASC had an ORR of 63.4% and a median PFS of 10.00 months. Our study shows that lung ASC with EGFR mutations respond effectively to EGFR-TKI treatment, albeit with a slightly lower efficacy compared to pure adenocarcinomas. The distinct biological behavior of ASC, which includes both squamous and glandular components, might contribute to the differences in treatment outcomes (3). The heterogeneity within the tumor may impact the response to

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TABLE 5 Association between clinical factors and the PFS.

	PFS (months)	Univariate analysis, P^a	Multivariate analysis, P^b
Data sources		0.257	0.279
Bicenter data	8.80		
Published data	11.00		
Age(years)		0.875	0.875
<60	9.00		
≥60	11.99		
Gender		0.467	0.442
Male	9.40		
Female	10.00		
Smoking status		0.907	0.863
Smoker (current/former)	9.40		
Non-smoker (never)	11.00		
EGFR mutation type		*P*1 = 0.005, *P*2 = 0.019, *P*3 = 0.771	0.181
19-DEL	11.00		
L858R	10.00		
G719X, L861Q	2.10		
Lines of EGFR-TKIs			0.127
1	8.05	0.116	
≥2	11.99		
EGFR-TKIs treatment		*P*4 = 0.101, *P*5 = 0.724, *P*6 = 0.087	0.864
Erlotinib	8.23		
Gefitinib	14.80		
Icotinib	11.77		

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; PFS, progression free survival; [a], Log-rank test; [b], Cox regression test; P1, P (19-DEL vs. G719X, L861Q); P2, P (L858R vs. G719X,L861Q); P3, P (19-DEL vs. L858R); P4, P (Erlotinib vs. Gefitinib); P5, P (Erlotinib vs. Icotinib); P6, P (Gefitinib vs. Icotinib).

TABLE 6 Association between clinical factors and the OS.

	OS (months)	Univariate analysis, P^a	Multivariate analysis, P^b
Data sources		0.154	0.133
Bicenter data	19.43		
Published data	25.00		
Age (years)		0.125	0.100
<60	19.43		
≥60	28.97		
Gender		0.300	0.214
Male	27.50		
Female	21.37		
Smoking status		0.522	0.371
Smoker (current/former)	19.37		
Non-smoker (never)	21.37		
EGFR mutation type		*P*7 = 0.973, *P*8 = 0.064, *P*9 = 0.213	0.530
19-DEL	23.67		
L858R	20.33		
G719X, L861Q	–		
Lines of EGFR-TKI		0.422	0.500
1	20.33		
≥2	25.00		
EGFR-TKI treatment		*P*10 = 0.613, *P*11 = 0.061, *P*12 = 0.009	0.168
Erlotinib	25.00		
Gefitinib	23.67		
Icotinib	15.01		

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; OS, overall survival; [a], Log-rank test; [b], Cox regression test; P7, P (19-DEL vs. L858R); P8, P (19-DEL vs. G719X, L861Q); P9, P (L858R vs. G719X, L861Q); P10, P (Erlotinib vs. Gefitinib); P11, P (Erlotinib vs. Icotinib); P12, P (Gefitinib vs. Icotinib).

EGFR-TKI, as adenocarcinoma and squamous components may respond differently to treatment. Besides, the variability in the molecular profile of ASC tumors, as compared to pure adenocarcinomas, might also be a factor (8). This variability could influence the tumor's response to EGFR-TKI therapy. Our study suggests a need for further research to explore the molecular mechanisms behind the differential response of ASC and pure adenocarcinomas to EGFR-TKI therapy.

According to our research, in patients with rare sensitive mutations (G719X, L861Q), the difference in PFS was statistically significant when compared to patients with deletion of exon 19 or exon 21 L858R mutations. However, because there were only 4 patients with rare sensitive mutation, the outcome needed to be further testified by more researches. Further, previous studies have shown that ADC patients with a L858R mutation in exon 21 of EGFR have significantly lower efficacy with EGFR-TKI treatment than patients in exon 19 of EGFR (23). However, patients with deletions in exon 19 and exon 21 L858R mutations had similar PFS (11.0 vs 10.0 months, P=0.771) and OS (23.67 vs. 20.33 months, P=0.973). The cause of this difference needs to further study. Our study primarily focused on the initial efficacy of EGFR-TKI in ASC patients. EGFR-TKI acquired resistance in lung ASC is gradually becoming a research hotspot (24). The progression and resistance mechanisms, including the frequency of T790M mutations, are undoubtedly crucial and future studies focusing on this aspect would indeed be valuable. Bsides, the efficacy in lung ASC of third generation TKI such as osimertinib and ceritinib still needs

further study (25, 26). Immunotherapy has shown promising prospects in the treatment of lung ASC (5).

In one published research, 55 ASC patients were demonstrated dual differentiation with varying proportions of ADC and SCC by using the microdissection (27). There is a pity that pathology was unable to determine which of 44 patients carried squamous cell carcinomatous and adenocarcinomatous components. Moreover, some researches discovered that the identical EGFR mutation patterns in the squamous cell carcinomatous and the adenocarcinomatous components in each patient, indicated the monoclonality of the two tumor components in ASC patients (28, 29). This conclusion was also testified by other researches (3). Since the identical EGFR mutation patterns occurred in the squamous cell carcinomatous and the adenocarcinomatous components of ASC, It may be the proportion of two tumor components in EGFR mutant ASC patients that determines the efficacy of EGFR-TKI in EGFR mutant ASC patients. The predominance of one component over the other could potentially affect the treatment outcomes. In addition, the therapeutic advantages on adenocarcinoma components of TKI may generate the withering of the adenocarcinomatous components of ASC, while the squamous cell carcinomatous of ASC gain a quantitative advantage (30). Researchers at our cancer center are investigating how the ratio of these two tumor components and EGFR-TKI efficacy are related.

In NSCLC, especially in metastatic disease, small biopsy samples can make it difficult to accurately differentiate between squamous cell carcinoma and ASC (1). This distinction is crucial as it impacts treatment decisions. Molecular testing, including EGFR mutation analysis, can play a critical role in identifying patients who might benefit from targeted therapies (31). This is particularly relevant in cases where histological classification is uncertain. Given the histological overlap between squamous tumors and ASC, molecular testing provides a more precise approach to identify the tumor's characteristics, thus guiding appropriate treatment (3). The American Society of Clinical Oncology (ASCO) emphasizes the need for comprehensive molecular profiling in NSCLC. By incorporating molecular testing, clinicians can better tailor treatment strategies to individual patient needs, especially for those with rare or atypical NSCLC subtypes like ASC.

It is necessary to illustrate the limitations of this study. Among the selected published studies, inclusion criteria and test methods for EGFR mutations were different, and clinical traits were not completely described. Moreover, the retrospective nature was another limitation of this research. The low incidence of ASC in lung cancer, however, makes our research quite significant as well.

In conclusion, this study which involved all available data, including data collected from our cancer centers of China and that pooled from previous studies, and identified the clinical profiles of EGFR-TKI application, suggested that EGFR-TKI was found to be an effective treatment in ASC harboring mutations in EGFR. Furthermore, the study recommends that EGFR mutation testing be conducted routinely on all lung ASC patients.

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 humans were approved by West China hospital's Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

XX: Conceptualization, Data curation, Writing – original draft. WD: Data curation, Writing – original draft. YZ: Supervision, Validation, Writing – original draft. YYL: Investigation, Software, Writing – original draft. MY: Formal analysis, Methodology, Resources, Writing – original draft. YML: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Neoadjuvant targeted therapy versus targeted combined with chemotherapy for resectable EGFR-mutant non–small cell lung cancer: a retrospective controlled real-world study

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Background: This study aimed to assess the role and effect of neoadjuvant targeted therapy (TT) versus targeted combined with chemotherapy (TC) for resectable EGFR-mutant non–small cell lung cancer (NSCLC).

Methods: Between March 2021 and June 2023, 20 patients with stage IA3-IIIB NSCLC were enrolled in the study. Eleven patients received EGFR-TKIs in the TT group, while nine patients received EGFR-TKIs and two cycles of cisplatin-based doublet chemotherapy (TC group). We compare the differences between the two groups through the following variables, including age, sex, surgical approach, postoperative complications, neoadjuvant therapy adverse events, complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD), objective response rate (ORR), major pathologic response (MPR), and pathologic complete response (pCR).

Results: Patients were predominantly female (75%) and never-smokers (95%). The average age was 59.2 years (range 46-79 years). Fifty-five percent harbored an exon 19 EGFR mutation and 45% an exon 21 mutation. The average targeted drug dosing time was 2.91 ± 1.7 (range 1-6) months in the TT group and 3.56 ± 3.54 (range 1-12) months in the TC group (P=0.598). The most common side effects were rash and diarrhea. No grade 5 events with neoadjuvant therapy were observed. The rate of R0 resection was 100% in all patients. Among the 11 patients in the TT group, 6 achieved a PR and 5 had SD, resulting in an ORR of 54.5%. Among the 9 patients in the TC group, 6 had PR and the remaining 3 had SD, resulting in an ORR of 66.6%. one patient (11.1%) in the TC group achieved pCR, while no patients in the TT group achieved pCR (P = 0.142). Two patients (18.2%) in the TT group reached MPR, and 2 patients (22.2%) in the TC group reached MPR (P = 0.257). The overall clinical downstage rate is 60%. Only 9 (45%) cases of yield clinical TNM (ycTNM) were consistent with yield pathologic TNM (ypTNM).

Conclusion: Results from this retrospective controlled research indicate that the neoadjuvant TT group is likely to be more effective outcomes and has safer profile in patients with EGFR-positive NSCLC than the neoadjuvant TC group. However, our results need to be validated in a multicenter, large sample prospective study.

KEYWORDS

NSCLC, EGFR-mutation, neoadjuvant targeted therapy, neoadjuvant targeted plus chemotherapy, MPR

Introduction

Lung cancer is the leading cause of cancer death in China and the world (1, 2). Previously, treatment options for potentially resectable patients with non-small cell lung cancer (NSCLC) include neoadjuvant therapy with cisplatin or carboplatin; and subsequent adjuvant chemotherapy and/or radiotherapy to prevent rapid recurrence (3). However, this benefit has been questioned (4). With the advent of Checkmate-816, immunotherapy alone or in combination with chemotherapy has created a wave of new research in the neoadjuvant setting (5). Clinical evidence has shown that patients with advanced, EGFRmutant NSCLC derive little or no benefit from cancer immunotherapy combining with or without targeted therapies (6, 7). In the past few decades, the increasing knowledge of cancer biology has led to the introduction of new targeted therapies for lung cancer (2). These new therapies target specific cancer processes and hence have the potential to be more effective and less toxic (8). Adjuvant targeted therapies (epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI)) have revolutionized the NSCLC care in the advanced disease setting (9). The recommended first-line treatment for patients with oncogene-addicted advanced NSCLC is targeted therapies. Targeted therapies could be beneficial in the neoadjuvant setting for this group of patients (10, 11). There are no completed targeted therapy clinical trials and no current neoadjuvant standard of care in the management of EGFR mutation-positive (EGFRm) NSCLC, but several studies (NCT01833572, NCT01217619, EMERGING-CTONG 1103, NEOS, and NCT03433469) are now recruiting (12–14). Considering the advantages of preoperative targeted therapy and the disadvantages of preoperative neoadjuvant chemotherapy, can we consider conducting separate neoadjuvant targeted therapy for this type of patient? Therefore, we conducted a retrospective controlled study of EGFR-TKIs combined with chemotherapy versus EGFR-TKIs alone as neoadjuvant therapy in the treatment of EGFR-mutation positive resectable NSCLC.

Patients and methods

Twenty treatment-naive patients with stageIA3-IIIB NSCLC were enrolled in the study from March 2021 to June 2023 at the Shandong Cancer Hospital and Institute. Inclusion criteria included: 1) age 18 years or older; 2) no previous treatment for lung cancer; 3) Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1 (15); 4) pathologically confirmed as adenocarcinoma; 5) with EGFR mutations in exon 19 or 21; 6) underwent surgery; and 7) neoadjuvant targeted therapy or targeted combined with chemotherapy. Exclusion criteria included: 1) history of malignancies in the past 5 years; 2) previous local radiotherapy or any systemic antitumor therapy;3) unstable systemic disease; and 4) history or current diagnosis of interstitial lung disease (ILD).

The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Shandong Cancer Hospital and Research Institute (No. SDTHEC2024002005).

Eleven patients received EGFR-TKIs as neoadjuvant therapy (TT group), while nine patients received EGFR-TKIs and two cycles of platinum-based doublet chemotherapy in combination with docetaxel or pemetrexed (TC group). Platinum-based drugs included carboplatin, with an area under the curve of 5, or cisplatin 25 mg/m2 on days 1–3. Pemetrexed (500 mg/m2) was then administered. Patients received 1–3 doses of preoperative chemotherapy every three weeks, and the average usage cycle was two in the TC groups. The target drugs included osimertinib, gefitinib, almonertinib, afatinib, furmonertinib, icotinib.

Patients underwent chest Computed Tomography (CT), abdomen CT, and Emission CT (ECT) scans; brain magnetic resonance imaging (MRI), or Positron Emission Tomography CT (PET-CT); bronchoscope or Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA); and examinations of cardiac function. In our routine clinical practice, simplified radiological evaluation (enhanced CT or PET-CT) was more common for N-stage patients. Only a few patients have undergone bronchoscopy or EBUS-TBNA.

The stages of the primary pulmonary tumor (T), lymph node (N), and metastasis (M) were evaluated based on the American Joint Committee on Cancer 8th edition TNM staging system for NSCLC (16). After neoadjuvant therapy, enhanced CT was performed to observe the response of the tumor to drugs, and the tumor size was evaluated according to RECIST 1.1 (response evaluation criteria in solid tumors version 1.1) (17). The evaluation of the target lesions was divided into complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD), and objective response (OR) including PR+CR. All the patients were monitored for adverse events, according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 5.0 (NCI-CTCAE 5.0) (18). Major pathologic response (MPR) was defined as residual viable tumor cells less than 10%, and pathologic complete response (pCR) was defined as no residual viable tumor (19).

Statistical analysis

Categoric variables were expressed as numbers or percentages and evaluated with λ^2 or Fisher's exact test. Continuous data were presented as mean ± SD and were compared using the two-sample Student t-test. All P-values were reported by 2-sided analyses, and the statistical significance level was set at less than 0.05. Statistical analyses were performed with SPSS 24.0 software (SPSS, Inc., Chicago, IL, USA) and R version 4.1 (R Foundation for Statistical Computing).

Results

Patient characteristics

Twenty NSCLC patients were recruited (Figure 1). Patients were predominantly female (75%) and never-smokers (95%), only one patient had ever-smoking. The average age was 59.2 years (range 46-79 years). Fifty-five percent had an exon 19 EGFR mutation and 45% had an exon 21 mutation. Most patients were stage clinical T2(cT2) NSCLC (TT group, 36.3%; TC group, 44.4%). The proportion of patients with cN2(55%) and cIIIA (55%) stages was the highest. Age, gender, smoking status, EGFR status, ECOG PS, dosing time, and clinical stage were balanced between arms. The average dosing time was 2.91 ± 1.7 (range 1-6) months in the TT group and 3.56 ± 3.54 (range 1-12) months in the TC group (P=0.598). The patient demographics and clinical characteristics are summarized in Table 1.

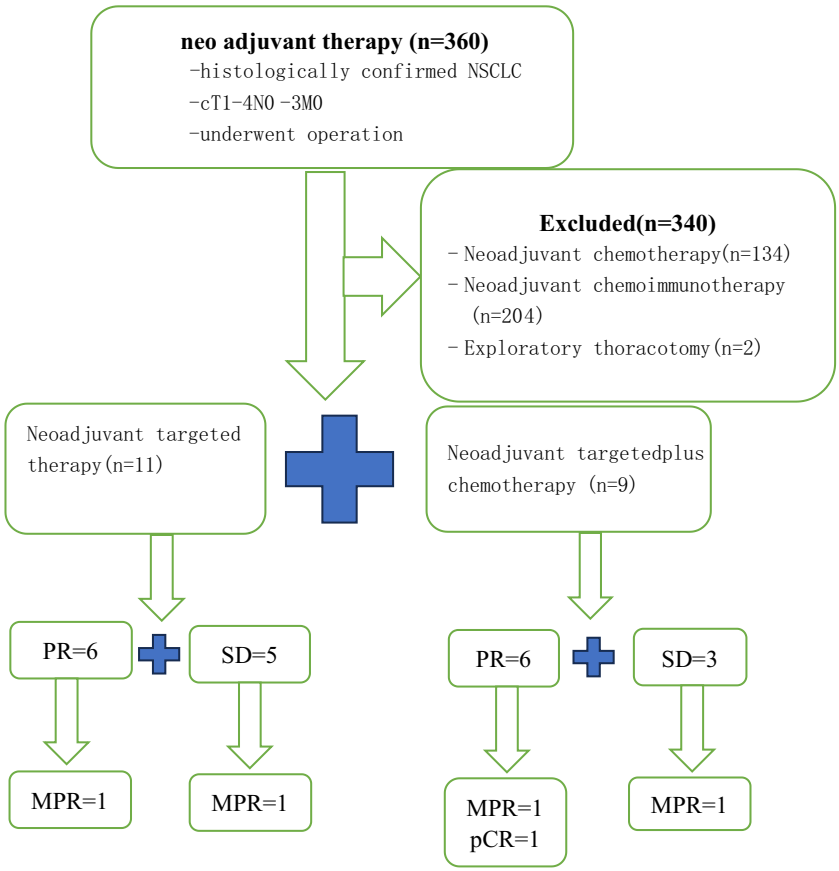

FIGURE 1
Flow diagram of study design.

TABLE 1 Patient demographics and clinical characteristics.

characteristic		total	TKI	TKI +Chemotehrapy	p-value
			11	9	
EGFR status	Exon 19 deletion	11	6	5	0.964
	L858R 21	9	5	4	
ECOG PS	0	13	8	5	0.423
	1	7	3	4	
Sex	male	5	2	3	0.436
	female	15	9	6	
smoking status	ever	1	1	0	0.353
	never	19	10	9	
cT	1	5	3	2	0.528
	2	8	4	4	
	3	2	2	0	
	4	5	2	3	
cN	0	4	2	2	0.067
	1	4	0	4	
	2	11	8	3	
	3	1	1	0	
cTNM	IA3	1	1	0	0.11
	IB	2	0	2	
	IIA	1	1	0	
	IIB	1	0	1	
	IIIA	11	5	6	
	IIIB	4	4	0	
age, years			60.91 ± 8.50	57.11 ± 8.08	0.323
dosing time, months			2.91 ± 1.7	3.56 ± 3.54	0.598

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; ECOG PS, Eastern Cooperative Oncology Group performance status.

Safety and tolerability

Overall, the two neoadjuvant treatment regimens were well tolerated. The most common side effects were rash and diarrhea. No grade 5 events with neoadjuvant therapy were observed. As shown in the Table 2, most patients underwent video-assisted thoracic surgery (VATS) (85%) and lobectomy (95%). No patient converted to pneumonectomy because of the complexity of the operation. The rate of R0 resection was 100% in all patients. The average operation time was 121.36 ± 62.45 minutes in the TT group and 91.11 ± 20.28 minutes in the TC group. Although the time of operation varies greatly, it is not statistically significant (p=0.155). We traced the operation time data and found that in the TT group, there were 2 cases with longer operation times (240min and 180min, respectively), which resulted in longer mean operation times. There were 2 cases in the TT group (postoperative chylothorax and hydrothorax). No perioperative mortality was found in either arm. There was no significant difference between the two arms. The follow-up time in the TC group (25 ± 5.15 months) was significantly longer than that in the TT group (18 ± 5.16 months) (P=0.007). One case in the TC group occurred brain metastasis 19 months after being diagnosed with lung cancer. After follow-up, further treatment is still underway.

Efficacy

Clinical responses to neoadjuvant therapies

As shown in the histogram (Figure 2) waterfall plot (Figure 3), among the 11 patients in the TT group, 6 acquired a PR and 5 had SD, resulting in an ORR of 54.5%. Of the 9 patients in the TC group, 6 had PR, and the remaining 3 had SD, resulting in an ORR of 66.6%. Our data show that patients in the TC group had a better ORR than those in the TT group, although the difference was not

TABLE 2 Patient surgical and postoperative characteristics.

characteristic		total	TKI	TKI+Chemotehrapy	p-value
operation methods	lobectomy	19	11	8	0.257
	bilobectomy	1	0	1	
surgery	thoractomy	3	1	2	0.413
	VATS	17	10	7	
complication	no	18	9	9	0.178
	yes	2	2	0	
operation time (min)			121.36 ± 62.45	91.11 ± 20.28	0.155
follow-up time (months)			18 ± 5.16	25 ± 5.15	0.007
discharge time (days)			5.91 ± 1.70	6.78 ± 2.59	0.378
status	alive	19	10	9	0.353
	death	1	1	0	

TKI, tyrosine kinase inhibitor.

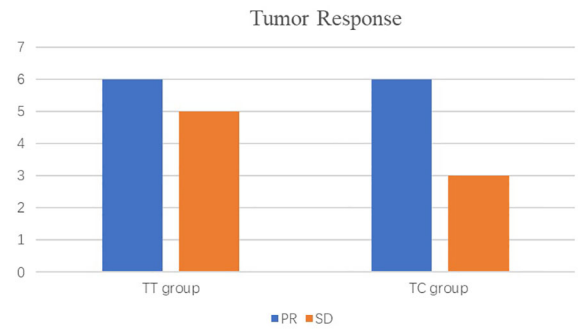

FIGURE 2
Clinical responses to neoadjuvant treatment.

statistically significant (P=0.582). After neoadjuvant treatment, the overall clinical downstage rate is 50%. The TT group and TC group were 54.5% and 44.4% (P=0.653), respectively (Table 3).

Pathological responses to neoadjuvant therapies

In the postoperative pathological analysis (Table 3), one patient (11.1%) in the TC group achieved pCR, while no patients in the TT group achieved pCR. The difference was not statistically significant (P = 0.142). Two patients (18.2%) in the TT group reached MPR, and 2 patients (22.2%) in the TC group reached MPR, the difference was not statistically significant (P = 0.257). From this, we can conclude that targeted drugs alone may achieve the effect of targeted plus chemotherapy. The overall clinical downstage rate is 60%. The TT group and TC group were 45.5% and 77.8% (P=0.142),

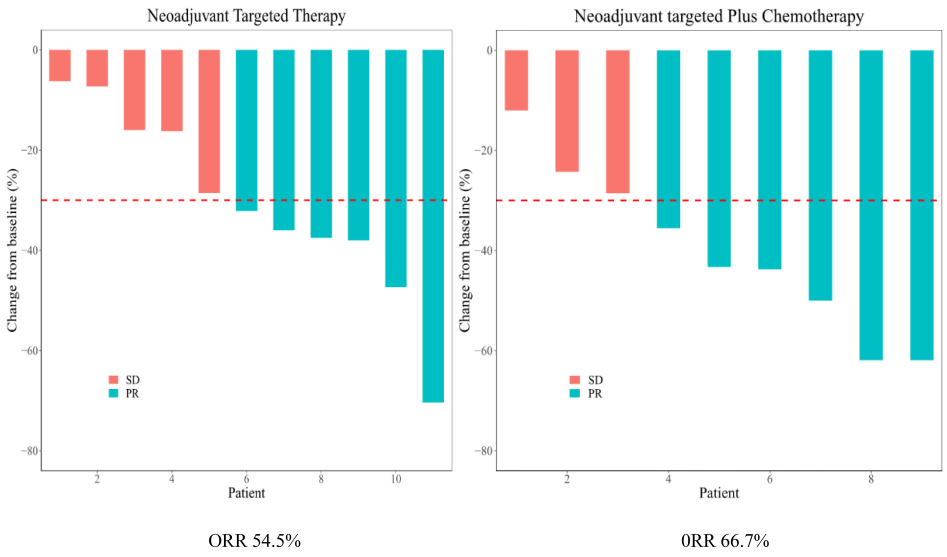

FIGURE 3
Waterfall plot of each arm.

TABLE 3 Evaluation of neoadjuvant therapy efficacy.

characteristic		total	TKI	TKI+Chemotehrapy	p-value
			11	9	
ycT	1	12	6	6	0.019
	2	5	5	0	
	3	3	0	3	
ycN	0	7	3	4	0.249
	1	7	3	4	
	2	6	5	1	
ycTNM	IA1	1	0	1	0.426
	IA2	2	1	1	
	IA3	1	0	2	
	1B	1	1	0	
	IIA	1	1	0	
	IIB	4	3	1	
	IIIA	9	5	4	
ORR	yes	12	6	6	0.582
	no	8	5	3	
PR	yes	12	6	6	0.582
	no	8	5	3	
SD	yes	8	5	3	0.582
	no	12	6	6	
cDownstage	yes	10	6	4	0.653
	no	10	5	5	
ypT	0	1	0	1	0.082
	IA	10	3	7	
	IB	5	4	1	
	IC	1	1	0	
	2A	3	3	0	
ypN	0	10	3	7	0.073
	1	1	1	0	
	2	9	7	2	
ypTNM	0	1	0	1	0.105
	IA1	8	2	6	
	IA2	1	1	0	
	IIB	1	1	0	
	IIIA	9	7	2	
pDownstage	yes	12	5	7	0.142
	no	8	6	2	
ycTNM=ypTNM	yes	9	7	2	0.064
	no	11	4	7	

(Continued)

TABLE 3 Continued

characteristic		total	TKI	TKI+Chemotehrapy	p-value
			11	9	
MPR	yes	4	2	2	0.822
	no	16	9	7	
CPR	yes	1	0	1	0.257
	no	19	11	8	

TKI, tyrosine kinase inhibitor; ORR, objective response rate; PR, partial response; SD, stable disease; MPR, major pathologic response; pCR, pathologic complete response; ycTNM, yield clinical TNM; ypTNM, yield pathological TNM.

respectively. We compared the yield clinical TNM (ycTNM) and yield pathological TNM (ypTNM) stages of each patient separately. Only 9 (45%) cases of ycTNM were consistent with ypTNM. The TT group and TC group have 7 (63.6%) and 2 (22.2%) (P=0.064), respectively. The remaining 11 patients had inconsistent staging, eight patients achieved better downstage than ycTNM. The TT group and TC group have 2 and 6, respectively. Both the TT group and TC group have two up-stage. The difference between post-treatment and postoperative stages reminds us of the limitations of the imaging assessment of staging.

Discussion

In a meta-analysis of patients with resectable NSCLC, neoadjuvant chemotherapy was found to improve 5-year overall survival (OS) by 5% compared with surgery alone (3). Therefore, there is a need for new and effective treatments to reduce the recurrence of disease, prolong the survival time, and improve the cure rates. Given the success of target therapy in the advanced disease setting, there is increasing research on neoadjuvant targeted therapy for mutation-driven resectable NSCLC (11, 13). Is targeted therapy combined with chemotherapy more effective as neoadjuvant therapy? Therefore, we conducted this retrospective controlled study.

In this study, patients who received TT or TC had similar clinical and pathological effects. Lara-Guerra H et al. demonstrated that gefitinib was a generally safe and feasible regimen for neoadjuvant therapy in unselected patients with stage I NSCLC, with a PR of 11%(NCT00188617) (13). Zhang Y et al. found that neoadjuvant gefitinib was a viable treatment option for stage II-IIIA NSCLC patients with EGFR-mutant, ORR, and MPR were 54.5% and 24.2%, respectively (NCT01833572) (11). Xiong L et al. reported that erlotinib resulted in a higher ORR (67%vs.19%) and pathologic response rate (67%vs.38%) than platinum-based adjuvant chemotherapy (PBAC) (NCT01217619) (20). The EMERGING-CTONG 1103 study found that the ORR for neoadjuvant erlotinib versus chemotherapy was 54.1% versus 34.3%. The MPR was 9.7% and 0%, respectively. No pCR was identified in either arm (10). The above studies were mostly single-arm studies of neoadjuvant TT or control studies of neoadjuvant TT compared to neoadjuvant chemotherapy, with varying clinical and pathological results obtained. Few studies directly compare neoadjuvant TT and TC. Combined with our results, or neoadjuvant targeted therapy alone, we could achieve the expected results. The reason why we propose this viewpoint is inspired by postoperative adjuvant therapy. For example, Tetsuya Isaka et al. showed that that patients with EGFR mutations (–) who received PBAC had better OS than those who did not receive PBAC, although EGFR mutation (+) patients who did and did not receive PBAC had no difference in OS. They concluded that neoadjuvant chemotherapy might not be necessary for EGFR mutation (+) patients with pathological stage II/III NSCLC (21). In another study from Japan, Yasuhiro Tsutani et al. evaluated the role and effect of adjuvant chemotherapy based on EGFR mutation status in patients with stage I lung adenocarcinoma. For patients with EGFR mutation (+), who received adjuvant chemotherapy or not, there was no significant difference in the 5-year recurrence-free survival (RFS) (74.3% vs 80.5%, P=0.573) and OS (91.7% vs 97.8%, P=0.183). For patients with EGFR mutation (-/unknown), received adjuvant chemotherapy or not, there were significant differences in the 5-year RFS (88.4% vs 63.6%, P=0.001) and OS (93.2% vs 77.9%, P=0.008) (22). There is another study from China, Wenyu Zhai et al. demonstrated that adjuvant chemotherapy was associated with improved OS and DFS outcomes in patients with EGFR mutation (–), but not benefit with EGFR mutation (+) (23). Harry B. Lengel et al. proved that preoperative targeted therapy was well tolerated and associated with good outcomes, regardless of induction chemotherapy (24). The ongoing clinical trials (NCT04470076, NCT04351555, NCT05011487, NCT05132985, NCT05430802) might have certain reference opinions for determining the choice of neoadjuvant treatment options (12).

A Phase II study of preoperative gefitinib in clinical stage I NSCLC showed that 83% of patients had consistent clinical and pathological staging (13). Ye Ning et al. retrospectively evaluated the survival rate of 10 patients with advanced NSCLC who underwent salvage surgery after EGFR-TKI neoadjuvant therapy and found that 70% of patients have consistent clinical and pathological staging. In the remaining 3 cases, 1 case was downstaged, and the other 2 cases were upstaged (25). NEOS is a prospective, multicenter, single-arm study designed to evaluate the efficacy and safety of osimertinib as a neoadjuvant treatment in resectable EGFR mutation (+) lung adenocarcinoma. The disease control rate (DCR) was 100% (15/15), 53.3% (8/15) of patients had

a pathological decline, and 42.9% (3/7) of the patients with confirmed N2 lymph nodes experienced downstaging to N0 disease after receiving neoadjuvant osimertinib (26).

The above studies are all single-arm studies. In the present study, there was a certain degree of decline in both the TT group and the TC group. However, the concordance rate of the clinical stage and postoperative stage in the TT group was higher than that in the TC group. In the TC group, after the neoadjuvant target was combined with chemotherapy, to some extent, the tumor shrank more significantly than that measured on CT. Compared to the TT group, chemotherapy might affect tumor shrinkage, but further molecular-level research is needed to determine the specific mechanism. Even if the tumor was somewhat responsive to drug, it still takes time for the necrotic lesion to absorb after targeted therapy, resulting in a reduction in the diameter measured by CT. This results in inconsistency between clinical remission and pathological outcome.

Compared with TC, TT has unique safety and tolerability in the neoadjuvant setting. It is important to consider whether there is any toxicity that may delay or prevent the efficacy of surgery during neoadjuvant therapy. Compared to neoadjuvant chemotherapy, the use of EGFR inhibitors has fewer severe respiratory adverse events (including pneumonitis and interstitial lung disease), which could limit the use of neoadjuvant therapy (27).

In our study, no grade 5 events were observed. The results demonstrate that most patients can tolerate these two schemes. Most patients underwent VATS and lobectomy. No patient received a pneumonectomy. The rate of R0 resection was 100%. Judging from these data, the two schemes have little impact on the operation. A narrative review similar to our results suggests that neoadjuvant target therapy is well tolerated in resectable NSCLC patients, with all patients undergoing surgery without delay or major complications (27, 28). Delays in surgery due to adverse events from oncology treatment, limitations in diagnostic services provided, and the possibility of progression during treatment may have challenged to the selection of neoadjuvant chemotherapy. It may be that our sample size is too small to show this difference between the two groups. The ongoing clinical trials will provide further information on the safety and tolerability of a wider range of TT for neoadjuvant treatment of resectable NSCLC.

The research on the neoadjuvant TT or TC is still early and less, and the optimal course of treatment is not yet known. Because of our retrospective study, it is difficult to control the preoperative medication time, which ranges from 1 month to 12 months. We also have no clear recommendations for the medication cycle. Perhaps according to the evaluation of CT (RECIST 1.1) after-treatment is a better choice.

There were several limitations to this research. Firstly, patient selection bias may have existed due to the retrospective nature of this real-world study. Secondly, given the limited number of patients in our cohort, larger multi-center or even prospective studies are necessary to confirm our findings. Finally, our results might be influenced by tumor characteristics and surgeon's techniques, experience, and preferences.

Conclusion

To our knowledge, few studies have conducted real-world studies on neoadjuvant targeted therapy versus targeted combined with chemotherapy for resectable EGFR-Mutant NSCLC. Results from this retrospective controlled research indicated that the neoadjuvant TT group was likely to be more effective outcomes and has safer profile in patients with EGFR-positive NSCLC than the TC group. Therefore, we recommend further investigation through a prospective study to validate the findings of this retrospective analysis.

Data availability statement

The original contributions presented in the study are included in the article/supplementary files, further inquiries can be directed to Weipeng Shao, shaoweipeng2015@163.com.

Ethics statement

This study was approved by the institutional review board of Shandong Cancer Hospital and Institute (SDTHEC2024002005). The requirement for written informed consent from individual patients for this retrospective analysis was waived by the institutional board.

Author contributions

WS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. BL: Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft. ZL: Conceptualization, Data curation, Formal analysis, Software, Writing – review & editing. FC: Investigation, Methodology, Project administration, Resources, Writing – review & editing. JL: Formal analysis, Methodology, Supervision, Writing – review & editing. HL: Formal analysis, Software, Supervision, Writing – review & editing. HG: Conceptualization, Investigation, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer DC declared a shared affiliation with the authors to the handling editor at the time of review.

Publisher's note

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