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Insights in thoracic oncology: 2021/2022

Edited by
Lizza E. L. Hendriks, Vamsi Velcheti, Alfredo Addeo and Kaushal Parikh

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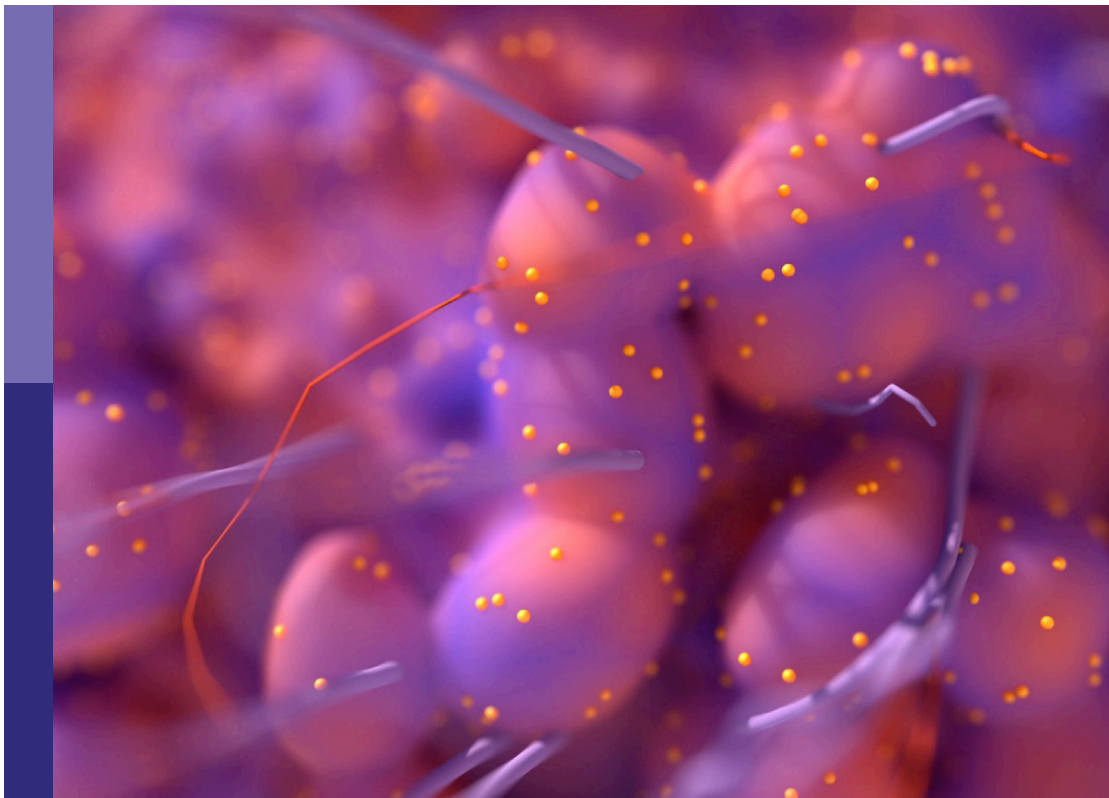

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Insights in thoracic oncology: 2021/2022

Topic editors

Lizza E. L. Hendriks — Maastricht University Medical Centre, Netherlands
Vamsi Velcheti — New York University, United States
Alfredo Addeo — Hôpitaux universitaires de Genève (HUG), Switzerland
Kaushal Parikh — Hackensack University Medical Center, United States

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EDITED AND REVIEWED BY
Marcello Migliore,
University of Catania, Italy

*CORRESPONDENCE
Alfredo Addeo
Alfredo.addeo@hcuge.ch

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Editorial: Insights in thoracic oncology: 2021/2022

Kaushal Parikh[1] and Alfredo Addeo[2]*

[1]Oncology Unit, Mayo Clinic, Rochester, NY, United States, [2]Oncology Department, University hospital Geneva, Geneva (CH) Swiss Cancer Centre Leman, Geneva, Switzerland

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NSCL, SCLC, driver mutations, surgery, biomarkers

Editorial on the Research Topic
Insights in thoracic oncology: 2021/2022

This Research Topic '*Insights in Thoracic Oncology: 2021/2022*', provide the readers a curated selection of articles to explore the current and future interests in the field of thoracic oncology. These articles encompass multiple disciplines of thoracic oncology and lay out new evidence in diagnosis, prognosis, and treatment of patients with thoracic malignancies. This series features both, high impact original research articles as well as state-of-the-art reviews, suitable for basic, translational and clinical scientists, trainees, and clinicians to stay abreast with this rapidly advancing field. The field is moving forward from genomics to 'multi'-omics, often using liquid biopsy specimens and minimally invasive techniques. Newer technologies have facilitated measurement and characterization of microRNA (miRNA) in cancer development, treatments, and prognosis and developed into the field of miRNAomics. Dezfuli et al evaluated the expression of miRNAs miR-146a and miR-155 in peripheral blood mononuclear cells. In this case-control study of 33 patients with NSCLC and 30 controls, they discovered significant downregulation of miR146a accompanied by TGF-β elevation. Further work can help develop these are markers for disease progression and outcomes. Arguably, biomarker development for thymic tumors is trailing other thoracic malignancies. Yuan et al elucidated the diagnostic and prognostic role of SOX9 expression in thymic epithelial tumors. They observed that SOX9 was highly expressed in TET cells and correlated with histologic subtypes of thymomas, potentially aiding in diagnosis. They also postulate that SOX9 expression serves as a negative prognostic marker for TETs and was associated with an immunosuppressive tumor microenvironment. The next article in the translational science segment is Johnson et al, wherein provide a comprehensive review on preclinical models and resources to aid research in mesothelioma. They also provide information on mesothelioma biobanks that are available globally to researchers.

For early stage or localized thoracic cancers, surgery with or without perioperative therapy remains the mainstay of treatment. The increased adoption of robotic and other minimally invasive surgical procedures warranted a randomized trial to assess its

effectiveness. The ROMAN study by Veronesi et al randomized 83 patients with early-stage NSCLC to robotic-assisted thoracic surgery (RATS) or video-assisted thoracic surgery (VATS) and observed that RATS was not superior to VATS with respect to perioperative outcomes, but it led to a greater degree of hilar and mediastinal nodal assessment compared to VATS. This is the first randomized study comparing two minimally invasive thoracic surgery methods for NSCLC and it sheds light on the need for more validation trials. The second surgical study in this series is a retrospective analysis by Testori et al, where they evaluate the efficacy of intraoperative hypertonic glucose solution administration on persistent air leak after pleurectomy/decortication for pleural mesothelioma[5]. Hypertonic glucose solution is hypothesized to be pro-inflammatory, leading to development of fibrous adhesion between lung and chest wall and resolution of air leak. This case-control study of 71 patients showed that intraoperative hypertonic glucose administration reduced duration of chest tube management after discharge without altering duration of hospitalization or duration of chest tube maintenance during hospitalization. Surgical resection is uncommon in small cell lung cancer, often due to the advanced stage at diagnosis. However, when feasible, surgical resection is the preferred treatment for localized disease and small cell carcinomas are also incidentally diagnosed during surgery at times. Li et al retrospectively analyzed the impact of adjuvant therapy in resected small cell lung cancer. Out of 153 patients included in this single center analysis, adjuvant radiation and adjuvant chemotherapy were associated with improved survival compared to no adjuvant therapy. The benefits adjuvant radiotherapy following chemotherapy were more pronounced for patients with pathologic nodal involvement. This study reiterates the role for surgery and adjuvant therapy in small cell lung cancer.

Non-small cell lung cancer has been the posterchild for targeted therapies and these agents have significantly improved patient outcomes. While osimertinib is the preferred EGFR tyrosine kinase inhibitor (TKI) in the US, several parts of the world have access issues to osimertinib, and earlier generation inhibitors such as afatinib, gefitinib or erlotinib remain standard of care. Passaro et al present a pooled analysis of three single arm phase IIIb studies of afatinib in patients with EGFR mutant NSCLC. Median PFS on afatinib in this analysis was 13.0 months and ORR was 55.0%. Afatinib was found to have no new toxicity signals and showed activity against uncommon EGFR mutations such as G719X, L761X, and S768IX).Patients with previously stable and or treated brain metastases (BM) were included and the efficacy of Afatinib was not affected by the presence of BM. Therefore afatinib could represent a viable frontline option in situations where osimertinib access is limited. EGFR mutations on exon 18 such are uncommon and there is limited evidence on the optimal treatment strategy in these cases. While afatinib is the most widely used TKI in this scenario, Xu et al retrospectively studied the outcome of 82 patients with EGFR exon 18 alterations. They observed no statistically significant difference in PFS between afatinib and the combination of a first generation TKI (erlotinib or gefitinib) with chemotherapy (p=0.709). From discovering and treating new targets, the focus is gradually shifting to overcoming resistance mechanisms and understanding tumor biology of patients with actionable targets. To this effect, Kamga et al studied 74 consecutive patients with EGFR mutant NSCLC and discovered that high circulating plasma levels of sonic hedgehog protein at diagnosis and on treatment with TKIs were associated with worse prognosis and treatment resistance. Another prospective study in this series by Raphael et al concluded that comprehensive genomic profiling of tumor or circulating tumor DNA impacted subsequent treatment in patients with ALK rearranged NSCLC who had disease progression following treatment with a second or third generation ALK TKI. This study further underlines the utility of comprehensive next-generation sequencing upon resistance to targeted therapies. Treatment options post-progression on TKI are limited. If treated with a TKI as frontline therapy, in the absence of targetable resistance mechanisms, treatment is often platinum-based chemotherapy with or without immune checkpoint inhibitors (ICIs). Patients with EGFR or ALK alterations typically respond poorly to ICIs while the responses are somewhat more heterogenous for patients with KRAS, BRAF V600E or MET exon 14 skipping alterations. The articles by Seegobin et al and Wiest et al comprehensively review the role of immune checkpoint inhibitors in treatment of NSCLC with EGFR and other actionable oncogenic driver alterations. In the theme of benefits of ICI in oncogenic-driven NSCLC, Hou et al studied the role of ICI in mice models harboring ATRX-deficient lung cancer cell lines. ATRX is a tumor suppressor gene and ATRX mutations are associated with poor prognosis in multiple cancers. The authors observed that ATRX mutations sensitized lung cancer cell line models to IBI and may serve as a biomarker for benefit with immunotherapy.

The above articles display the tremendous advancements in care of patients with lung cancer. The ultimate goals of cancer care are improving quality of life and prolonging survival. The final two articles in this series focus on these pivotal questions. Guo et al asked the most important question from a patient's perspective, '*How long have I got?*' and retrospectively analyzed 998 patients with metastatic NSCLC to derive an answer. In this real-world analysis, 1-, 2-, and 5-year survival rates were 74%, 49% and 16%, respectively. Their multivariate regression analysis suggested that histopathology, performance score, number of chemotherapy cycles received, and targeted therapy receipt were independently prognostic. However, among patients who survived greater than 3 months, the authors could not identify predictors to differentiate between long-term (>38 months) and short term (<12 months) survivors.

This effort highlights our ongoing challenges to accurately predict patient outcomes in lung cancer. Performance status (PS) has been a valuable indicator to guide therapy and prognosis. Performance status, however, is variable and often not an objective assessment. An objective assessment of muscle mass and strength may complement performance status in predicting outcomes. Yang et al prospectively recruited 639 patients with advanced NSCLC and evaluated sarcopenia using CT scan-based skeletal muscle index (SMI) and handgrip strength. They observed that CT-defined sarcopenia alone and in combination with poor handgrip strength were more strongly associated with a poor prognosis than Eastern Cooperative Oncology Group (ECOG) PS≥2 alone (HR 2.0, 95% CI 1.65-2.43; HR 2.00, 95% CI 1.59-2.49). Furthermore, CT-defined sarcopenia, poor handgrip strength and ECOG PS≥2, together defined as severe sarcopenia, was also more strongly associated with poor prognosis compared to ECOG PS≥2 (HR 1.37, 95% CI 1.10-1.73). As we continue to use ECOG PS as a strong prognostic indicator, this prospective study calls for further improvements in our ability to predict patient outcomes.

In conclusion, the articles in this series provide the reader with new and ongoing research in thoracic oncology, review current management strategies and updates, and encourage further contributions in this field to improve lives of patients.

Author contributions

KP drafted the editorial and for the rest the authors contributed equally. All authors contributed to the article and approved the submitted version.

Conflict of interest

KP reports advisory board fees from Guardant Health and Jazz Pharmaceuticals. AA reports advisory board fees from Merck Sharpe Dohme, Roche, Takeda, Pfizer, Bristol-Myers Squibb, AstraZeneca, Eli-Lilly; speaker's bureau fees from Eli-Lilly, AstraZeneca, Amgen;

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Afatinib in EGFR TKI-Naïve Patients with Locally Advanced or Metastatic EGFR Mutation-Positive Non-Small Cell Lung Cancer: A Pooled Analysis of Three Phase IIIb Studies

Antonio Passaro[1], Filippo de Marinis[1], Hai-Yan Tu[2], Konstantin K. Laktionov[3], Jifeng Feng[4], Artem Poltoratskiy[5], Jun Zhao[6], Eng Huat Tan[7], Maya Gottfried[8], Victor Lee[9], Dariusz Kowalski[10], Cheng Ta Yang[11], BJ Srinivasa[12], Laura Clementi[13], Tejaswini Jalikop[14], Dennis Chin Lun Huang[15], Agnieszka Cseh[16], Keunchil Park[17] and Yi-Long Wu[2]*

[1] Division of Thoracic Oncology, IEO, European Institute of Oncology IRCCS, Milan, Italy, [2] Guangdong Lung Cancer Institute, Guangdong Provincial People's Hospital and Guangdong Academy of Medical Sciences, Guangzhou, China, [3] Russian Academy of Medical Sciences, Moscow, Russia, [4] Jiangsu Provincial Tumor Hospital, Nanjing, China, [5] Petrov Research Institute of Oncology, St Petersburg, Russia, [6] Peking University Cancer Hospital & Institute, Beijing, China, [7] National Cancer Centre, Singapore, Singapore, [8] Tel Aviv University, Tel Aviv, Israel, [9] Department of Clinical Oncology, Queen Mary Hospital, The University of Hong Kong, Hong Kong, China, [10] Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw, Poland, [11] Chang Gung Memorial Hospital, Guishan, Taoyuan, Taiwan, [12] HCG Hospital, Bangalore, India, [13] Boehringer Ingelheim Italia S.p.A., Milan, Italy, [14] Syneos Health, Raleigh, NC, United States, [15] Boehringer Ingelheim Taiwan Limited, Taipei, Taiwan, [16] Boehringer Ingelheim International GmbH, Ingelheim am Rhein, Germany, [17] Division of Hematology Oncology, Samsung Medical Center Sungkyunkwan University School of Medicine, Seoul, South Korea

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Edited by:
Alfredo Addeo, Geneva University Hospitals (HUG), Switzerland

Reviewed by:
Giuseppe Luigi Banna, United Lincolnshire Hospitals NHS Trust, United Kingdom
Alex Friedlaender, Geneva University Hospitals (HUG), Switzerland

***Correspondence:**
Antonio Passaro
Antonio.Passaro@ieo.it

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Background: Afatinib is approved for first-line treatment of patients with epidermal growth factor receptor mutation-positive (*EGFR*m+) non-small-cell lung cancer (NSCLC). Here, we report findings from a combined analysis of three phase IIIb studies of afatinib in EGFR tyrosine kinase inhibitor (TKI)-naïve patients.

Methods: EGFR-TKI-naïve patients with *EGFR*m+ NSCLC received afatinib 40 mg/day. Dose reductions were permitted for adverse events (AEs). Efficacy endpoints included progression-free survival (PFS), time to symptomatic progression (TTSP), and tumor response. Subgroup analyses were performed by Eastern Cooperative Oncology Group performance status (ECOG PS), presence of brain metastasis, age and common/uncommon *EGFR* mutations (plus other factors).

Results: 1108 patients were treated. Median age was 61 years (range, 25–89); 19.2% had baseline brain metastases, 4.4% had ECOG PS ≥2, and 17.9% had tumors harboring uncommon mutations. Treatment-related AEs (TRAEs) were reported in 97.2%, most commonly diarrhea and rash. 41.6% had AEs leading to dose reduction. Median PFS was 13.0 months [95% confidence interval (CI): 12.0–13.8]; median TTSP was 14.8 months (95% CI: 13.9–16.1). Objective response rate (ORR) was 55.0%. Age, presence of baseline brain metastases, major (G719X, L861Q, S768I) or compound uncommon

mutations had little/no effect on PFS, TTSP, or ORR, while outcomes were poorer in patients with ECOG PS 2 or exon 20 insertion/T790M mutations.

Conclusions: Afatinib was tolerable with no new safety signals. Afatinib demonstrated encouraging efficacy in a broad patient population, including those with brain metastases or uncommon *EGFR* mutations.

Keywords: afatinib, real world, safety, *EGFR* mutation, EGFR TKI-naïve, NSCLC

INTRODUCTION

Activating mutations in the epidermal growth factor receptor (*EGFR*) gene, leading to aberrant EGFR signaling, render non-small cell lung cancer (NSCLC) tumors highly sensitive to targeted treatment with EGFR tyrosine kinase inhibitors (TKIs) (1). Based on seminal randomized controlled trials (RCTs) (1), EGFR TKIs are the first-line treatment of choice in patients with advanced *EGFR* mutation-positive (EGFRm+) NSCLC, with five TKIs currently approved. These are: the first-generation reversible EGFR TKIs, gefitinib and erlotinib; the second-generation irreversible ErbB family blockers, afatinib and dacomitinib; and the third-generation irreversible EGFR TKI, osimertinib (2–5).

As an ErbB family blocker, afatinib inhibits signaling *via* all hetero- and homodimers formed by ErbB1 (EGFR), ErbB2 [human epidermal growth factor receptor 2 (HER2)], ErbB3 (HER3), and ErbB4 (HER4) (6, 7). In RCTs, afatinib significantly improved progression-free survival (PFS) versus standard chemotherapy (8, 9). Furthermore, in the LUX-Lung 3 and 6 trials, afatinib significantly improved overall survival (OS) *versus* chemotherapy in patients with tumors harboring Del19 mutations (10). In LUX-Lung 7, afatinib conferred statistically significant improvement in PFS (although there was minimal difference in medians) and time-to-treatment failure versus gefitinib (11). There was no significant difference in OS (12). Across these RCTs, afatinib was tolerable, with few treatment discontinuations due to toxicity. Treatment-related adverse events (TRAEs) were managed effectively with tolerability-guided dose reductions.

RCTs are conducted under highly controlled settings, often with strict inclusion criteria. Consequently, certain patient subgroups are generally under-represented in clinical trials, such as the very elderly and patients with brain metastases, uncommon mutations, prior chemotherapy treatment, or Eastern Cooperative Oncology Group performance status (ECOG PS) ≥2. Accordingly, the importance of assessing the efficacy and tolerability of recently developed drugs in 'real world' settings is becoming increasingly recognized (13). To date, available real-world evidence suggests that afatinib is effective and tolerable in diverse patient populations treated in routine clinical practice (14–18). Here, in order to assess outcomes in a larger cohort, we report a combined analysis of three phase IIIb studies of afatinib in EGFR TKI-naïve patients with *EGFR*m+ NSCLC treated in a setting similar to daily clinical practice (19, 20).

METHODS

Study Designs

Study 1200.55 (NCT01853826; conducted in Europe, Australia, Russia, and Israel), Study 1200.66 (NCT01953913; conducted in Asia), and Study 1200.193 (NCT01931306; conducted in South Korea) were all phase IIIb, open-label, multicenter, single-arm trials of afatinib in EGFR TKI-naïve patients with locally advanced or metastatic *EGFR*m+ NSCLC (**Supplementary Figure 1**). All studies were approved by the Institutional Review Board or Independent Ethics Committee of each participating center, and were carried out in accordance with the Declaration of Helsinki, the International Conference on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, Good Clinical Practice, and local laws. All patients provided written, informed consent.

Patients and Treatment

Patients were aged ≥18 years with histologically-confirmed, locally advanced or metastatic *EGFR*m+ NSCLC, adequate organ function, and an ECOG PS of 0–2. Exclusion criteria included: previous use of an EGFR TKI; use of any anti-cancer treatment (or hormonal anti-cancer treatment for Study 1200.193) <2 weeks, radiotherapy (except palliative) <14 days (or <4 weeks for Study 1200.66), and major surgery <4 weeks before the first dose of afatinib; history or presence of cardiovascular abnormalities; pre-existing interstitial lung disease; and symptomatic brain metastases.

Patients received afatinib (starting dose 40 mg once daily) until disease progression, lack of tolerability or other reasons necessitating withdrawal. Investigators could continue afatinib beyond radiological progression for as long as they judged that the patient was benefiting. TRAEs were managed using tolerability-guided dose modifications. In the event of any drug-related grade ≥3 AE, persistent grade 2 diarrhea, or grade ≥2 renal dysfunction, treatment was paused until the severity recovered to grade ≤1 or baseline severity. Treatment could then be resumed at a lower dose (reduced by 10 mg decrements) to a minimum of 20 mg/day. If the patient could not tolerate 20 mg/day, or the patient did not recover to grade ≤1 or baseline within 6 weeks, treatment was discontinued.

Endpoints and Assessments

The primary objective of each study was to evaluate the safety of afatinib; the secondary objective was to assess the efficacy of afatinib. AEs were graded using the National Cancer Institute

Common Terminology Criteria for Adverse Events version 3.0. Efficacy endpoints were chosen to reflect real-world clinical practice and current treatment guidelines, and included: PFS (defined as time from first administration of afatinib to the date of progression or to the date of death, whichever occurred first); time to symptomatic progression (TTSP; defined as the time from first administration of afatinib to the date of first documented clinically significant symptomatic progression); and tumor response. Efficacy analyses were based on the assessment of cancer-related symptoms and, if available, radiologic assessments as per standard of care at the participating institution and determined by Response Evaluation Criteria in Solid Tumors (RECIST). Tumor assessments, and the version of RECIST criteria used in the three studies were undertaken according to local standard of care at each participating site. PFS and ORR were judged by investigator. *EGFR* mutations were detected according to the methodology used at each participating institution.

Statistical Analyses

All patients who received ≥1 dose of afatinib (treated set) were included in the safety and efficacy analyses. Subgroup analyses were conducted according to: *EGFR* mutation status (common/uncommon); presence of brain metastases at baseline (yes/no); age (<65 years/≥65 years and <75 years/≥75 years); ECOG PS (0–1/2); and line of therapy (first/second/>second). Patients with tumors harboring uncommon *EGFR* mutations were further subdivided into the following five groups: 1) T790M; 2) exon 20 insertions; 3) 'major' uncommon mutations (G719X, L861Q, and S768I, with or without any other mutation except T790M or exon 20 insertion); 4) compound mutations; and 5) other uncommon mutations. Outcomes were also assessed for compound mutations including major mutations. Descriptive statistics are presented; no hypotheses testing was planned, and all analyses were exploratory.

RESULTS

Patients, Disposition, and Treatment Exposure

Of the 1163 patients enrolled, 1109 entered and 1108 had been treated with afatinib (**Supplementary Figure 1**). Overall, 1081 (97.6%) patients discontinued treatment, the most common reason being progressive disease, in 739 (66.7%) patients. Median age was 61 years (range, 25–89), 38.2% of patients were aged ≥65 years, with 10.7% aged ≥75 years. Most patients (58.3%) were female and were predominantly either Asian (57.7%) or white (42.0%; **Table 1**). An ECOG PS of 2 was reported in 49 (4.4%) patients, and 213 (19.2%) patients had brain metastases. The most common histological classification was adenocarcinoma, in 95.8% of patients.

In total, 909 (82.0%) patients had tumors harboring common *EGFR* mutations, while 198 (17.9%) had tumors harboring uncommon mutations only; the most frequent uncommon *EGFR* mutations were insertions in exon 20, which were detected in 70 patients (6.3% overall). Nearly a third of patients (33.1%) had previously received systemic chemotherapy. The median duration of treatment across all lines of afatinib was 12.7 months (range, 0.07–56.1 months). Dose reductions from 40 mg/day to 30 mg/day were performed in 462 (41.7%) patients, 145 of whom (13.1% overall) had a further dose reduction to 20 mg/day.

Safety

Most patients (1100; 99.3%) experienced an AE, and 620 (56.0%) patients experienced grade ≥3 AEs (**Table 2**). Any-grade TRAEs were reported in 1077 (97.2%) patients, and grade ≥3 TRAEs were reported in 412 (37.2%) patients. The most common TRAEs (any grade/grade ≥3) were diarrhea (89.1%/14.0%), rash (61.6%/9.1%), and paronychia (39.7%/3.6%; **Table 2**). Serious AEs (SAEs) were reported in 403 (36.4%) patients, the most common being malignant neoplasm progression in 53 (4.8%) patients, and pleural effusion in 38 (3.4%) patients; 81 (7.3%) patients had a treatment-related SAE, the most common being diarrhea in 28 (2.5%) patients. AEs leading to dose reduction of afatinib were reported in 461 (41.6%) of patients. The most common reasons for dose reduction were diarrhea in 199 (18%) patients, and rash in 108 (9.7%) patients. AEs leading to discontinuation of afatinib were reported in 160 (14.4%) patients, among whom 58 (5.2%) patients experienced TRAEs leading to drug discontinuation; the most frequent of these was diarrhea in 17 patients (1.5%). A total of 122 patients (11.0%) had an AE that led to death, including malignant neoplasm progression in 41 (3.7%) patients, and respiratory failure in 14 (1.3%) patients. There were five TRAEs resulting in death (decreased appetite, dyspnea, pneumonitis, respiratory failure, intestinal infarction).

Efficacy

PFS

Median PFS was 13.0 months overall and was 13.9 months among patients with tumors harboring common mutations (**Table 3**; **Figures 1A, B**). Median PFS was longer in patients with ECOG PS 0/1 compared to those with ECOG PS 2 (median: 13.4 and 7.7 months, respectively), and this was also the case among only those patients with tumors harboring common mutations (median, 14.1 and 8.8 months; **Table 3**; **Figures 1C, D**). Median PFS was slightly longer in patients without compared to those with brain metastases at baseline (median, 13.7 and 10.6 months; **Figure 1E**), and in patients treated with first-line afatinib compared to second- or later-line afatinib (median, 13.7, 12.9 and 8.3 months, respectively; **Figure 1F**), while age had little or no effect on PFS (**Table 3**; **Figures 1G, H**).

TTSP

Median TTSP was 14.8 months overall and was 16.1 months in patients with tumors harboring common mutations (**Table 3**; **Supplementary Figures 2A, B**). Median TTSP was numerically longer in patients with ECOG PS 0/1 *versus* 2 (median, 15.2 and 9.9 months) including among only those with common mutations (median, 16.6 and 9.9 months; **Table 3**; **Supplementary Figures 2C, D**). Median TTSP was slightly longer in patients without baseline brain metastases compared

TABLE 1 | Baseline demographics and disease characteristics in the treated set.

Characteristic	Afatinib (n = 1108)
Sex, n (%)	
Female	646 (58.3)
Median age, years (range)	61 (25–89)
≥65 years, n (%)	423 (38.2)
≥75 years, n (%)	119 (10.7)
Race, n (%)	
Asian	639 (57.7)
White	465 (42.0)
Other[†]	4 (0.4)
Smoking status, n (%)	
Never smoked	735 (66.3)
Ex-smoker	307 (27.7)
Current smoker	66 (6.0)
Histological classification, n (%)	
Predominantly adenocarcinoma	1061 (95.8)
Predominantly squamous cell carcinoma	20 (1.8)
Large cell/undifferentiated carcinoma	11 (1.0)
NOS/missing	16 (1.4)
Prior therapy	
Any	578 (52.2)
Chemotherapy/other systemic therapy	373 (33.7)
Radiotherapy	213 (19.2)
Surgery	278 (25.1)
EGFR mutation, n (%)	
Common only (del19 and/or L858R)	909 (82.0)
Del 19	556 (50.2)
L858R	429 (38.7)
Uncommon only	198 (17.9)
Missing	1 (0.1)
Baseline ECOG PS, n (%)	
0	285 (25.7)
1	773 (69.8)
2	49 (4.4)
Missing	1 (0.1)
Baseline brain metastases,[‡] n (%)	213 (19.2)
Prior systemic chemotherapy, n (%)	367 (33.1)

ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; NOS, not otherwise specified. [†]Other: one Native Hawaiian or other Pacific Islander; three Black/African American. [‡]Asymptomatic.

TABLE 2 | Overall summary of AEs, and most common TRAEs (occurring in ≥10% of patients).

AE, n (%)	Treated set (n = 1108)	
Any AE	1100 (99.3)	
Any grade ≥3 AE	620 (56.0)	
Any TRAE	1077 (97.2)	
Any grade ≥3 TRAE	412 (37.2)	
Any SAE	403 (36.4)	
AEs leading to dose reduction	461 (41.6)	
AEs leading to discontinuation	160 (14.4)	
TRAEs leading to discontinuation	58 (5.2)	
AEs leading to death	122 (11.0)	
Most common TRAEs	All grades	Grade ≥3
Diarrhea	987 (89.1)	155 (14.0)
Rash	683 (61.6)	101 (9.1)
Paronychia	440 (39.7)	40 (3.6)
Stomatitis	243 (21.9)	27 (2.4)
Mucosal inflammation	170 (15.3)	20 (1.8)
Mouth ulceration	149 (13.4)	10 (0.9)
Dry skin	144 (13.0)	2 (0.2)
Pruritus	135 (12.2)	3 (0.3)

AE, adverse event; SAE, serious adverse event; TRAE, treatment-related adverse event.

to those with brain metastases at baseline (median, 15.5 and 13.7 months; **Supplementary Figure 1E**), and in patients treated with afatinib in first line compared with second or later lines (median, 16.0, 13.8 and 10.6 months, respectively; **Supplementary Figure 2F**). Age had little or no effect on TTSP (**Table 3** and **Supplementary Figures 2G, H**).

Tumor Response

Overall, 609 of the 1108 treated patients (55.0%) had an objective response, including 40 (3.6%) complete responses and 569 (51.4%) partial responses. An additional 368 (33.2%) patients had stable disease, for a disease control rate of 88.2% (n=977). Median duration of objective response (DOR) in the overall treated set was 13.2 months (95% CI: 12.2–14.4), and median duration of disease control was 14.1 months (95% CI: 13.6–14.8; **Supplementary Table 1**).

Patients with Uncommon Mutations

Baseline characteristics of patients with uncommon mutations were generally consistent with the overall treated set (**Table 4**). Compared with the T790M and exon 20 mutation subgroups (median PFS, 3.9 and 5.6 months, respectively), median PFS was longer in the compound, 'major' and 'other' mutation subgroups (11.0, 9.2, and 8.6 months, respectively), particularly in the subgroup with compound mutations with a 'major' uncommon mutation (15.6 months; **Figure 2A**). Median TTSP was also longest in the 'compound with major mutation' subgroup (18.5 months), followed by the compound mutation (13.9 months), 'major' mutation (11.1 months), 'other' mutation (9.7 months), exon 20 mutation (5.9 months), and T790M (3.8 months) subgroups (**Figure 2B**). Objective response rates were higher in the compound/'compound with major', and 'major' uncommon mutation subgroups compared with the exon 20 mutation and T790M subgroups, as was the corresponding DOR (**Supplementary Table 2**).

DISCUSSION

This study was a combined analysis of three phase IIIb, open-label, multicenter, single-arm trials in which EGFR TKI-naïve patients with locally advanced or metastatic *EGFR*m+ NSCLC received afatinib. Patient characteristics were comparable to those previously reported in studies of EGFR TKIs used in routine clinical practice, both globally and in Asia (14–17). The patient population included subsets that are generally under-represented in clinical trials, including the elderly (38.2% aged ≥65 years; 10.7% aged ≥75 years), patients with brain metastases (19.2%), patients with ECOG PS 2 (4.4%), and those with tumors harboring uncommon *EGFR* mutations (17.9%).

In this diverse patient population, afatinib was generally tolerable with no new or unexpected safety findings. The most common AEs were EGFR TKI class-related toxicities (diarrhea, rash/acne, stomatitis, and paronychia) consistent with findings

TABLE 3 | Post-hoc analysis of TTSP and PFS for specified subgroups.

Category	Patient subgroup		
All patients			
N	1108		
Median PFS, months (95% CI)	13.0 (12.0–13.8)		
Median TTSP, months (95% CI)	14.8 (13.9–16.1)		
EGFR mutation type[†]	Common[†]		Uncommon[‡]
N	909		198
Median PFS, months (95% CI)	13.9 (13.2–14.7)		7.4 (6.0–9.0)
Median TTSP, months (95% CI)	16.1 (14.8–17.7)		8.3 (7.2–11.0)
Common mutation type	Del19		L858R
N	531		378
Median PFS, months (95% CI)	14.5 (13.8–15.9)		12.6 (11.1–13.8)
Median TTSP, months (95% CI)	17.2 (15.5–19.3)		14.5 (13.1–16.5)
ECOG PS	0/1		2
N	1058		49
Median PFS, months (95% CI)	13.4 (12.4–14.1)		7.7 (5.7–11.6)
Median TTSP, months (95% CI)	15.2 (14.1–16.6)		9.9 (7.6–13.9)
ECOG PS (patients with common mutations)[†]	0/1		2
N	869		40
Median PFS, months (95% CI)	14.1 (13.5–14.8)		8.8 (5.7–13.9)
Median TTSP, months (95% CI)	16.6 (15.1–18.1)		9.9 (7.6–14.5)
Afatinib line of therapy	First-line	Second-line	>Second-line
N	770	261	77
Median PFS, months (95% CI)	13.7 (12.6–14.5)	12.9 (11.3–13.8)	8.3 (6.6–12.6)
Median TTSP, months (95% CI)	16.0 (14.4–17.7)	13.8 (12.7–15.4)	10.6 (7.6–14.8)
Brain metastases at screening[§]	Yes		No
N	213		894
Median PFS, months (95% CI)	10.6 (9.1–12.8)		13.7 (12.8–14.4)
Median TTSP, months (95% CI)	13.7 (11.0–14.8)		15.5 (14.1–16.9)
Age, years	<75 years		≥75 years
N	989		119
Median PFS, months (95% CI)	13.0 (12.0–13.9)		13.0 (9.1–14.8)
Median TTSP, months (95% CI)	14.8 (13.8–16.1)		14.8 (13.1–22.3)
Age, years	<65 years		≥65 years
N	685		423
Median PFS, months (95% CI)	12.6 (11.3–13.6)		13.9 (12.7–15.2)
Median TTSP, months (95% CI)	13.8 (12.9–15.1)		17.5 (15.0–20.6)

CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; NE, not evaluable; PFS, progression-free survival; TTSP, time to symptomatic progression. [†]Patients with EGFR mutation categories of Del19 only or L858R only. [‡]Patients with EGFR mutation categories other than Exon19 only and L858R only. [§]Asymptomatic.

from the LUX-Lung 3, 6, and 7 studies (8, 9, 11). The overall rate of dose reductions due to AEs (41.6%) was similar to that reported in the LUX-Lung 3 and 7 studies (52% and 39%, respectively) (8, 11), but were more frequent than in LUX-Lung 6 (28%) (9), possibly reflecting differences in side effect management in different populations. However, consistent with RCT data (21, 22), and real-world studies (23), TRAEs rarely led to afatinib discontinuation in everyday clinical practice.

PFS and objective response rates (ORR) in this study are comparable to afatinib real-world studies (median PFS: 11.8–19.1 months; ORR: 67.1-76.5%) (14, 16, 17) and in the LUX-Lung trials, (median PFS 11.0–11.1 months; ORR: 56–70%) (8, 9, 11). At 14.8 months, median TTSP was almost 2 months longer than the median PFS, indicating that, following tumor progression, patients obtained clinical benefit from afatinib for another ~2 months on average, before clinically significant symptomatic progression was identified and treatment was suspended. Of note, the constituent studies in this analysis were largely undertaken before osimertinib was widely available as a second-line treatment option in patients with T790M-mediated acquired resistance to EGFR TKIs. Therefore, as it is estimated that 50–70% of patients treated with afatinib acquire the T790M mutation (24), the observation of widespread treatment beyond progression in this study probably does not reflect contemporary treatment practices, especially as tumor re-biopsies at the point of radiological progression are becoming more commonplace (25). In patients who acquire the T790M mutation, treatment with osimertinib should not be delayed. Nevertheless, in patients with *EGFR*m+ NSCLC and no obvious targeted second-line treatment options after failure of afatinib, continuing treatment beyond radiological progression could be an appropriate strategy in the absence of clinical deterioration.

Limited data are available to guide treatment choices in older patients with NSCLC, which can be complicated by age-related factors such as comorbidities and polypharmacy (26). Consistent with previous studies (26), afatinib appeared to be generally effective, and tolerable, in the elderly patients included in this analysis. Indeed, when using an age cut-off of 65 years, outcomes were actually slightly improved in older compared to younger patients, which is consistent with accumulating evidence that

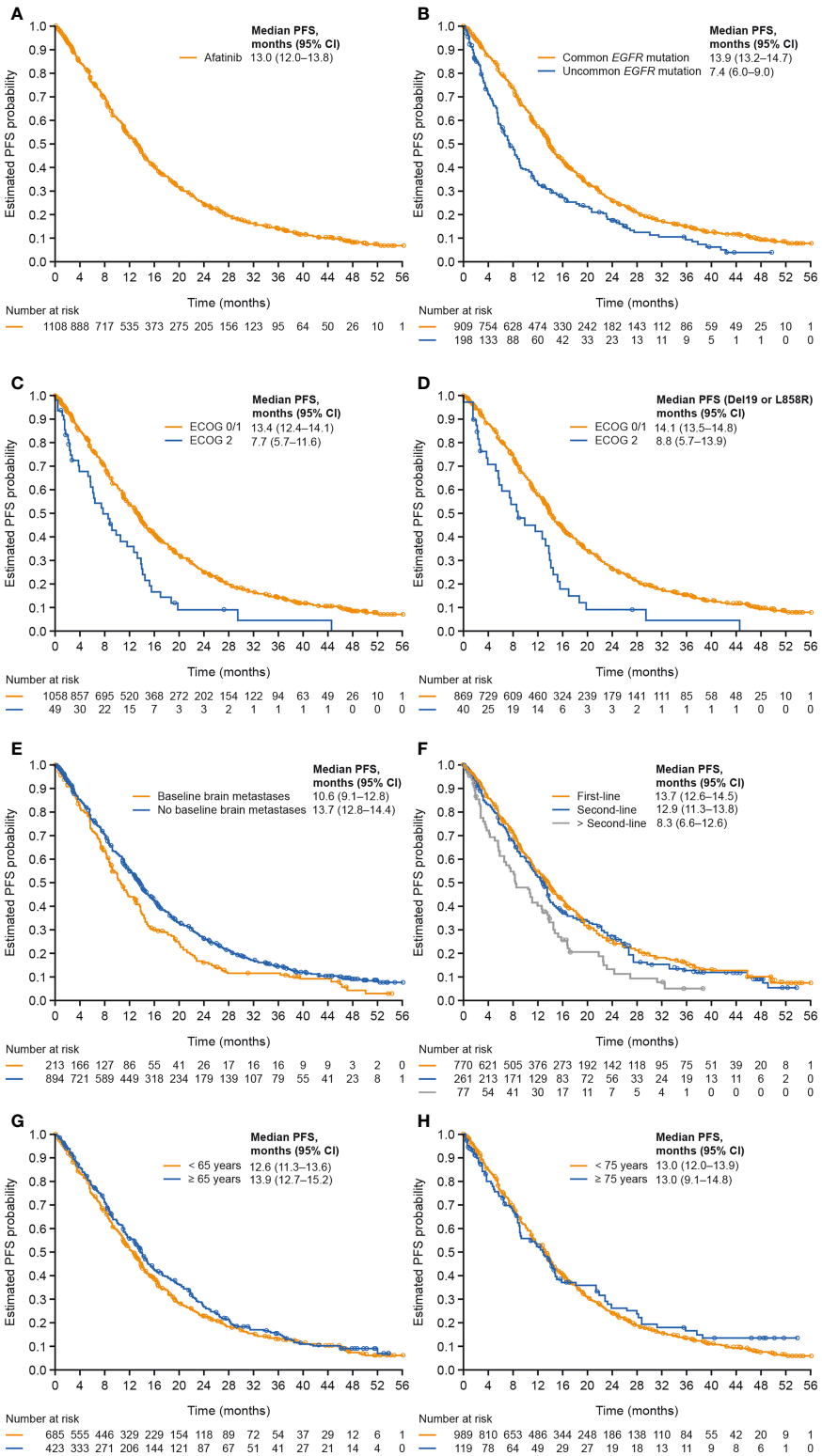

FIGURE 1 | PFS in **(A)** all patients, **(B)** patients with tumors harboring common versus uncommon mutations, **(C)** patients with ECOG PS 0/1 versus 2, **(D)** patients with common mutations and ECOG PS 0/1 versus 2, **(E)** patients with versus without baseline brain metastases, **(F)** patients treated with afatinib in first, second and later lines of therapy, **(G)** patients aged <65 or ≥65 years, and **(H)** patients aged <75 or ≥75 years. CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; PFS, progression-free survival.

TABLE 4 | Baseline demographics and disease characteristics according to the type of uncommon *EGFR* mutation.

Characteristic	T790M (n = 8)	Exon 20 (n = 36)	Major (n = 62)	Compound (n = 12)	Compound with major (n = 8)	Other (n = 5)
Sex, n (%)						
Female	3 (37.5)	22 (61.1)	31 (50.0)	8 (66.7)	5 (62.5)	4 (80.0)
Race, n (%)						
Asian	1 (12.5)	3 (8.3)	41 (66.1)	8 (66.7)	6 (75.0)	0
White	7 (87.5)	33 (91.7)	20 (32.3)	4 (33.3)	2 (25.0)	5 (100)
Other[†]	0	0	1 (1.6)	0	0	0
Prior lines of therapy						
First	6 (75.0)	23 (63.9)	43 (69.4)	6 (50.0)	5 (62.5)	3 (60.0)
Second	1 (12.5)	6 (16.7)	18 (29.0)	6 (50.0)	3 (37.5)	1 (20.0)
Third	0	5 (13.9)	1 (1.6)	0	0	1 (20.0)
≥Fourth	1 (12.5)	2 (5.6)	0	0	0	0
Baseline ECOG PS, n (%)						
0	3 (37.5)	15 (41.7)	16 (25.8)	3 (25.0)	3 (37.5)	1 (20.0)
1	4 (50.0)	9 (25.0)	42 (67.7)	8 (66.7)	4 (50.0)	4 (80.0)
2	1 (12.5)	1 (2.8)	4 (6.5)	1 (8.3)	1 (12.5)	0
Missing	0	1 (2.8)	0	0	0	0
Baseline brain metastases,[‡] n (%)	0	8 (22.2)	11 (17.7)	1 (8.3)	1 (12.5)	1 (20.0)

ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor. [†]other: One Black/African American. [‡]Asymptomatic.

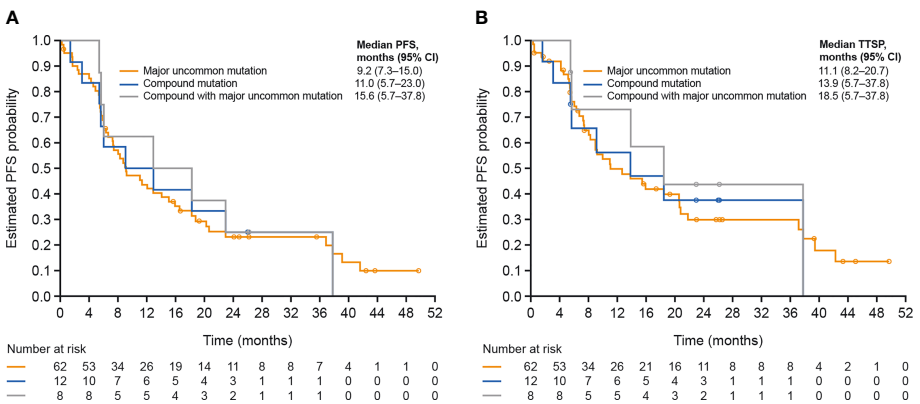

FIGURE 2 | **(A)** PFS and **(B)** TTSP according to type of uncommon *EGFR* mutation. CI, confidence interval; EGFR, epidermal growth factor receptor; PFS, progression-free survival; TTSP, time to symptomatic progression.

EGFR TKIs may actually be more effective in prolonging PFS in older patients compared with younger patients (27, 28). We found that poor performance status (ECOG PS ≥2) was associated with worse efficacy outcomes with afatinib than in patients with ECOG PS 0/1; however, this analysis was based on only 4.4% of the patient population with ECOG PS 2, therefore limiting robust analysis of these findings. These findings illustrate that chronological age alone should not determine the choice of treatment in elderly patients with NSCLC, and that biological age is more relevant for predicting treatment efficacy and safety.

Patients with *EGFR*m+ NSCLC are particularly susceptible to developing brain metastases, both at diagnosis and during the disease course (15, 29). Consistent with previous studies (18, 30), the efficacy and safety of afatinib was not affected by the presence of stable brain metastases. Other studies have indicated that afatinib can cross the blood-brain-barrier, is active against symptomatic brain metastases and mitigates the risk of CNS progression (15, 31). Overall, therefore, afatinib appears to be a treatment option in patients with CNS involvement or at risk of CNS progression.

Consistent with previous findings (32, 33), this analysis demonstrated that afatinib was effective against 'major' uncommon mutations (G719, L761, and S768) and compound mutations. Contrary to results demonstrated in a previous study (34), efficacy was observed with afatinib across all treatment lines, including in patients with previous chemotherapy or EGFR TKI failure. Afatinib was also active in some patients with tumors harboring exon 20 insertions or 'other' *EGFR* mutations; however, novel therapies including mobocertinib (35), poziotinib (36) and the recently approved amivantamab (37) have shown promising activity in early phase clinical trials in tumors harboring exon 20 insertions, and may prove to be more effective for this subgroup of patients. Nevertheless, while new

effective treatment options are becoming available, it is unclear whether all exon 20 insertion mutations respond to amivantamab and other agents. More detailed data are therefore required to assess the sensitivity of individual mutations but it may be that EGFR TKIs could be an option in a subset of this highly heterogeneous group.

This broad activity reflects preclinical findings showing that many uncommon *EGFR* mutations, including compound and very rare mutations, are sensitive to afatinib (38). The finding that compound *EGFR* mutations (where an EGFR-TKI sensitizing or other mutation is identified together with a mutation of unknown clinical significance) (39) are particularly sensitive to treatment with afatinib is notable, as these mutations are identified in up to one quarter of *EGFR* mutation-positive NSCLC tumors and are associated with poor prognosis (39–41). Our findings suggest that afatinib may be considered as a treatment option if a compound mutation is detected, particularly for compound mutations that include a major mutation.

This study had several limitations. Its open-label design means that the results should be interpreted with caution, particularly regarding the impact of afatinib on survival outcomes. Additionally, next-generation sequencing was unavailable for all samples, therefore limiting the scope of analysis for known negative predictive factors such as concurrent non-*EGFR* co-mutations and the effect of allele frequency (42, 43). Furthermore, all radiological assessments and *EGFR* mutation detection were performed locally according to the methodology used at each participating institution. Finally, exploratory subgroup analyses were conducted post-hoc, meaning that no formal statistical comparisons could be conducted, thus limiting the strength of the conclusions

In summary, the safety and efficacy results from this combined analysis of three large phase IIIb studies are generally consistent with findings from subanalyses of previous RCTs and real-world studies of afatinib in *EGFR*m+ NSCLC. Afatinib was tolerable and demonstrated encouraging efficacy across different patient subgroups, including patients with brain metastases and those with tumors harboring uncommon *EGFR* mutations.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All studies were approved by the Institutional Review Board or Independent Ethics Committee of each participating center, and were carried out in accordance with the Declaration of Helsinki, the International Conference on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, Good Clinical Practice, and local laws. This is not a primary study but a pooled analysis of previous studies. All patients provided written, informed consent.

AUTHOR CONTRIBUTIONS

APa: Formal analysis, investigation, resources, and manuscript writing. FdM: Formal analysis, investigation, resources, and manuscript writing. H-YT: Investigation and manuscript writing (review and editing). KKL: Formal analysis, investigation, resources, and manuscript writing (review and editing). JF: Investigation and manuscript writing (review and editing). APo: Formal analysis, investigation, resources, and manuscript writing (review and editing). JZ: Investigation and manuscript writing (review and editing). EHT: Investigation and manuscript writing (review and editing). MG: Manuscript writing (review and editing). VL: Conceptualization, methodology, validation, investigation, resources, and manuscript writing (original draft, review, and editing). DK: Formal analysis, investigation, data curation and manuscript writing (original draft, review, and editing). CY: Investigation and manuscript writing (review and editing). BS: Investigation and manuscript writing (review and editing). LC: Manuscript writing (review and editing). TJ: Investigation and manuscript writing (review and editing). DCLH: Investigation and manuscript writing (review and editing). AC: Conceptualization, methodology and manuscript writing (original draft, review, and editing). KP: Formal analysis, investigation, resources, and manuscript writing. Y-LW: Formal analysis, investigation, resources, and manuscript writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.709877/full#supplementary-material

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EGFR Exon 18 Mutations in Advanced Non-Small Cell Lung Cancer: A Real-World Study on Diverse Treatment Patterns and Clinical Outcomes

Haiyan Xu[1†], Guangjian Yang[2†], Weihua Li[3], Junling Li[2], Xuezhi Hao[2], Puyuan Xing[2], Yaning Yang[2] and Yan Wang[2]*

[1] Department of Comprehensive Oncology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China, [2] Department of Medical Oncology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China, [3] Department of Pathology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

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***Correspondence:**
Yan Wang
wangyanyifu@163.com

[†]These authors have contributed equally to this work and share first authorship

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Background: Approximately 3–5% of patients with epidermal growth factor receptor (*EGFR*) mutation-positive non-small cell lung cancer (NSCLC) harbor exon 18 mutations. The appropriate treatment for such patients has not been clarified. The aim of this study was to investigate the response of patients with NSCLC harboring *EGFR* exon 18 mutations to different therapeutic options.

Methods: Between May 2014 and September 2020, the clinical outcomes of 82 patients harboring *EGFR* exon 18 mutations who received first-generation (1G) *EGFR*-tyrosine kinase inhibitor (TKI), second-generation (2G) *EGFR*-TKI afatinib, chemotherapy, and 1G TKI in combination with chemotherapy as the initial therapy were retrospectively analyzed.

Results: A total of 82 NSCLC patients harboring *EGFR* 18 mutations with whose treatment and survival outcomes were available were analyzed. The median age was 59 years, and 47 (57.3%) were female. The most common kind of *EGFR* exon 18 mutation was G719X (75.6%), followed by E709X (15.9%), E709_T710delinsD (3.6%), and other subtypes (4.9%). There was a significant difference in median progression-free survival (mPFS) by therapeutic strategy (P = 0.017). The mPFS of 1G TKI, 2G TKI afatinib, chemotherapy, and 1G TKI in combination with chemotherapy were 7.7 (95% CI, 4.2–11.2), 11.3 (95% CI, 5.6–17.0), 5.0 (95% CI, 2.3–17.7), and 11.1 (95% CI, 5.9–16.4) months, respectively. No significant difference in PFS was observed between afatinib and 1G TKI in combination with chemotherapy (P = 0.709).

Conclusions: Like afatinib, 1G TKI in combination with chemotherapy might be an effective treatment option for patients harboring *EGFR* exon 18 mutations.

Keywords: non-small cell lung cancer, epidermal growth factor receptor, uncommon mutation, targeted therapy, efficacy

INTRODUCTION

Epidermal growth factor receptor (*EGFR*) is a transmembrane glycoprotein with cytoplasmic kinase activity that can transduce essential growth factor signals from extracellular cues to cellular responses, thereby regulating cellular proliferation, differentiation, angiogenesis, and metastasis. *EGFR* mutations mainly occur in exons 18–21, which encode in the intracellular tyrosine kinase domain of *EGFR* (1). In the Asian population, the overall proportion of *EGFR* mutations was 49.1%, which is higher than that in the global population (11.9%) (2), that an in-frame deletion in exon 19 and the L858R missense mutation in exon 21 are the two most common *EGFR* mutations, which are called as the classic or sensitizing *EGFR* mutations. In contrast, 10–20% of patients with non-small cell lung cancer (NSCLC) harbor uncommon or rare *EGFR* mutation (3–6). The most prevalent of the uncommon *EGFR* mutations are point mutation and duplication in exons 18–21, *de novo* T790M mutations in exon 20, and exon 20 insertions (7). Exon 18 mutations involve missense mutations G719X and E709X, insertion-deletion (indel) mutation E709_T710delinsX, and other molecular subtypes, comprising approximately 3–5% of all the *EGFR* alterations (8, 9). Compared to patients with tyrosine kinase inhibitor (TKI)-sensitizing *EGFR* mutations, NSCLC patients with *EGFR* exon 18 mutations generally respond slightly worse to first-generation (1G) *EGFR*-TKI (7, 10, 11).

With the discovery of the oncogenic function of *EGFR*, TKIs have dramatically changed the treatment landscape of advanced NSCLC from conventional cytotoxic chemotherapy to targeted therapy in recent years. *EGFR*-TKIs are currently recognized as the first-line standard treatment for advanced NSCLC patients with *EGFR* mutations. At present, three generations of *EGFR*-TKIs include 1G reversible *EGFR*-TKIs (erlotinib, gefitinib, and icotinib), second-generation (2G) irreversible ErbB blocks (afatinib and dacomitinib), and third-generation (3G) irreversible *EGFR*-TKIs (osimertinib). Resulting from their molecular structures and biochemical differences among different *EGFR*-TKIs, their sensitivities to different *EGFR*-TKIs vary widely. A series of clinical studies have reported a response rate of 14–53.3% to 1G *EGFR*-TKIs for uncommon *EGFR* mutations, with a median progression-free survival (mPFS) of 5.98–11.6 months and a median overall survival (mOS) of 19.8–25.2 months (11–15). G719X mutations have also been demonstrated to be responsive to the 2G *EGFR*-TKI afatinib and neratinib. The overall response rates to afatinib and neratinib in NSCLC patients with G719X mutations were 77.8% and 75%, respectively, with mPFS of 13.8 months and 12.1 months and mOS of 26.9 months (9, 16), which were similar to those for classic *EGFR* mutations. At the 21st World Conference on Lung Cancer (WCLC) in 2020, the results of a phase II SUMMIT basket study revealed that pretreated NSCLC patients with *EGFR* exon 18 mutations had an objective response rate (ORR) of 40% and a duration of response (DoR) of 7.5 months to neratinib, and the ORR and DoR were better than those of other *EGFR*-TKIs in previous studies (17). As neratinib was not available, on the basis of the results of the clinical trial LUX-Lung 2, LUX-Lung 3, and LUX-Lung6, involving 32 patients, the 2G *EGFR*-TKI afatinib was expanded the label by the U.S. Food and Drug Administration (FDA) in 2018 for patients with advanced NSCLC with uncommon *EGFR* mutations. In addition, a recent single-arm prospective phase II study from South Korea reported that the 3G *EGFR*-TKI osimertinib also had clinical activity in patients with uncommon *EGFR* mutations, including G719X, L861Q, and S768I, with an ORR of 53% and mPFS of 8.2 months (18).

Due to the small total sample size that exists because of the lack of randomized clinical trials and the exclusion of NSCLC patients harboring uncommon *EGFR* mutations from previous studies, the clinical outcomes of diverse treatment modalities for *EGFR*-exon- 18 mutated NSCLC have not been fully elucidated. Further study is still required to determine which treatment modality is the most effective in advanced NSCLC with uncommon *EGFR* mutations.

Therefore, we initiated a real-world study to investigate the therapeutic responses and disease progression patterns in advanced NSCLC patients harboring *EGFR* exon 18 mutations who were treated with four diverse therapy strategies: 1G *EGFR*-TKI (gefitinib, erlotinib, or icotinib), the 2G *EGFR*-TKI afatinib, chemotherapy, and a 1G *EGFR*-TKI in combination with chemotherapy.

MATERIALS AND METHODS

Patients and Data Collection

This retrospective, single-center study was analyzed in 82 advanced NSCLC patients harboring *EGFR* exon 18 mutations who received monotherapy with a 1G- or 2G- *EGFR*-TKI, chemotherapy or a 1G *EGFR*-TKI in combination with chemotherapy in a first-line setting between May 2014 and September 2020. *EGFR* mutation testing was confirmed by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) assay or next-generation sequencing (NGS). All the gene capture panels are used in this study including 168 cancer-related NGS panel, and interrogated whole exons and critical introns for the 8 classic NSCLC oncogenic drivers, which includes *EGFR*, *KRAS*, *ALK*, *ROS1*, *BRAF*, *ERBB2*, *MET*, and *RET*. The study flow chart is presented in the **Supplementary Appendix** (**Supplementary Figure S1**).

Patients who met the following criteria were included in the analysis: age ≥18 years, Eastern Cooperative Oncology Group performance status (ECOG PS) score of 2 or less, and histologically or cytologically confirmed unresectable stage IIIB–IV or recurrent NSCLC with *EGFR* exon 18 single mutation or compound mutations. Compound mutations were defined as an exon 18 mutation in combination with another, common or uncommon, mutation in exons 18–21.

Exclusion criteria included prior treatment with concurrent chemotherapy and radiotherapy, anti-angiogenic treatment combined with *EGFR*-TKI, immunotherapy, or uncontrolled symptomatic brain metastasis.

Treatment and Response Evaluation

EGFR-TKI monotherapy included the 1G TKI gefitinib (a dose of 250 mg once daily), erlotinib (a dose of 150 mg once daily), or icotinib (a dose of 125 mg three times daily), and the 2G TKI

afatinib at a dose of 40 mg once daily. The chemotherapy regimens were intravenous pemetrexed (500 mg/m^2, day 1) plus cisplatin (n = 12,75 mg/m^2, d1), with or without anti-*VEGF* monoclonal antibody (bevacizumab 7.5 mg/kg, day 1) every 21 days as one cycle, followed by maintenance treatment with bevacizumab or pemetrexed monotherapy or a combination of bevacizumab plus pemetrexed after 6 cycles. Ten patients received carboplatin with area under the curve (AUC) equal to 5 if they were intolerable with cisplatin. Other patients received a 1G *EGFR*-TKI combined with chemotherapy every 21 days for four to six cycles, followed by maintenance treatment with pemetrexed with 1G *EGFR*-TKIs. All patients continued treatment until radiographic progression imaging examination or unacceptable toxicity as determined by their physicians.

Imaging examination at baseline was used to confirm the stage of disease, with measurable target lesions documented by computed tomography (CT) of the chest and abdomen, brain magnetic resonance imaging (MRI), or whole-body bone scans. Responses were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. The radiological images of the patients were reviewed by one radiologist, and another senior radiologist reviewed the images again in real time until an agreement was reached. Then, the imaging results were sent to hospital's order system. Efficacy was evaluated in the first month of treatment initiation and then scanned approximately every 2 months to assess treatment response. Tumor responses were evaluated as a complete response (CR), a partial response (PR), stable disease (SD), or progressive disease (PD) for at least 6 months by the investigator according to the RECIST version 1.1.

The primary endpoint was the duration of PFS. PFS was calculated from the time of treatment initiation to the date of documented disease progression or death. The ORR was defined as the percentage of patients with confirmed CR or PR, and the disease control rate (DCR) was defined as the percentage of those with CR, PR, or SD. Overall survival (OS) was calculated from the date of first-line treatment to death or last follow-up. We recorded the pattern of first documented treatment failure according to RECIST version 1.1. Smokers were defined as current or former smokers who had smoked continuously or cumulatively in their lifetime for 6 months or more, and nonsmokers were defined as individuals who had smoked fewer than 100 cigarettes in their lifetime. All clinical data were extracted from electronic medical records in our cancer center. As an observational real-world study, it was exempted from obtaining patients' informed consent and was approved by the institutional Ethics Review Board of National Cancer Center/ Cancer Hospital, Chinese Academy of Medical Sciences, and Peking Union Medical College (approval 18-070/1648).

Statistical Analysis

Statistical analyses were carried out by SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Patients' baseline characteristics are presented as descriptive statistics. Dichotomous variables are presented as percentages and were analyzed with the chi-square test (or Fisher's exact test when there was an expected frequency of less than 5). The Kaplan–Meier method with the long-rank test was used to compare PFS in different groups, which is expressed as the median value and corresponding 95% confidence index (CI). Univariate and multivariate Cox proportional hazards regression were used to evaluate predictive factors associated with PFS. A two-tailed test with $P < 0.05$ was considered statistically significant. Variables included age, sex, smoking history, clinical stage, ECOG score, histological type, molecular subtype of *EGFR* exon 18 mutation, and treatment pattern. GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) was used to generate survival curves and forest plots of subtype analysis.

RESULTS

Baseline Characteristics

A total of 82 patients with advanced lung adenocarcinoma harboring *EGFR* exon 18 mutations were included in the final analysis. A total of 47 (57.3%) females and 35 (42.7%) males were included, and the median age at diagnosis was 59 years old (range: 33–76 years). Forty-eight patients (58.5%) had a good ECOG PS score of 0, and 79 (96.3%) patients with lung adenocarcinoma were identified. Most patients had no smoking history (n = 57, 69.5%). Nearly a quarter of patients (n = 20) presented the central nervous system (CNS) metastasis at baseline. Sixty-nine cases (84.1%) were identified by NGS and 13(15.9%) were found by ARMS-PCR assay. All specimens were available for genetic testing *via* tissue biopsy (n = 77) or peripheral blood samples (n = 5). Of them, tissue samples originated from the lung and pleural effusion (n = 63), lymph nodes (n = 9), and other sites (n = 5). The baseline characteristics of patients were well balanced among the different treatment arms (in **Table 1**).

Subtypes of *EGFR* Exon 18 Mutations Among All Patients With or Without CNS Metastasis or Coexisting Genetic Alterations

Among the 82 patients with *EGFR* exon 18 mutations, the most common *EGFR* exon 18 mutation was G719X (n = 62, 75.6%), followed by E709X (n = 13, 15.9%), E709_T710delinsD (n = 3, 3.6%), and G724S (n = 4, 4.9%) (**Figure 1**). G719X substitutions G719A, G719C, and G719S and unknown subtypes were found in 62 patients, among them 34.1% harbored single G719X mutation and 41.5% harbored a compound G719X mutation. Thirteen patients carried E709X mutation. Nine out of 13 patients harbored E709K with G719A/C/S as well, and the other 4 patients carried E709K/A/Q with L858R. The detailed molecular subtypes of *EGFR* exon 18 mutations were shown in **Table 2**.

Among the 20 patients who presented with baseline CNS metastasis at the time of the primary diagnosis, the most common *EGFR* exon 18 mutation subtypes were G719X (n = 16, 80%) and E709_T710delinsD (n = 2, 10%). Other molecular subtypes were E709X and G724S mutations (n = 2, 10%). With a limited number of cases, no specific *EGFR* exon 18 subtype had an increased tendency for CNS metastasis at diagnosis **(Figure 1)**.

TABLE 1 | Baseline characteristics of patients.

Baseline Characteristics	1G TKI (n = 24)	2G TKI (n = 21)	CT (n = 22)	1G TKI + CT (n = 15)	*P*
Age					0.428
≤60	12 (50.0)	8 (38.1)	14 (63.6)	6 (40.0)	
>60	12 (50.0)	13 (61.9)	8 (36.4)	9 (60.0)	
Sex					0.412
Female	15 (62.5)	14 (66.7)	12 (54.5)	6 (40.0)	
Male	9 (37.5)	7 (33.3)	10 (45.5)	9 (60.0)	
Smoking					0.136
Yes	4 (16.7)	5 (23.8)	9 (40.9)	7 (46.7)	
No	20 (83.3)	16 (76.2)	13 (59.1)	8 (53.3)	
ECOG score					0.265
0	13 (54.2)	16 (76.2)	12 (54.5)	7 (46.7)	
1–2	11 (45.8)	5 (23.8)	10 (45.5)	8 (53.3)	
Histological types					0.622
ADC	22 (91.7)	21 (100)	21 (95.5)	15 (100)	
Non-ADC	2 (8.3)	0 (0)	1 (4.5)	0 (0)	
Tumor stage					0.569
IIIb	1 (4.2)	3 (14.3)	3 (13.6)	1 (6.7)	
IV	23 (95.8)	18 (85.7)	19 (86.4)	14 (93.3)	
Brain metastases					0.139
Yes	9 (37.5)	6 (28.6)	4 (18.2)	1 (6.7)	
No	15 (62.6)	15 (71.4)	18 (81.8)	14 (93.3)	
Molecular subtype					0.289
G719X	20 (83.3)	16 (76.1)	12 (54.6)	14 (93.3)	
E709X	3 (12.5)	3 (14.3)	6 (27.3)	1 (6.7)	
E709_T710 delinsD	1 (4.2)	1 (4.8)	1 (4.5)	0 (0)	
G724S	0 (0)	1 (4.8)	3 (13.6)	0 (0)	

1G, first-generation; 2G, second-generation; TKI, tyrosine kinase inhibitors; CT, chemotherapy; ECOG, Eastern Cooperative Oncology Group; ADC, adenocarcinoma; EGFR, epidermal growth factor receptor.

Sixty-nine cases were tested by NGS, and 10 (12.2%) had coexisting genetic alterations. *TP53* mutation was detected in four cases, and *EGFR* amplification was detected in two samples. In addition, *MET* amplification and mutations of *PTEN*, *FGFR3*, and *HER2* were detected in one case each.

Therapeutic Responses and Survival Analysis After First-Line Therapy in Patients With *EGFR* Exon 18 Mutations

At the time of the cutoff date (December 31, 2020), the median follow-up time since the diagnosis of advanced or metastatic disease was 30.8 months (range: 1.8–81.0 months). Of the 82 patients, 1G *EGFR*-TKI was administered in 24 patients, another 21 patients received afatinib, 22 patients received chemotherapy, and 15 patients received a 1G *EGFR*-TKI combined with chemotherapy. The response rates to 1G *EGFR*-TKI, 2G *EGFR*-TKI afatinib, chemotherapy, and 1G *EGFR*-TKI combined with chemotherapy were 25.0%, 52.4%, 40.9%, and 46.7% ($P = 0.276$), and the DCRs were 78.2%, 76.1%, 47.8%, and 86.7%, respectively ($P = 0.021$).

Most patients harboring *EGFR* exon 18 mutations receive *EGFR*-TKI treatment involving a 1G, 2G, or 3G TKI in the first-line setting, though these make up a small sample in all. A summary of the different therapeutic strategies for *EGFR* exon 18 mutations from various studies is shown in **Table 3**. In our study, the 2G TKI afatinib had relatively long PFS for NSCLC patients with *EGFR* exon 18 mutations. The mPFS differed significantly

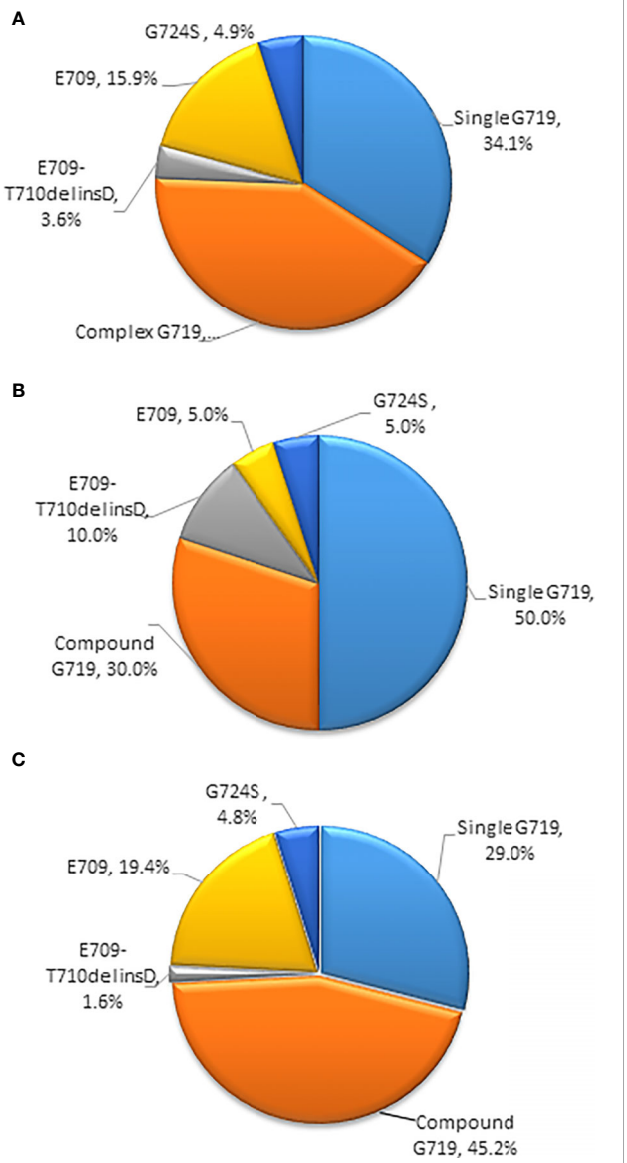

FIGURE 1 | **(A)** Percentages of subtypes of *EGFR* exon 18 mutations among all patients **(B)** Percentages of subtypes of *EGFR* exon 18 mutations with brain metastases **(C)** Percentages of subtypes of *EGFR* exon 18 mutations without brain metastases. (*EGFR*, epidermal growth factor receptor).

between patients treated with different treatment strategies ($P = 0.017$). The mPFS of 1G *EGFR*-TKI, the 2G EGFR-TKI afatinib, chemotherapy, and 1G *EGFR*-TKI in combination with chemotherapy were 7.7 (95% CI, 4.2–11.2), 11.3 (95% CI, 5.6–17.0), 5.0 (95% CI, 2.3–17.7), and 11.1months (95% CI, 5.9–16.4), respectively (**Figure 2**).

We further analyzed the clinical outcomes in advanced NSCLC patients harboring *EGFR* exon 18 mutations treated with four diverse therapeutic strategies. Compared to the 2G *EGFR*-TKI afatinib as the standard of care, a significant survival drop was observed with chemotherapy alone (HR = 2.261, 95%

TABLE 2 | Different subtypes of *EGFR* exon 18 mutation found in this sample.

Subtypes of *EGFR* exon 18 mutation	Number (n = 82, %)
G719X	62 (75.6)
G719A	15
G719C	1
G719S	3
G719 unknown	9
G719A + S768I/S720F/L861R/R766	3/1/1/1
G719C + S768I/L861Q/L861R/K714N	11/1/1/1
G719S + S768I/L858R	7/3
G719 unknown + S768I	4
E709X	13 (15.9)
E709K + G719A/C/S	3/2/4
E709K/A/Q + L858R	4
E709_T710delinsD	3 (3.6)
G724S + *EGFR*19/S768I	3/1 (4.9)
Total	82 (100)

EGFR, epidermal growth factor receptor.

CI: 1.108–4.611, P = 0.025), while there was no significant difference in PFS between 1G *EGFR*-TKI or 1G *EGFR*-TKI combined with chemotherapy and afatinib (HR = 1.632, 95% CI: 0.806–3.304, P = 0.173 and HR = 0.774, 95% CI: 0.323–1.854, P = 0.566, respectively), and the mOS was not reached in any subgroup.

Univariate and Multivariate Analyses for PFS by the Cox Regression Model

Univariate analysis showed that the PFS of advanced NSCLC patients with *EGFR* exon 18 mutations was significantly associated with molecular subtype (P < 0.001) and treatment pattern (P = 0.023) (**Table 4**). Although there was no statistically significant difference in PFS between those with and without brain metastasis due to the small sample size, the status of brain metastasis might have affected the outcome of PFS in a previous study (20). Therefore, the status of brain metastasis, molecular subtype, and treatment pattern was entered into the multivariate Cox regression model. Multivariate analyses confirmed that *EGFR* exon 18 molecular subtype and treatment pattern were independent predictors of PFS in advanced NSCLC patients with *EGFR* exon 18 (P < 0.05, **Table 5**).

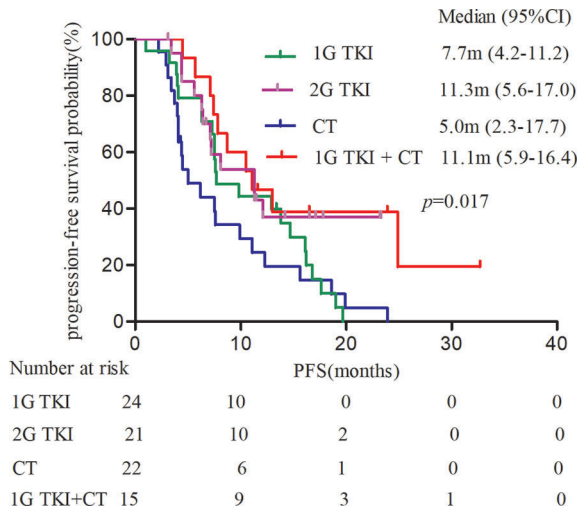

FIGURE 2 | Kaplan-Meier curves of PFS in patients harboring *EGFR* exon 18 mutations treated with different treatment modalities. (PFS, progression-free survival).

Subgroup Analysis by Different Treatment Pattern

Subgroup analyses of PFS based on investigator assessment are presented according to baseline characteristics. The PFS of patients in the afatinib cohort was significantly better than that in the chemotherapy cohort among females and among patients without brain metastases, regardless of age (P < 0.05, **Figure 3A**). In contrast, no significant PFS benefit was observed between the afatinib cohort and the 1G TKI or in the 1G TKI in combination with chemotherapy cohort in any subgroup, including the age, sex, smoking history, ECOG score, brain metastasis, and molecular type subgroups (P > 0.05, **Figures 3B, C**).

Disease Progression Patterns in Patients With *EGFR* Exon 18 Mutations

At the cutoff date, 65 (79.3%) of 82 patients presented disease progression. Intrathoracic metastases were the most common

TABLE 3 | Comparisons of different treatments for *EGFR* exon 18 mutations from various studies.

Study	N	*EGFR* exon 18 subtype	Treatment	ORR	PFS
This study	82	G719X	A	52.4%	11.3 (5.6–17.0)
		E709X	G/E/I + CT	46.7%	11.1 (5.9–16.4)
		DelE709_T710ins D	G/E/I	26.1%	7.7 (4.3–11.1)
		Complex G724S	CT	39.1%	6.2 (1.8–10.6)
Passaro et al. (19)	42	Single 18 mutation	G/E/A	31.0%	8.3 (4.9–11.7)
Zhang et al. (13)	22	Single G719X	G/E/I	22.7%	7.6 (4.9–10.4)
		Complex G719 mutations			
Chui et al. (14)	78	Single G719X mutation	G/E	36.8%	6.3
	9	G719X + L861Q		88.9%	NR
	10	G719X + S768I		50.0%	NR
Yang et al. (7)	8	SingleG719X	A	77.8%	13.8 (6.8–NE)
	10	Complex G719 mutation			
Cho et al. (18)	19	G719X	O	53.0%	8.2

A, afatinib; G, gefitinib; E, erlotinib; I, icotinib; O, osimertinib; CT, chemotherapy; NR, not reached.

TABLE 4 | Univariate survival analyses for PFS.

Variable	B	SE	HR	95% CI	*P*
Age (≥60 *vs.* <60)	-0.064	0.250	0.938	0.575–1.530	0.798
Sex (male *vs.* female)	0.507	0.265	1.061	0.988–2.792	0.056
Smoking history (yes *vs.* no)	0.209	0.276	0.450	0.717–2.117	0.450
Histological types (ADC *vs.* Non-ADC)	-0.043	0.595	0.958	0.299–3.074	0.943
Clinical stage (IIIb *vs.* IV)	-0.023	0.431	0.978	0.420–2.275	0.958
ECOG score (0 *vs.* 1–2 points)	-0.034	0.254	0.966	0.587–1.590	0.893
Brain metastases at baseline (yes *vs.* no)	0.489	0.296	1.631	0.913–2.913	0.099
Molecular subtype					<0.001
(E709-T710delinsD *vs.* G719X)	0.865	0.730	2.376	0.568–9.937	0.236
(E709X *vs.* G719X)	-0.619	0.384	0.539	0.254–1.144	0.107
(G724S *vs.* G719X)	2.675	0.623	14.515	4.277–49.259	<0.001
Treatment patterns					0.023
(CT *vs.* 2G TKI)	0.816	0.364	2.261	1.108–4.611	0.025
(1G TKI *vs.* 2G TKI)	0.490	0.360	1.632	0.806–3.304	0.173
(1G TKI+CT *vs.* 2G TKI)	-0.256	0.445	0.774	0.323–1.854	0.566

HR, hazard ratio; CI, confidence interval; ADC, adenocarcinoma; ECOG, Eastern Cooperative Oncology Group; TKI, tyrosine kinase inhibitor; CT, chemotherapy; 1G, first-generation; 2G, second-generation.

progressive pattern in patients harboring *EGFR* exon 18 mutations, accounting for 55.3% (n = 36), followed by 24.6% (n = 16) in the brain and 20.1% (n = 13) in other organs, including the liver, bone, adrenal, and lymph nodes, among all patients.

Different treatment modalities had a tendency to develop distinct progressive patterns (P = 0.037). Intrathoracic metastases were observed in 26.1% of progressive patients treated with chemotherapy, 12.3% of those treated with 1G *EGFR*-TKI, 9.2% of those treated with 1G *EGF*R-TKI in combination with chemotherapy, and only 7.7% of those treated with afatinib. The proportion of brain metastases was the highest in patients treated with 1G *EGFR*-TKIs (n = 10, 15.4% of progressive patients). The rates of brain metastases in patients treated with afatinib, chemotherapy, and 1G *EGFR*-TKI in combination with chemotherapy were 4.5% (n = 3), 3.0% (n = 2), and 1.5% (n = 1), respectively. The disease progression patterns are listed in the **Supplementary Appendix** (**Supplementary Figure S2**).

DISCUSSIONS

With the development of comprehensive molecular profiling of NSCLC, an increasing number of uncommon *EGFR* mutations have been revealed other than the known *EGFR*-sensitive mutations, including *EGFR* exon 19 deletion and the exon 21 L858R missense mutation. *EGFR* exon 18 mutations are listed as uncommon mutations involving missense and deletion/insertion mutations. However, patients with *EGFR* exon 18 mutations have heterogeneous outcomes after taking different *EGFR*-TKIs (9, 19, 21). To date, this is the largest of treatment outcome reported in NSCLC patients harboring *EGFR* exon 18 mutation. In this study, we analyzed the distribution of subtypes of *EGFR* exon 18 mutation and the clinical outcomes of advanced NSCLC patients with *EGFR* exon 18 mutations receiving different treatment strategies as first-line therapy. In line with previous studies (22, 23), the most common *EGFR* exon 18 mutation was G719X (75.6%), followed by E709X (15.9%), E709_T710delinsD (3.6%), and other subtypes (4.9%). Single G719X mutation involved different subtypes (G719A, C, D, S, and V), the most common of which was G719A (n = 15, 53.6%). A total of 41.5% of *EGFR* G719X mutations were identified as part of compound mutations (n = 34), and approximately one-third of G719X mutations presented in combination with the S768I mutation.

Given the low prevalence of *EGFR* exon 18 mutations and the lack of prospective head-to-head research data, NSCLC patients harbor uncommon *EGFR* exon 18 mutations. The results of different treatment options are still relatively sparse. Our study provides real-world therapeutic responses in advanced NSCLC patients with *EGFR* exon 18 mutation treated with different

TABLE 5 | Predictors of PFS analyzed by multivariate Cox regression.

Variable	B	SE	HR	95% CI	*P*
Brain metastases at baseline (yes *vs.* no)	0.130	0.341	1.138	0.583–2.221	0.704
Molecular subtype					<0.001
(E709-T710delinsD *vs.* G719X)	0.864	0.804	2.372	0.491–11.460	0.283
(E709X *vs.* G719X)	-0.802	0.403	0.448	0.204–0.987	0.046
(G724S *vs.* G719X)	2.219	0.659	9.199	2.528–33.470	0.001
Treatment patterns					0.023
(CT *vs.* 2G TKI)	0.874	0.382	2.398	1.135–5.066	0.022
(1G TKI *vs.* 2G TKI)	0.479	0.381	1.614	0.765–3.406	0.209
(1G TKI + CT *vs.* 2G TKI)	-0.245	0.459	0.783	0.318–1.926	0.594

HR, hazard ratio; CI, confidence interval; TKI, tyrosine kinase inhibitors; CT, chemotherapy; 1G, first-generation; 2G, second-generation.

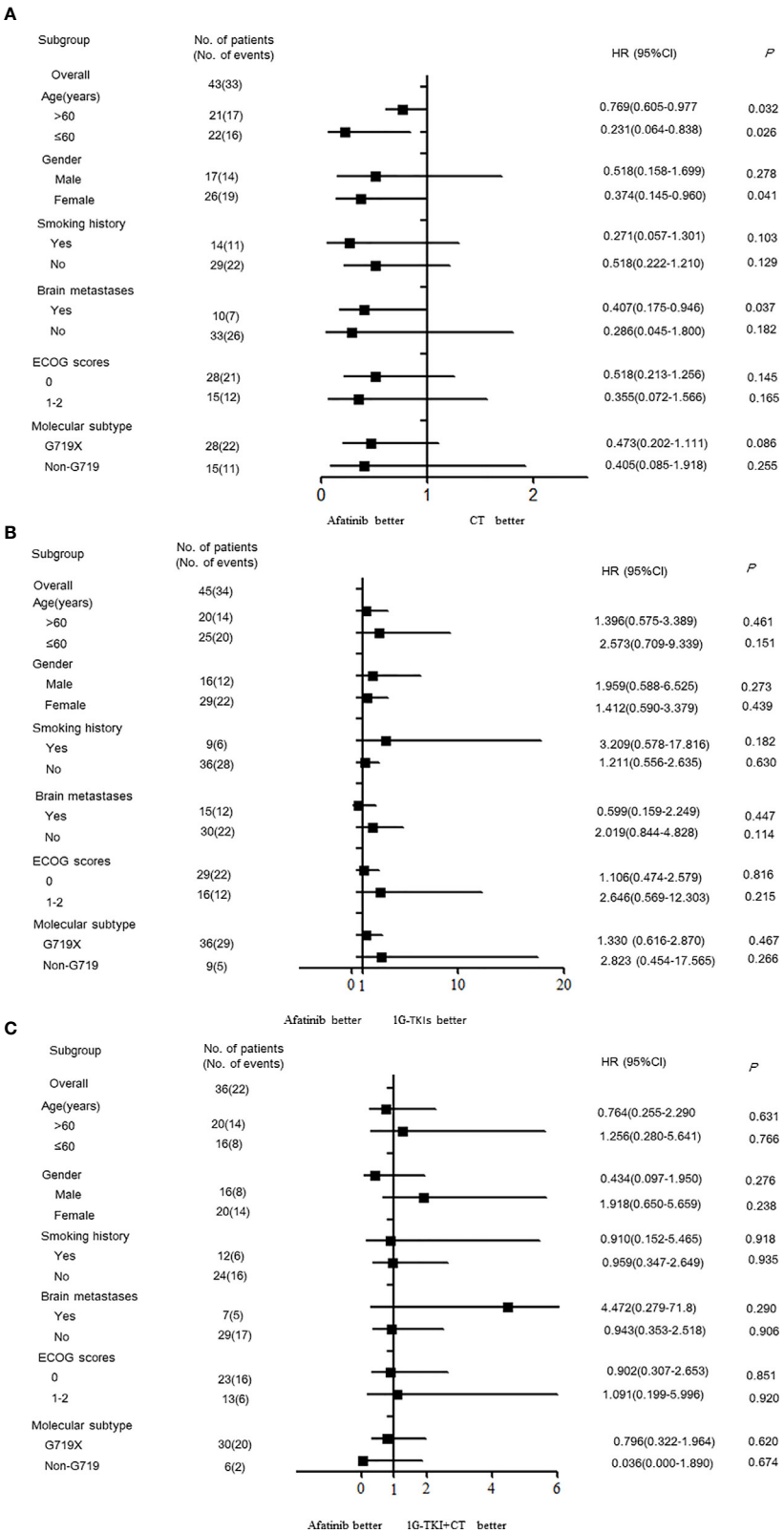

FIGURE 3 | Forest plots by the various demographics and disease characteristics. **(A)** afatinib *vs.* CT **(B)** afatinib *vs.* 1GTKI **(C)** afatinib *vs.* 1G TKI+CT. (CT, chemotherapy; 1G-TKI, first-generation tyrosine kinase inhibitor).

treatment strategies in the first-line setting. Patients treated with a 2G *EGFR*-TKI (afatinib) or a 1G *EGFR*-TKI in combination with chemotherapy had a relatively better response rate than those treated with a 1G *EGFR*-TKI or chemotherapy alone. The response rates to 1G *EGFR*-TKI, afatinib, chemotherapy, and 1G *EGFR*-TKI combined with chemotherapy were 25.0%, 52.4%, 40.9%, and 46.7% ($P = 0.276$), and the DCRs were 78.2%,76.1%, 47.8%, and 86.7%, respectively ($P = 0.021$).

We also analyzed the clinical outcomes in advanced NSCLC patients harboring *EGFR* exon 18 mutations treated with the four different treatment strategies. The mPFS of patients treated with different treatment strategies was significantly different ($P = 0.017$). Taking the 2G *EGFR*-TKI afatinib as the standard of care, a significant deficit in PFS was observed in patients who had chemotherapy alone ($P = 0.023$): The mPFS of patients treated with afatinib was 11.3 months, compared with 5.5 months in those treated with chemotherapy. There was no significant difference in PFS between the 1G *EGFR*-TKI group or the 1G *EGFR*-TKI combined with chemotherapy group and afatinib ($P > 0.05$). The mPFS of the 1G *EGFR*-TKI group and the 1G *EGFR*-TKI in combination with chemotherapy group was 7.7 months and 11.1 months, respectively. Furthermore, multivariate analyses demonstrated that treatment pattern was an independent predictor of PFS in NSCLC patients with *EGFR* exon 18 mutations. Similarly, Kobayashi et al. (24) reported that patients with specific exon 18 mutations were more sensitive to 2G *EGFR*-TKI than 1G *EGFR*-TKI or the 3G *EGFR*-TKI osimertinib compared with *EGFR* exon 19 deletion patients, and to some degree, they generally tended to be resistant to gefitinib or erlotinib. An *in vitro* study in cell lines also showed a better response to afatinib and neratinib than to gefitinib and erlotinib, with respective IC90s of 0.9 nM and 1.1 nM, in cell with G719A (25).

Based on the LUX-Lung clinical trials, afatinib was approved for the treatment of metastatic NSCLC harboring *EGFR* S768I, L861Q, and/or G719X by the U.S. FDA in 2018. However, afatinib cannot cover all uncommon *EGFR* mutations and severe dose-limiting toxicities were observed in these trials, so those patients should explore alternative treatment strategies. A phase III clinical trial (NEJ009) (26) demonstrated that gefitinib in combination with pemetrexed and carboplatin improved PFS and OS compared with *EGFR*-TKIs alone for untreated advanced NSCLC with classic *EGFR* mutations. However, the efficacy of gefitinib in combination with pemetrexed and carboplatin in uncommon *EGFR* mutations is unknown. Our study confirms the results of NEJ009, indicating that 1G in combination with chemotherapy has a good PFS outcome for patients not only with common *EGFR* mutations but also with uncommon *EGFR* exon 18 mutations. Compared to afatinib treatment as the standard of care, there was a similar survival time in PFS of 1G *EGFR*-TKI in combination with chemotherapy ($P = 0.709$), which indicated that 1G *EGFR*-TKI in combination with chemotherapy might be a potentially effective option for the treatment of NSCLC patients with *EGFR* exon 18 mutations. It was of note that a subgroup included in the present analysis had received 1G *EGFR*-TKI plus chemotherapy, which enriched the treatment data for uncommon *EGFR* mutation. In addition to treatment strategy, molecular subtype was associated with PFS in our NSCLC patients with *EGFR* exon 18 mutation. Therefore, comprehensive and detailed molecular subtype testing is of great importance to clinicians to evaluate survival outcomes.

Several limitations of our study must be identified. Firstly, this was a retrospective study with a small sample size, which might have induced potential bias. Secondly, patients received 1G *EGFR*-TKI heterogeneity treatment involving gefitinib, erlotinib, and icotinib, and selection bias was inevitable. Thirdly, given the limited sample number and the efficacy of these two *EGFR*-TKIs against various exon 18 mutation subtypes, we did not analyze the efficacy of another 2G *EGFR*-TKI, dacotinib, or the 3G *EGFR*-TKI osimertinib. Finally, this study lacks the incidence of T790M beyond 1G *EGFR*-TKI and 2G *EGFR*-TKI resistance due to the low rate of re-biopsy or insufficient tissue specimens.

In conclusion, our data indicate that the combination of 1G *EGFR*-TKIs with chemotherapy was associated with a good response rate and a promising PFS outcome for NSCLC patients with uncommon *EGFR* exon 18 mutations. 1G *EGFR*-TKIs in combination with chemotherapy might be a feasible first-line treatment option, like afatinib. In clinical practice, when patients cannot tolerate the toxicity of afatinib, clinicians might use 1G *EGFR*-TKI in combination with chemotherapy for treatment of uncommon *EGFR* exon 18 mutations. Although the small sample size of patients with *EGFR* exon 18 mutations is a limitation, this trend in PFS that we found provides clues for further research and treatment. Future studies should determine the most appropriate treatment recommendation for NSCLC patients harboring uncommon *EGFR* exon 18 mutations.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional Ethics Review Board of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (approval 18-070/1648). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HX and GY have contributed equally to this work and share first authorship. All authors have no conflicts of interest to declare.

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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.2021.713483/full#supplementary-material

Supplementary Figure 1 | The study flow chart.

Supplementary Figure 2 | (A) Disease progressive sites among all *EGFR* exon 18 mutations **(B)** Intrathoracic metastases with different treatment modalities **(C)** Brain metastases with different treatment modalities. (*EGFR*, epidermal growth factor receptor).

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Perioperative Outcome of Robotic Approach *Versus* Manual Videothoracoscopic Major Resection in Patients Affected by Early Lung Cancer: Results of a Randomized Multicentric Study (ROMAN Study)

Giulia Veronesi[1,2], Abbas El-Sayed Abbas[3,4], Piergiorgio Muriana[1], Rosalba Lembo[5], Edoardo Bottoni[6], Gianluca Perroni[1], Alberto Testori[6], Elisa Dieci[1], Charles T. Bakhos[3,4], Shamus Car[7], Luca Luzzi[8], Marco Alloisio[6,9] and Pierluigi Novellis[1]*

[1] Department of Thoracic Surgery, IRCCS San Raffaele Scientific Institute, Milan, Italy, [2] Faculty of Medicine and Surgery, Vita-Salute San Raffaele University, Milan, Italy, [3] Department of Thoracic Medicine and Surgery, Lewis Katz School of Medicine, Temple University Hospital, Philadelphia, PA, United States, [4] Department of Surgery, Lewis Katz School of Medicine, Temple University Hospital, Philadelphia, PA, United States, [5] Department of Anesthesia and Intensive Care, IRCCS San Raffaele Scientific Institute, Milan, Italy, [6] Division of Thoracic and General Surgery, Humanitas Clinical and Research Center, Rozzano, Italy, [7] Division of Thoracic Surgery, Department of Surgery, University of Maryland School of Medicine, Baltimore, MD, United States, [8] Thoracic Surgery Unit, Department of Medicine, Surgery and Neuro Sciences, Diagnostic Imaging, University of Siena, Azienda Ospedaliera Universitaria Senese, Siena, Italy, [9] Department of Biomedical Science, Humanitas University, Rozzano, Italy

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Alfredo Addeo, Geneva University Hospitals (HUG), Switzerland

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Paul Emile Van Schil, Antwerp University Hospital, Belgium
Agathe Seguin-Givelet, L'Institut Mutualiste Montsouris, France
Jari Räsänen, Helsinki University Library, Finland

***Correspondence:**
Giulia Veronesi
veronesi.giulia@hsr.it

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Introduction: We report the results of the first prospective international randomized control trial to compare the perioperative outcome and surgical radicality of the robotic approach with those of traditional video-assisted surgery in the treatment of early-stage lung cancer.

Methods: Patients with clinical stage T1–T2, N0–N1 non-small cell lung cancer (NSCLC) were randomly assigned to robotic-assisted thoracoscopic surgery (RATS) or video-assisted thoracic surgery (VATS) resection arms. The primary objective was the incidence of adverse events including complications and conversion to thoracotomy. The secondary objectives included extent of lymph node (LN) dissection and other indicators.

Results: This trial was closed at 83 cases as the probability of concluding in favor of the robot arm for the primary outcome was null according to the observed trend. In this study, we report the results of the analysis conducted on the patients enrolled until trial suspension. Thirty-nine cases were randomized in the VATS arm and 38 in the robotic arm. Six patients were excluded from analysis. Despite finding no difference between the two arms in perioperative complications, conversions, duration of surgery, or duration of postoperative stay, a significantly greater degree of LN assessment by the robotic technique was observed in regards to the median number of sampled LN stations [6,

interquartile range (IQR) 4–6 *vs*. 4, IQR 3–5; $p = 0.0002$], hilar LNs (7, IQR 5–10 *vs*. 4, IQR 2–7; $p = 0.0003$), and mediastinal LNs (7, IQR 5–10 *vs*. 5, IQR 3–7; $p = 0.0001$).

Conclusions: The results of this trial demonstrated that RATS was not superior to VATS considering the perioperative outcome for early-stage NSCLC, but the robotic approach allowed an improvement of LN dissection. Further studies are suggested to validate the results of this trial.

Clinical Trial Registration: clinicaltrials.gov, identifier NCT02804893.

Keywords: non-small cell lung cancer (NSCLC), surgery, robotic surgery, VATS, randomized study

INTRODUCTION

The robotic-assisted thoracoscopic surgery (RATS) approach has emerged as a valid alternative to the traditional minimally invasive video-assisted thoracoscopic surgery (VATS) (1–3). Thanks to significant technical advantages and stereoscopic visualization, it has become the preferred technique of an increasing number of thoracic surgeons (4). Many studies have shown that robotic-assisted pulmonary resection is both feasible and safe for the treatment of lung cancer (1–3, 5–7), with long-term outcomes comparable to that reported for open and VATS approaches (8, 9).

Some retrospective analyses of population-based database showed that RATS was associated with improved perioperative outcomes compared to the open approach but had comparable results to VATS (10, 11). In 2016, Agzarian et al. conducted a comparative meta-analysis of robotic pulmonary resection and other modalities. There were no significant differences in conversion rates, prolonged air leaks, blood loss, or length of stay between RATS and VATS (12). Different results were shown in 2017 by Oh et al., who analyzed the Premier Healthcare Database to compare perioperative clinical outcomes from elective lobectomy by RATS, VATS, and thoracotomy, with propensity score matching (1:1). Compared with the VATS and open approaches, RATS lobectomy was associated with a shorter length of stay, lower complication rates, and lower conversion rate (10).

Recently, Kneuertz et al. showed that lymph node upstaging with RATS was superior to VATS and comparable to the open approach (13). Novellis *et al.* also reported a retrospective comparative analysis of RATS *versus* open and VATS approaches for lung lobectomies, with a significant difference in perioperative outcome in favor of the robotic approach (11). Their study also observed that more lymph node (LN) stations were removed by RATS when compared with VATS and thoracotomy (11).

To date, no randomized trials comparing the early- and long-term outcome of VATS *versus* RATS lobectomy have been reported. We therefore designed a multicenter randomized controlled trial with the primary objective to assess the overall perioperative complication rate, including conversion to thoracotomy and 30-day complication rate. As a secondary objective, we explored the extent of LN dissection, postoperative hospital stay, duration of surgery, long-term assessment of pain, quality of life (QoL), and recurrence rate. In this paper, we report the results of the early outcomes.

MATERIALS AND METHODS

Ethics Committee Approval

The study protocol was evaluated by the Humanitas Clinical and Research Center Ethic Committee (no. 1566) and approved by the local internal review boards of all participating centers. It was registered at ClinicalTrials.gov (NCT02804893). All participants gave written informed consent to participate in the study.

Study Design

We designed a prospective, randomized, multicenter study on 300 patients (150 VATS lobectomies and 150 RATS lobectomies) affected by early-stage non-small cell lung cancer (NSCLC). The expected time period for recruitment was 1 year, and that for follow-up was 2 years. For participation in the study, trial surgeons needed a minimum of 30 major lung resections performed using one or each of the two techniques. Every participating center needed the ability to offer both techniques (RATS and VATS).

Randomization was performed through a dedicated Internet-based system with a balance software for center stratification (validated by FDA, Title 21 of the Code of Federal Regulations, Part 11) within 4 weeks prior to the planned operation date once the eligibility of the patient had been confirmed and consent was given. This interval allowed a sufficient time to schedule the date of surgery.

Study Objectives

The aim of this study was to compare VATS and RATS approaches in the treatment of early-stage NSCLC in terms of operative and perioperative results. We identified as primary endpoints the rate of conversions, bleeding, and perioperative complications (assessed by modified Clavien–Dindo scale). The secondary endpoints were duration of surgery, number of resected LNs, number of dissected LN stations, postoperative hospital stay, postoperative pain with daily evaluation, quality of life by EORTC QoL-C30, postoperative respiratory function, and rate of local or distant recurrence at 2 years.

Inclusion/Exclusion Criteria

The inclusion criteria were as follows: age older than 18 years old and known or suspected NSCLC. In case of suspected lung cancer with no preoperative diagnosis, frozen section was indicated during surgery in order to confirm the disease. If a benign lesion was diagnosed, the patient was considered a dropout of the study. Other inclusion criteria include the following: patients in clinical stage T1–T2–T3, N0–N1, candidate for lobectomy, anatomical segmentectomy, or bilobectomy; patients with multiple lung tumors could be included if they could be resected with a lobectomy, lobectomy plus segmentectomy, or bilobectomy and each tumor should be staged separately; and American Society of Anesthesiologists score 1–3. Written informed consent was signed prior to performing any study procedures.

The exclusion criteria were also as follows: metastatic cancer, extrapulmonary primary cancers in the past 2 years, severe heart disease, alcohol abuse, renal impairment (creatinine >2.5 mg/dl), and other serious comorbidities that contraindicate surgery.

Preoperative Evaluation

Preoperative analysis included staging studies such as chest CT scan and PET scan. For stages higher than IA, brain CT with contrast or MRI was required, while brain MRI was done in case of suspicious brain lesions. Standard functional evaluation included EKG, cardiological evaluation, pulmonary function tests, and anesthesia evaluation. When required by the physician, additional tests were introduced, such as cardiac stress test, echocardiography, and pulmonary scintigraphy.

Staging and functional exams were done within 6 weeks of surgery. In case of suspicious mediastinal nodes, endobronchial ultrasound or mediastinoscopy was done before resection.

During the operation, frozen section for confirmation of diagnosis was done in cases of lesions with no preoperative diagnosis. All operations were performed under general anesthesia, with the patients in the lateral decubitus position.

Operative Approaches

VATS lobectomy or segmentectomy was performed through one to four thoracoscopic incisions without rib spreading. The procedure was performed with videoscopic visualization without direct vision. The hilar structures were dissected, stapled, and divided. Endoscopic ligation of pulmonary arterial branches was occasionally performed. The fissure was completed, and the lobe of lung was resected. This definition of VATS lobectomy is a modification of CALGB 39802 (14).

Robotic lobectomy or segmentectomy was performed through four to five thoracoscopic incisions without rib spreading. The Da Vinci Robotic System (Intuitive, Sunnyvale, USA) was used. Under 3D vision, the hilar structures (vein, artery, and bronchus) were dissected, ligated, and divided in sequence using ligatures, by oversewing, or with staplers. The surgical approach for robotic resection was chosen according to the preference of the operator. In complete portal robotic lobectomy, all the ports were placed along a single intercostal space, and dissection was carried in a posterior to anterior direction with carbon dioxide use. The surgical specimen was then removed through a trans- or supradiaphragmatic incision (2). The robotic-assisted lobectomy approach was carried out through a utility incision at the fourth intercostal space and three additional ports without CO_2 use. In this case, pulmonary hilum was approached from its anterior aspect. The specimen was extracted through the utility incision at the end of operation (1, 3).

LN dissection, both in VATS and RATS, was undertaken in accordance with the International Association of the Study of Lung Cancer recommendations of a minimum of six LN stations removed, of which three are from the mediastinum that includes the subcarinal station (15).

Postoperative Care

The bladder catheter, if used, was removed when the urine output was adequate (>40 ml/h after surgery), without a known prostate disease. The chest tube was removed when the amount of drainage was less than 350 cc over 24 h (regardless of postoperative day) and in the absence of air leak. If prolonged air leak was observed, Heimlich valve was applied, and discharge was scheduled in the absence of clinical contraindications.

Statistical Analysis

The primary objective was the incidence of adverse events including complications and conversions. At least one of these events was considered a failure of surgery. To have 80% power and a significance level of 5% to demonstrate a reduction of 15% rate of adverse events starting from 35% with VATS to 20% with robotic approach, a sample size of 300 subjects was initially calculated, 150 in each arm, with an expected dropout of less than 1% of the enrolled subjects. This sample size also had a power of 95% to detect a difference of 0.4 in the mean number of mediastinal lymph node stations, starting from 2.5, with a common standard deviation of 1.

Intention-to-treat and per-protocol analyses were performed. No imputation for missing data was planned. We also performed a planned *post-hoc* power analysis for secondary outcome, specifically for the number of hilar and mediastinal lymph nodes, and lymph node stations were harvested.

Categorical data were presented as absolute number and percentages and were compared by two-tailed χ^2 test or Fisher's exact test when appropriate. Means and standard deviations were used when the variables were normally distributed, while medians and interquartile ranges were used with nonnormally distributed variables. Continuous measurements were compared using a nonparametric test or Student's *t*-test if data were normally distributed. A logistic regression model with stepwise selection was used to identify predictors of primary outcome. Clinical data collected before randomization were entered into the model if they had a univariate *P*-value of less than 0.25. The trial group (robot *vs.* VATS) was forced into the multivariate model. Collinearity and overfitting were assessed with the use of a stepwise regression model and a Pearson correlation test. In the multivariate analyses, clinical factors or potential confounding variables were expressed as odds ratios with 95% confidence intervals. Statistical

significance was set at the two-tailed 0.05 alpha level. All statistical analyses were performed with the Stata software (ver. 16; Texas USA).

RESULTS

From April 2017 to November 2018, we screened 83 patients in four centers for eligibility (49 in center no. 1, 30 in center no. 2, and two patients both in center no. 3 and no. 4). Six patients were excluded from randomization: in detail, three patients did not undergo surgery because of contraindications encountered during the preoperative evaluation and three patients for other reasons. Seventy-seven patients provided informed consent and were randomized; 39 (51%) were assigned to the VATS group and 38 (49%) to the robot group (**Figure 1**). Patient demographics and disease characteristics were well balanced by treatment and are summarized in **Table 1**. The intraoperative results are reported in **Table 2**.

The study was closed as part of the periodic analyses by the independent data monitoring committee because, during the review, any difference observed between arms in terms of adverse events and the probability of concluding in favor of the robot arm was 0% (futility reason) if the observed trend had continued. In detail, conversion to thoracotomy was required in three cases of the RATS group and in two patients of the VATS group (p = 0.64). Early postoperative complications occurred in 13 cases (34%) in the robotic group and in nine cases (23%) in the VATS

TABLE 1 | Baseline characteristics of the patients enrolled in the VATS and ROBOT groups.

	Group VATS, *N* = 39	Group ROBOT, *N* = 38	*P*-value
Age, years (mean ± SD)	69 ± 7.3	69 ± 8.3	0.87
Female (%)	16 (41)	17 (45)	0.82
BMI (mean ± SD)	26 ± 4.1	27 ± 4.0	0.44
Smoking status			
Nonsmokers (%)	10 (38)	10 (45)	0.77
Former (%)	13 (45)	16 (57)	0.43
Stop smoking, years median (IQR)	15 (5–25)	20 (5–30)	0.90
Smokers (%)	16 (62)	12 (55)	0.77
Number of cigarettes/day median (IQR)	20 (20–30)	20 (10–30)	0.21
Pulmonary function evaluation			
FEV1, L (mean ± SD)	91 ± 24.8	86 ± 25.0	0.37
DLCO, mmol/min/KPa/L (mean ± SD)	76 ± 19.6	76 ± 20.5	0.91
ASA score (%)[a]			
I – II	24 (62)	19 (54)	0.64
III	15 (38)	16 (46)	
Clinical stage (%)[b]			
IA	25 (71)	28 (76)	0.48
IB	7 (20)	7 (19)	
IIA	1 (3)	2 (5)	
IIB	2 (6)	0 (0)	

SD, standard deviation; IQR, interquartile range; BMI, body mass index; FEV1, forced expiratory volume in the first second; DLCO, diffusing capacity of the lung for carbon monoxide; ASA score, American Society of Anesthesiology score.
[a]Data were available for analysis in 74 patients.
[b]Data were available for analysis in 72 patients.

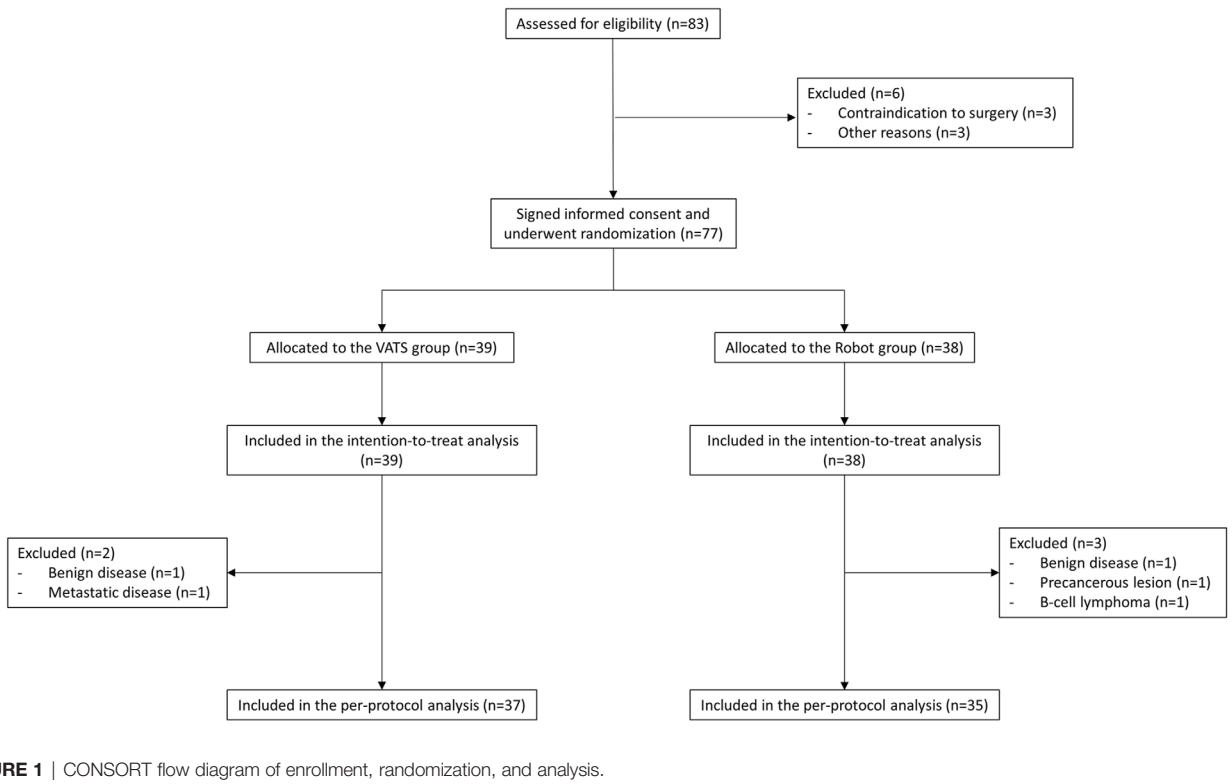

FIGURE 1 | CONSORT flow diagram of enrollment, randomization, and analysis.

TABLE 2 | Intraoperative characteristics in the VATS and ROBOT groups of patients.

	Group VATS, *N* = 39	Group ROBOT, *N* = 38	*P*-value
Left side (%)	16 (41)	14 (37)	0.71
Lobe (%)			
Lower	16 (41)	16 (42)	0.92
Middle	1 (2.6)	6 (16)	0.056
Upper	22 (56)	16 (42)	0.21
Number of incisions, median (IQR)	2 (2–3)	4 (4–4)	<0.0001
Utility incision size, cm (mean ± SD)	3.3 ± 0.67	2.7 ± 0.86	0.01
Pleural adhesions (%)[a]			
Light	16 (76)	14 (64)	0.37
Moderate	5 (24)	3 (14)	0.39
Strong	0 (0)	5 (22)	0.02
Resection (%)			
Lobectomy	37 (95)	36 (95)	0.99
Segmentectomy	2 (5.1)	2 (5.3)	
R0 (%)[b]	38 (97)	38 (100)	0.15
R1 (%)	1 (3)	0 (0)	0.32
Operative time (skin to skin), min (mean ± SD)	183 ± 40.9	179 ± 54.2	0.71

SD, standard deviation; IQR, interquartile range.
[a]Data were available for analysis in 43 patients
[b]Radicality (R) was assessed following the definition proposed by the International Association of the Study of Lung Cancer (16, 17).

TABLE 3 | Postoperative outcomes and pathological results in the VATS and ROBOT groups of patients.

	Group VATS, *N* = 39	Group ROBOT, *N* = 38	*P*-value
Final pathology report (%)			
Adenocarcinoma	31 (79)	26 (70)	0.43
Squamous cell carcinoma	3 (8)	6 (16)	0.25
Other	5 (13)	5 (14)	0.66
Pathological stage (%)[a]			
IA	20 (58)	24 (69)	0.58
IB	7 (20)	4 (11)	
IIA	0 (0)	1 (3)	
IIB	4 (11)	4 (11)	
IIIA	4 (11)	2 (6)	
Size, mm median (IQR)	21 (14–30)	20 (15–28)	0.42
Number of hilar lymph nodes			
Mean ± SD	4.5 ± 3.6	7.8 ± 4.3	0.0006
Median (IQR)	4 (2–7)	7 (5–10)	0.0003
Number of mediastinal lymph nodes			
Mean ± SD	5.7 ± 3.7	8.1 ± 5.4	0.0001
Median (IQR)	5 (3–7)	7 (5–10)	0.0001
Number of lymph node stations sampled			
Mean ± SD	3.9 ± 1.2	5.2 ± 1.4	0.0001
Median (IQR)	4 (3–5)	6 (4–6)	0.0002
ICU recovery (%)	5 (13)	4 (11)	0.79
ICU stay, days median (IQR)	1 (1–1)	1 (1–1)	0.88
Chest tube duration, days median (IQR)	4 (3–6)	4 (3–6)	0.48
Hospital stay, days median (IQR)	4 (3–6)	5 (4–8)	0.27
Primary outcome[b] (%)	11 (28)	16 (42)	0.24
Conversion to OPEN (%)	2 (5)	3 (8)	0.64
Early post-operative complications (%)	9 (23)	13 (34)	0.28
Complication grade (%)			
I–II	4 (12)	11 (32)	0.04
III	3 (9)	2 (8)	0.85
Most frequent early complication (%)			
Air leak	4 (10)	6 (16)	0.47
Atrial fibrillation	3 (7.7)	4 (11)	0.71
Serous drainage	1 (3)	1 (3)	0.99
Pneumonia	1 (3)	4 (11)	0.16
Pneumothorax	1 (3)	0 (0)	0.32
Atelectasis	1 (3)	3 (8)	0.29
Urinary tract infection	0 (0)	1 (3)	0.31
Other complications	2 (5)	3 (8)	0.62
Follow-up			
Adjuvant therapy[c]	4 (12)	3 (9)	0.69
Chemotherapy	4 (12)	3 (9)	0.69
Radiotherapy	2 (6)	2 (6)	0.99
Readmission (%)	0 (0)	4 (16)	0.08
Later complication (%)	2 (11)	5 (23)	0.33

SD, standard deviation; IQR, interquartile range; ICU, intensive care unit.
[a]Data were available for analysis in 70 patients.
[b]Composite outcome: conversion to open and/or any early postoperative complication.
[c]Data were available for analysis in 68 patients.

group (p = 0.28). Other post-procedural data are shown in **Table 3**.

There was a substantial efficacy improvement in the robot arm for the secondary outcome especially for LN dissection parameters. The *post-hoc* analysis for this secondary outcome showed a power of 99% when comparing the mean number of LN station harvest and 94 and 60% for hilar and mediastinal LNs, respectively. A significant difference was found between the groups when the numbers of LNs and nodal stations harvested were considered. RATS was superior to VATS in terms of hilar (7, IQR 5–10 *vs*. 4, IQR 2–7; p = 0.0003) and mediastinal (7, IQR 5–10 *vs*. 5, IQR 3–7; p = 0.0001) LNs and in terms of nodal stations harvested (6, IQR 4–6 *vs*. 4, IQR 3–5; p = 0.0002).

Overall, the pathological examination showed a higher stage of disease than those predicted by preoperative evaluation in 15 patients; three additional patients were downstaged. Among patients that were upstaged, nine (25.7%) were enrolled in the VATS group and six (17.1%) in the robotic arm (p = 0.56). Nodal upstaging resulted evident in five patients (14.3%) treated by VATS (two from cN0 to pN1 and three from cN0 to pN2) and in four (11.4%) robotic cases (two from cN0 to pN1 and two from cN0 to pN2). No technique was found to be superior in terms of nodal upstaging (p = 0.72).

A univariate association between baseline variables on the primary outcome (perioperative complication including conversions) was performed; former smoker status, duration of smoking, and preoperative forced expiratory volume in the first second were statistically significant and were included in the multivariate analysis along with the randomization group. The logistic regression model showed that only the former smoker status was a statistically significant predictor of the primary outcome (OR 4.6; p = 0.03). However, this result was not further confirmed by a per-protocol analysis, probably due to the sample size (**Supplementary Table S1**).

Data on QoL, pain, and recurrence require a longer follow-up time to have a complete recording and are not reported in this initial analysis. The results of the per-protocol analysis are reported in **Supplementary Tables S1–S3**

DISCUSSION

In their systematic review of perioperative and oncological outcomes of patients undergoing surgical treatment of lung cancer, Azgarian and colleagues advocated the need of a prospective randomized trial to compare open surgery, VATS, and RATS to overcome biased results introduced by selection (12). On the other hand, Korst and Lee considered a randomized study between these approaches useless, as it would be a mere comparison of surgical instrumentation (18). Nevertheless, we believe that, in our present study, the risk of bias due to patient selection and preferences of the surgeon could be limited because all the enrolled individuals were treated in experienced centers offering both VATS and robotic surgery and after completion of the respective learning curves.

Two main results have been obtained by this prospective, multicentric, randomized trial: First, no statistical differences were found between RATS and VATS in terms of conversion rate and postoperative complications. Second, the robotic approach allowed an enhanced lymph node dissection compared to VATS.

Regarding the first objective of the study, the data are in line with previous retrospective nonrandomized trials (7). In a previous analysis by Novellis et al., a superiority of RATS *versus* VATS was reported mainly due to the different level of learning curve when the study was conducted (11). In this study, in order to avoid disparities in surgical experience, we defined a threshold of surgical procedures for each eligible thoracic surgeon with a minimum 30 cases of RATS and/or VATS based on learning curve thresholds previously described for those approaches (3, 19, 20). Despite the number of recruited subjects in the trial being lower than the expected target, the statistical analysis confirmed that the *post-hoc* power analysis based on the preliminary results was adequate to confirm similar outcome and safety of patients treated in the two arms.

This randomized study demonstrates that, for standard lobectomy, experienced surgeons can obtain similar results with both VATS and RATS approaches in terms of safety of the procedure. A prior meta-analysis of 12 retrospective studies showed no significant difference in conversion rate, pneumonia incidence, prolonged air leak, or arrhythmia between the two techniques (21). Swanson et al. performed a multihospital database analysis involving 15,502 patients: they compared wedge resection and lobectomy performed either by RATS or VATS after propensity score matching and found no differences in terms of complications up to 30 days between groups (22). Conversion from RATS to thoracotomy occurs, on average, in 6.7% of cases, with higher rates in left upper lobectomy (17.5%) and overall complication rate accounting for 42% (23, 24). As the probability of concluding in favor of the robot arm was 0% if the observed trend continued, we decided to close the study to new patient entry for "futility reasons", upon the recommendation of the independent data monitoring committee.

Another result of this study relates to one of the secondary outcomes, observing a substantial improvement of efficacy in the RATS arm for the number of hilar LNs and LN stations harvested with a *post-hoc* power analysis of 94 and 99%, respectively. This finding is the first observation in a randomized trial of the superiority in number of hilar LNs and nodal stations ($p = 0.0003$ and $p = 0.0002$) harvested with the robotic approach compared with VATS. The mediastinal LN harvest was also significantly improved by the robotic technique ($p = 0.0001$), but a *post-hoc* analysis of 60% suggests that further investigation is needed.

In recent years, with the advent of minimally invasive surgery for lung cancer, the role of systematic mediastinal and hilar LN dissection has been investigated in depth. In fact, the presence of lymphatic involvement is one of the most impacting factors on the long-term survival of patients receiving surgery for NSCLC (25). In the Italian registry of VATS lobectomy, the number of resected LNs was noted as the only technical predictor of a nearly twofold probability of nodal upstaging in patients with clinical T1–T3, N0 NSCLC (26).

The term to describe the identification of unforeseen LN metastases at postoperative pathologic examination is nodal upstaging, which may be an indirect indicator of the oncological efficacy of the surgical technique. In our study cohort, both thoracoscopic and robotic techniques showed similar rates of nodal upstaging (14.3 *vs*. 11.4%, respectively), without a significant difference at statistical analysis ($p = 0.72$). Moreover, both approaches showed comparable ability to identify unanticipated hilar (N1) or mediastinal (N2) lymph node metastasis, yet there is no consensus on the performance of robotic surgery compared with VATS in terms of nodal upstaging.

In two propensity-matched analyses based on large samples including patients with clinical stage I tumors, contrasting results have been obtained (13, 27). In fact, in the study by Hennon et al. evaluating the impact of surgical approach on nodal upstaging in patients undergoing pulmonary lobectomy, the robotic technique was associated with slightly inferior results compared with VATS (11.2 *vs*. 11.7%, respectively) (27). On the other hand, in the study by Kneuertz and colleagues, robotic surgery had a significantly higher number of nodal upstaging than VATS (16.2 *vs*. 12.3%, $p = 0.03$) (13). According to our results, we cannot conclude about the superiority of one technique in terms of nodal upstaging due to the limited number of events. Future studies specifically designed to address this topic should be recommended in the future.

A large meta-analysis by Zhang et al. showed that VATS lymphadenectomy harvested a lower overall number of lymph nodes compared with patients treated by open thoracotomy, along with the resection of a lower number of N2 lymph nodes (28). According to the authors, such disparity may be caused by VATS surgeons wishing to avoid possible complications during mediastinal dissection.

Another previous retrospective series comparing LN dissection in VATS and RATS had controversial results. In a 2016 retrospective analysis, Toker et al. demonstrated a

superiority of RATS in the number of N1 LNs harvested above station 11. However, no difference was found when N2 or station 10 was considered nor in the number of nodal stations dissected (29). Conversely, a recent meta-analysis involving 20 retrospective studies found no difference in the number of removed LNs (30).

In the present study, we found a median number of seven (IQR 5–10) hilar lymph nodes with RATS and four (IQR 2–7) with VATS ($p = 0.0003$) and seven (IQR 5–10) mediastinal lymph nodes *vs.* five (IQR 3–7) in VATS ($p = 0.0001$). Our data confirm previous retrospective studies, possibly related to the technical benefits of 3D vision and wristed instrumentation of the robotic platform over VATS (11, 15). Compared with VATS, the robotic system offers the possibility of better dissection of lymphatic structures despite the presence of fibrosis and enhanced control of hemostasis and lymphatic leakage (31). In the study by Merritt *et al.*, it was demonstrated that experienced surgeons are able to resect a higher number of overall and N2 lymph nodes by RATS compared with a group of patients treated by VATS (32). Nevertheless, the increased rate of lymph node dissection obtained in the robotic group was not associated to a higher incidence of complications, with particular regard to postoperative air leaks ($p = 0.47$) and serous chest drain ($p = 0.99$), despite a higher number of patients affected by strong pleural adhesions (22%) compared to cases treated by VATS (0%, $p = 0.02$). Moreover, complications occurring in the robotic group required no intervention in most cases (Clavien–Dindo grade I–II, $p = 0.04$). These results were consistent with a recent large meta-analysis by Ma et al. that showed better lymph node assessment, a reduction of 50% of the risk of conversion, and lower overall postoperative complication rate in patients undergoing pulmonary lobectomy by the robotic technique than VATS (33).

The technical advantages of robotic surgery have also been demonstrated for the treatment of locally advanced disease. In 2018, Veronesi et al. presented the results of a multicentric study of patients with stage IIIA disease who underwent robotic lung resection (34). Interestingly, in patients who had undergone preoperative induction therapy, the mean number of LNs harvested during the procedure as well as the rate of conversion to thoracotomy and postoperative complications did not differ from the upfront surgery group.

A growing number of studies have analyzed the oncological efficacy of parenchymal sparing resections in patients with early-stage NSCLC (35). The results of our study gain importance if they also translate to sublobar anatomical resections. It has been recently demonstrated that long-term survival in patients undergoing segmentectomy or lobectomy is still overlapping even in the presence of lymphatic metastases if an appropriate systematic LN dissection is performed, which allows patients to receive adjuvant therapies when nodal metastasis exists (36).

In the study by Zhang *et al.*, two cohorts of patients with early-stage NSCLC, treated by either robotic or VATS anatomical segmentectomy, underwent propensity matching and showed that a significantly higher number of hilar (N1) LNs was harvested in the robotic group (28). Consequently, the robotic system has the potential to improve lymph node dissection, in particular, in peripheral stations, a point that will gain increasing attention if anatomical segmentectomy is demonstrated to be equivalent to lobectomy for stage IA NSCLC. Therefore, dedicated studies on robotic approach for anatomical segmentectomies are required.

This study does have limitations. The trial was closed with a significantly lower number of patients than planned in the design of the study, and no difference between the two arms was demonstrated with regard to the primary outcome (conversion rate and early complications). Additionally, the dropout rate was higher than predicted, probably because in some centers the patients did not undergo preoperative biopsy. Some unavoidable intrinsic characteristics of randomized surgical studies (*i.e.*, operator skills) and of the surgical techniques (*e.g.*, number of ports) could induce additional bias in the interpretation of the results.

Despite these aspects, the analysis did show adequate statistical power with regard to secondary outcomes. This result, however, should be considered with caution in the light of the negative result of primary outcome. In the future, we suggest further studies specifically designed to evaluate the performance of minimally invasive techniques for lymph node dissection and the potential improvement of oncological outcome.

In conclusion, we performed the first randomized trial to evaluate the performance of VATS and RATS in the treatment of patients affected by NSCLC. Despite that RATS was not superior to VATS in perioperative outcomes, the robotic technique showed a better performance in LN dissection, which may have potential implications on its oncological efficacy. Further follow-up will be reported in the future regarding long-term outcomes. Larger studies are needed to confirm our results and to compare the role of robotic approach in patients treated with anatomical segmentectomy for early-stage disease.

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 Ethic Committee of Humanitas Clinical and Research Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GV contributed to conception and design of the study. ED and PM organized the database. GV, AA, EB, AT, CB, SC, LL, MA and PN contributed to data collection. RL and PM performed the statistical analysis. GV and PM wrote the first draft of the manuscript. AA,

PN, GP and RL wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.726408/full#supplementary-material

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Impact of Adjuvant Therapy on Survival in Surgically Resected Limited-Stage Small Cell Lung Cancer

Di Li[1,2,3†], Chaoqiang Deng[1,2,3†], Qiang Zheng[2,3,4†], Fangqiu Fu[1,2,3†], Shengping Wang[2,3,5], Yuan Li[2,3,4], Haiquan Chen[1,2,3] and Yang Zhang[1,2,3]*

[1] Department of Thoracic Surgery and State Key Laboratory of Genetic Engineering, Fudan University Shanghai Cancer Center, Shanghai, China, [2] Institute of Thoracic Oncology, Fudan University, Shanghai, China, [3] Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China, [4] Department of Pathology, Fudan University Shanghai Cancer Center, Shanghai, China, [5] Department of Radiology, Fudan University Shanghai Cancer Center, Shanghai, China

Background: Data on efficacy of adjuvant therapy for surgically resected small cell lung cancer are scant. This study was determined to reveal the survival benefits of different adjuvant treatment modalities for limited-stage small cell lung cancer patients following surgical resection.

Methods: Data of patients with histologically confirmed small cell lung cancer after surgical resection were collected from November 2006 to June 2019. Survival analyses were calculated by Kaplan–Meier method, with log-rank test to evaluate statistical significance. Prognostic factors were identified by multivariate analysis using cox proportional hazards model. Further survival analysis and cox regression analysis stratified by clinicopathologic features were conducted to evaluate the survival benefits of different adjuvant treatment modalities.

Results: In total, 153 out of 157 patients were analyzed. Multivariate analysis showed male sex, lymph node metastasis, residual tumor, VPI and non-adjuvant therapy were independently associated with poor prognosis. Subgroup analyses revealed both adjuvant chemotherapy and adjuvant chemoradiotherapy were significantly associated with superior survival for stage pT2-4 (HR=0.176, 95%CI:0.053-0.578, p=0.004; and HR=0.115, 95%CI:0.033-0.405, p=0.001) and pure SCLC patients (HR=0.182, 95% CI:0.067-0.494, p=0.001; and HR=0.181, 95%CI:0.071-0.465, p<0.001). For pN0 patients, adjuvant chemotherapy was associated with better survival (HR=0.219, 95% CI:0.054-0.891, p=0.034), while adjuvant chemoradiotherapy was associated with improved survival for pN+ patients (HR=0.324, 95%CI:0.138-0.760, p=0.010).

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Christine Garcia, Weill Cornell Medical Center, United States

***Correspondence:**
Yang Zhang
fduzhangyang1987@hotmail.com

[†]These authors have contributed equally to this work

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Conclusions: For patients without pathologic lymph node metastasis, there is a survival benefit with adjuvant chemotherapy. However, for patients with pathologic lymph node metastasis, adjuvant chemoradiotherapy might achieve a significant survival benefit. Further prospective studies are needed to validate the results.

Keywords: small cell lung cancer (SCLC), adjuvant chemotherapy (ACT), adjuvant chemoradiotherapy (ACRT), overall survival (OS), prognosis

INTRODUCTION

Lung cancer accounts for 12% of new cases of cancer worldwide and is the leading cause of cancer-related deaths (1). Small cell lung cancer (SCLC) is increasing by more than 180,000 cases per year, accounting for approximately 15% of all newly diagnosed cases of lung cancer worldwide (2–4). Although rare cases have been reported among non-smokers, almost all SCLC patients are current or former smokers (5, 6). In countries with high smoking rates, such as China, the incidence of SCLC is expected to remain elevated (3). SCLC progresses rapidly, and more than 70% of SCLC patients already have lymph nodes diseases or distant metastases at the time of diagnosis (7).

Platinum-based chemotherapy combined with radiotherapy remains the predominant treatment modality for SCLC patients (8–11). According to the National Comprehensive Cancer Network guidelines, surgical resection is recommended for early-stage SCLC patients (cT1-2N0M0) (9, 11). Nevertheless, due to the highly aggressive nature of SCLC, the number of operable SCLC patients is quite small. Consequently, data on efficacy of adjuvant therapy for resected SCLC are scarce. Previous studies have focused on the survival benefit of patients with SCLC after surgical resection, and the results have shown that patients with surgical resection have favorable survival (12–15). In recent years, there have been several studies concerning the benefits of specific adjuvant treatment modalities (16–22). However, few studies have comprehensively analyzed the benefits of different adjuvant treatment modalities for patients with surgically resected SCLC.

In this study, therefore, we analyzed the survival of SCLC patients with surgical resection and investigated the prognostic factors of these patients. Furthermore, we evaluated the impact of adjuvant chemotherapy and adjuvant chemoradiotherapy on the overall survival stratified by clinicopathologic features.

PATIENTS AND METHODS

Patients

From November 2006 to June 2019, patients who 1) had a preoperative diagnosis of lung cancer, 2) met the indications for surgery, 3) and with histologically confirmed SCLC after surgical resection were collected from the Department of Thoracic Surgery, Fudan University Shanghai Cancer Center (FUSCC), Shanghai, China. A total of 157 patients were enrolled. Following clinicopathologic characteristics were prospectively collected including gender, age, smoking history, ECOG scores, time of surgery, surgery procedures, pathology reports, and postoperative adjuvant therapy regimens. To minimize selection bias, four patients (2.5%) were excluded because they died within 30 days after surgery, as these patients would not have received adjuvant therapy (17). Ultimately, 153 patients were included in the analysis. This study was approved by the Ethics Committee and Institutional Review Boards, and all patients were exempt from an informed consent due to the retrospective nature of the study. Surgical resections were classified as sublobectomy, lobectomy and greater than lobectomy according to the extent of resection. All pathologic sections were re-reviewed by 2 pathologists. The clinical and pathological staging were reevaluated according to the American Joint Committee on Cancer (AJCC) eighth edition TNM and the Veterans Administration Lung Study Group (VALSG) staging systems (23, 24).

Adjuvant Treatment Modalities

Etoposide with cisplatin for 4 to 6 cycles was the predominant adjuvant chemotherapy regimen. For patients with chest radiation, most of them received 50 Gy in 30 days. Patients with prophylactic cranial irradiation (PCI) most frequently received radiation dose of 25 Gy in 10 days. Since all patients in our cohort who underwent chest radiation or PCI had previously received adjuvant chemotherapy and no patients received radiotherapy alone, we divided the patients into non-adjuvant therapy group, adjuvant chemotherapy group and adjuvant chemoradiotherapy group according to the adjuvant treatment modalities. The adjuvant chemoradiotherapy was defined as receiving chemotherapy followed by radiotherapy after surgery, and radiotherapy including chest radiation, PCI or both.

Statistical Analysis

Overall survival (OS) was defined as the time from surgery to death from any cause or last follow-up. The overall survival was analyzed using the Kaplan–Meier method, with the log-rank test performed to evaluate survival variances. Cox proportional hazard model was used to identify the independent prognostic factors of surgically resected SCLC patients. To evaluate the survival benefits of different adjuvant treatment modalities, further survival analysis and multivariate analysis stratified by

Abbreviations: AJCC, the American Joint Committee on Cancer; CI, Confidence interval; FUSCC, Fudan University Shanghai Cancer Center; HR, Hazard ratio; LS-SCLS, Limited-stage small cell lung cancer; LVI, Lymphovascular invasion; OS, Overall survival; PCI, Prophylactic cranial irradiation; RFS, Recurrence-free survival; SCLC, Small cell lung cancer; VALSG, the Veterans Administration Lung Study Group; VATS, Video-assisted thoracic surgery; VPI, Visceral pleural invasion.

clinicopathologic features were conducted. For the multivariate model, all factors with a p<0.05 in the univariate analysis was included. All tests were bilateral, and statistical significance was set at p<0.05. All statistical analysis was performed using IBM SPSS 26.0 (IBM-SPS Inc, Armonk, NY) and RStudio software version 1.3.1093 (RStudio Inc, Boston, Mass).

RESULTS

Patient Characteristic

In total, 153 surgically resected SCLC patients were reviewed (**Table 1**). The majority of these patients are older than 60 (65%), male sex (84%), and ever smokers (76%). Most of them underwent lobectomy by muscle-sparing method. Of all patients, 77 (50%), 59 (39%), 12 (8%), and 5 (3%) patients with stage pT1, pT2, pT3 and pT4, respectively. Postoperative pathologic evaluation revealed 70 (46%) patients without lymph node metastases, while 83 (54%) patients with lymph node metastases. There were 108 (71%) patients with pure SCLC and 45 (29%) patients with combined SCLC. In terms of postoperative treatment modalities, 59 (39%) patients only received adjuvant chemotherapy, 60 (39%) patients received adjuvant radiotherapy after chemotherapy (28 cases of chest radiation, 14 cases of PCI and 18 cases of both chest radiation and PCI), whereas 34 (22%) patients did not undergo any adjuvant therapy after surgery because of preference or the intolerance of side effects. The median follow-up time was 56.6 months (95%CI: 44.43-68.77). Median OS was 60.8 months (95%CI: 32.26-89.33).

Prognostic Factors for Surgically Resected SCLC Patients

Survival analysis showed both adjuvant chemotherapy group (p=0.003) and adjuvant chemoradiotherapy (p<0.001) had superior prognosis compared with non-adjuvant therapy group (**Figure 1**). Five-year survival rate of non-adjuvant therapy group, adjuvant chemotherapy group and adjuvant chemoradiotherapy group were 20.4% (95% CI: 2.2%-38.6%), 57.9% (95% CI: 43.4%-72.4%), and 58.6% (95% CI: 43.9%-73.3%), respectively. After cox multivariate regression analysis, we found both adjuvant chemotherapy (HR=0.303, 95% CI:0.141-0.654, p=0.002) and adjuvant chemoradiotherapy (HR=0.267, 95%CI:0.122-0.583, p=0.001) were significantly associated with better survival compared with non-adjuvant therapy. In addition, multivariate analysis showed male sex, lymph node metastases, residual tumor, and VPI were associated with poor prognosis (**Table 2**).

Impact of Adjuvant Therapy on Overall Survival

To further investigate the impact of adjuvant therapy on survival, we conducted subgroup analyses to assess overall survival stratified by pathologic T stage, lymph node metastasis, and histologic subtypes. For stage pT1 or combined SCLC patients (**Figure 2A** and **Figure 3B**), survival analysis showed overall survival was not significantly different among non-adjuvant therapy, adjuvant chemotherapy, and adjuvant chemoradiotherapy group. However, both adjuvant chemotherapy and adjuvant chemoradiotherapy significantly improved survival for patients with stage pT2-4 (p<0.001 and p<0.001, **Figure 2B**) and pure SCLC (p=0.039 and p<0.001, **Figure 3A**). Meanwhile, survival analysis revealed adjuvant chemotherapy group and adjuvant chemoradiotherapy group had superior survival for pN0 and pN+ subgroups, respectively (p=0.017, **Figure 2C** and p<0.001, **Figure 2D**).

Cox multivariate regression analysis revealed consistent results with survival analysis. After multivariate analysis, both adjuvant chemotherapy and adjuvant chemoradiotherapy were associated with better survival for pT2-4 (HR=0.176, 95% CI:0.053-0.578, p=0.004; and HR=0.115, 95%CI: 0.033-0.405, p=0.001) and pure SCLC patients (HR=0.182, 95%CI: 0.067-0.494, p=0.001; and HR=0.181, 95%CI: 0.071-0.465, p<0.001). For patients with pN0 disease, adjuvant chemotherapy was independently associated with survival (HR=0.219, 95%CI: 0.054-0.891, p=0.034), while adjuvant chemoradiotherapy was

TABLE 1 | Characteristics and adjuvant therapy regimens.

Clinical Characteristic	LS-SCLS (n = 153) (%)
Gender	
Male	129 (84)
Female	24 (16)
Age	
≥60	100 (65)
<60	53 (35)
Smoking history	
Never Smoker	36 (24)
Ever Smoker	117 (76)
Extent of resection	
Lobectomy	110 (72)
>Lobectomy	32 (21)
<Lobectomy	11 (7)
Pathological T stage	
pT1	77 (50)
pT2-4	76 (50)
Pathological N stage	
pN0	70 (46)
pN+	83 (54)
VPI	
Absent	115 (75)
Present	26 (17)
Unknown	12 (8)
LVI	
Absent	71 (46)
Present	64 (42)
Unknown	18 (12)
Residual tumor	
R0	144 (94)
R1	9 (6)
Histology	
Pure	108 (71)
Combined	45 (29)
Postoperative therapy	
Non-adjuvant therapy	34 (22)
Adjuvant chemotherapy	59 (39)
Adjuvant chemoradiotherapy	60 (39)

Values are presented as n (%).
Unknown: Data was not available.
LS-SCLS, Limited-stage small cell lung cancer; VATS, Video-assisted thoracic surgery; VPI, visceral pleural invasion; LVI, lymphovascular invasion.

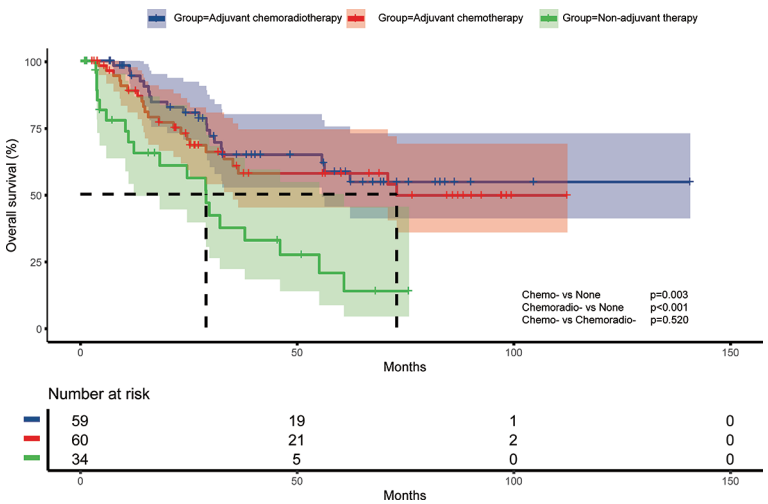

FIGURE 1 | Overall survival of different adjuvant treatment modalities in surgically resected small cell lung cancer patients.

TABLE 2 | *Cox* regression analysis of factors associated with overall survival for surgically resected SCLC patients (n=153).

Variable	Univariate		Multivariate	
	P	HR (95% CI)	*P*	HR (95% CI)
Gender (female *vs* male)	**0.010**	0.264 (0.095-0.730)	**0.003**	0.108 (0.024-0.478)
Age (≥60 *vs* <60)	0.506	1.199 (0.702-2.049)		
Smoking history (never *vs* ever)	**0.027**	0.446 (0.218-0.911)	0.735	0.842 (0.310-2.287)
Operation mode (open *vs* VATS)	0.840	0.930 (0.458-1.887)		
Extent of resection				
Lobe *vs* <Lobe	0.320	2.054 (0.497-8.489)		
>Lobe *vs* <Lobe	0.105	3.356 (0.775-14.536)		
Pathological T stage (pT2-4 *vs* pT1)	**0.031**	1.740 (1.050-2.883)	0.840	1.081 (0.507-2.304)
Pathological N stage (pN+ *vs* pN0)	**<0.001**	2.767 (1.591-4.812)	**<0.001**	3.256 (1.709-6.204)
LVI (present *vs* absent)	**0.002**	2.583 (1.426-4.680)	0.128	1.765 (0.850-3.668)
VPI (present *vs* absent)	**<0.001**	2.986 (1.613-5.526)	**<0.001**	3.730 (1.803-7.718)
Residual tumor (R1 *vs* R0)	**0.022**	2.696 (1.156-6.290)	**0.015**	5.077 (1.369-18.836)
Histologic subtype (combined *vs* pure)	0.628	0.868 (0.490-1.537)		
Adjuvant therapy				
Chemotherapy *vs* None	**0.004**	0.400 (0.215-0.743)	**0.002**	0.303 (0.141-0.654)
Chemoradiotherapy *vs* None	**0.001**	0.328 (0.175-0.616)	**0.001**	0.267 (0.122-0.583)

VATS, Video-assisted thoracic surgery; VPI, visceral pleural invasion; LVI, lymphovascular invasion.
P < 0.05 is indicated by bold.

associated with improved survival for patients with pN+ disease (HR=0.324, 95%CI:0.138-0.760, p=0.010) (**Table 3**).

DISCUSSION

Up to now, the treatment modality of SCLC is still dominated by platinum-based chemotherapy combined with radiotherapy (8–11). For early-stage SCLC, surgical resection is recommended (8, 11). In our clinical work, we found a proportion of relatively advanced SCLC patients who underwent surgery incidentally had good survival after adjuvant therapy. Thus, based on a retrospective analysis of a relatively large population, we comprehensively investigated the impact of adjuvant therapy on the survival of surgically resected SCLC patients with pI-III stage, and corroborated the survival benefit of adjuvant chemotherapy and adjuvant chemoradiotherapy for these patients. Furthermore, by means of subgroup analyses, we found other interesting results. Both adjuvant chemotherapy and adjuvant chemoradiotherapy were independently associated with superior survival for pT2-4 and pure SCLC patients. For patients without pathologic lymph node metastasis, adjuvant chemotherapy was independently associated with better survival, while adjuvant chemoradiotherapy was significantly associated with improved survival for patients with pathologic lymph node metastasis.

Previous studies have separately evaluated the efficacy of different adjuvant treatment modalities (17–21, 25, 26). Yao et al. found that SCLC patients with stage pN0 and pN1 who

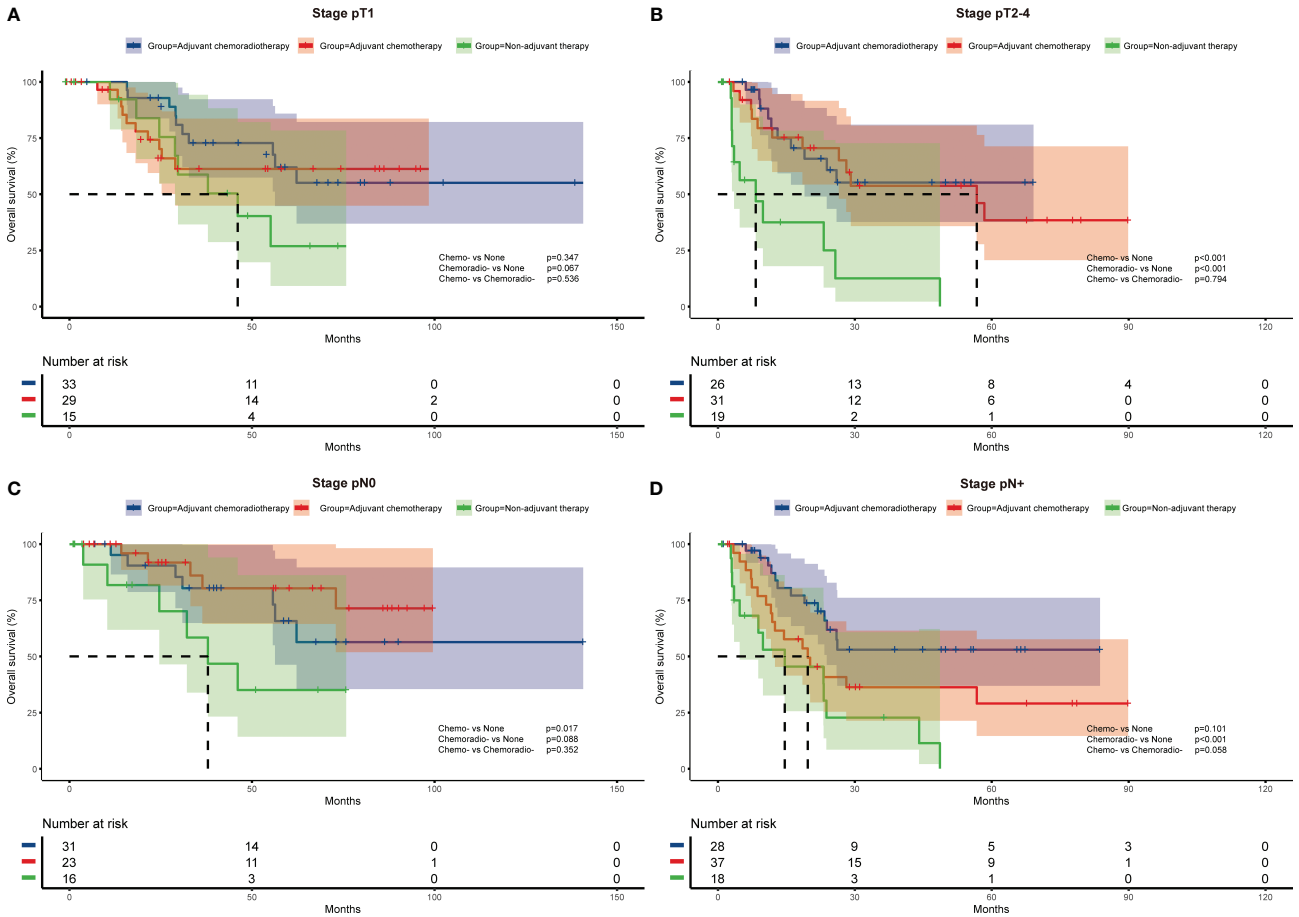

FIGURE 2 | Overall survival of different adjuvant treatment modalities in patients with specific pathologic T stages **(A, B)** and N stages **(C, D)**.

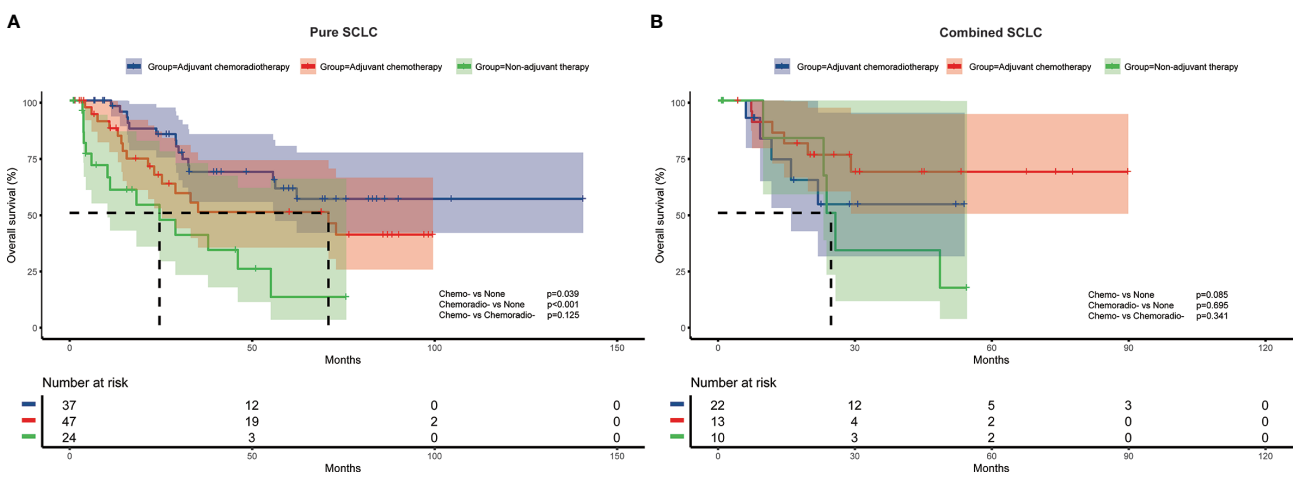

FIGURE 3 | Overall survival of different adjuvant treatment modalities in patients with pure SCLC **(A)** and combined SCLC **(B)**.

TABLE 3 | Multivariate analysis of factors associated with overall survival for surgically resected SCLC patients with pT2-4 (n=76), histologic pure SCLC (n=108), pN0 disease (n=70) and pN+ disease (n=83).

Variable	pT2-4		pure SCLC		pN0		pN+	
	P	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)
Gender (female *vs* male)			0.137	0.323 (0.073-1.430)			0.065	0.314 (0.092-1.074)
Smoking history (never *vs* ever)			0.704	0.823 (0.301-2.250)				
Pathological T stage (pT2-4 *vs* pT1)			0.147	1.715 (0.827-3.554)				
Pathological N stage (pN+ *vs* pN0)	**0.019**	3.218 (1.207-8.581)	0.246	1.645 (0.709-3.814)				
LVI (present *vs* absent)	0.120	2.346 (0.800-6.878)	**0.002**	3.594 (1.575-8.205)				
VPI (present *vs* absent)	0.084	2.210 (0.900-5.425)			**0.048**	4.149 (1.012-17.013)	**0.001**	3.756 (1.711-8.248)
Residual tumor (R1 *vs* R0)	0.877	0.820 (0.066-10.137)					**0.005**	6.607 (1.749-24.951)
Histologic subtype (combined *vs* pure)					0.085	0.253 (0.053-1.212)		
Adjuvant therapy								
Chemotherapy *vs* None	**0.004**	0.176 (0.053-0.578)	**0.001**	0.182 (0.067-0.494)	**0.034**	0.219 (0.054-0.891)	0.261	0.636 (0.289-1.400)
Radio-chemotherapy *vs* None	**0.001**	0.115 (0.033-0.405)	**<0.001**	0.181 (0.071-0.465)	0.149	0.411 (0.123-1.375)	**0.010**	0.324 (0.138-0.760)

VATS, Video-assisted thoracic surgery; VPI, visceral pleural invasion; LVI, lymphovascular invasion. P < 0.05 is indicated by bold.

received adjuvant chemotherapy after surgery had a longer overall survival compared to surgery alone (HR=0.57, 95%CI: 0.36-0.91, p= 0.019) (19). Yang and colleagues found that among patients with early-stage (pT1-2N0M0) SCLC after surgery resection, those who received adjuvant chemotherapy had better survival compared with non-adjuvant therapy (HR=0.78, 95%CI: 0.63-0.95, p=0.02) (17). Wakeam E et al. demonstrated a survival benefit of adjuvant radiotherapy for both pN1 (HR=0.79, 95% CI: 0.62-1.00, p=0.05) and pN2 (HR=0.60, 95% CI: 0.48-0.75, p<0.001) diseases, whereas there was no survival benefit for pN0 disease (HR=1.05, 95%CI: 0.83-1.34, p=0.68) (21). In the study by Xu and colleagues, PCI had a survival benefit for resected SCLC patients with stage pII (HR=0.54, 95% CI:0.30-0.99, p=0.047) and pIII (HR=0.54, 95% CI:0.34-0.86, p=0.009), but not for stage pI patients (HR=1.61, 95% CI:0.68-3.83, p=0.282) (18). These studies focused on the impact of the specific adjuvant treatment modalities on the survival at specific pathologic stages. In our cohort, 119 out of 153 patients underwent adjuvant chemotherapy. Of these patients, 59 patients only received adjuvant chemotherapy and 60 patients received adjuvant radiotherapy after chemotherapy. Using *Cox* multivariate regression analysis, we found both adjuvant chemotherapy (HR=0.303, 95%CI: 0.141-0.654, p=0.002) and adjuvant chemoradiotherapy (HR=0.267, 95%CI: 0.122-0.583, p=0.001) significantly improved the survival of SCLC patients after surgical resection. This mutually corroborates with the results of several previous studies. In addition, we found male sex, lymph node metastases, residual tumor, and VPI were associated with poor prognosis.

Previous studies suggested that adjuvant chemotherapy has a survival benefit in relatively early-stage patients (17, 19), and chest radiation and PCI tend to have a survival benefit in relatively advanced patients (18, 21, 22). In a previous study, Nicolas Zhou et al. performed a similar grouping, classifying patients into two subgroups, pN0 and pN+, to investigate the effects of adjuvant chemotherapy, PORT, and PCI on RFS in both groups, respectively, and multivariate analysis found that adjuvant chemotherapy significantly prolonged RFS in both groups (HR=0.49, 95%CI: 0.27-0.91, p=0.024 and HR=0.41, 95%CI: 0.18-0.94, p=0.035, respectively) (20). To further investigate the impact of different adjuvant treatment modalities on survival, we conducted subgroup analyses to assess overall survival stratified by pathologic T stage, lymph node metastasis, and histologic subtypes. Survival analysis showed both adjuvant chemotherapy and adjuvant chemoradiotherapy significantly improved survival for patients with stage pT2-4 and pure SCLC. Meanwhile, adjuvant chemotherapy group and adjuvant chemoradiotherapy group had superior survival for pN0 and pN+ subgroups, respectively. Multivariate analysis revealed consistent results with survival analysis. There was no statistically significant survival benefit of adjuvant therapy for patients with tumor diameters less than 3 cm (pT1) compared to greater than 3 cm (pT2-4), which suggested that the role of adjuvant therapy was limited in very early-stage SCLC. Due to its highly aggressive character, SCLC is more sensitive to adjuvant therapy than NSCLC. Therefore, for pure SCLC, there was a survival benefit of adjuvant therapy. In contrast, for combined SCLC with non-small cell component, the effect of adjuvant therapy was less pronounced. In terms of the results of lymph node metastasis status subgroup analysis, we speculate that SCLC is a very aggressive disease with the potential for metastasis in early stage, therefore, even for patients with R0 resection and pN0 disease, systemic chemotherapy is needed. Meanwhile, patients with pN+ disease have a higher degree of disease progression and are more probable to have local spread and distant metastases than patients with pN0 disease. Thus, pN+ patients are more likely to have a survival benefit from additional chest radiation around the resected lesion or PCI on the basis of adjuvant chemotherapy. Hence, the status of lymph node metastases has a significant impact on the selection of adjuvant treatment options for postoperative patients with SCLC.

This study has several limitations. As a single-center retrospective study, selection and institutional bias are inevitable. In our clinical work, to expand the study population, in addition to patients with early limited-stage SCLC (cT1-2N0M0), we also included patients who had a diagnosis of lung cancer before surgery but not suspected of SCLC and underwent surgery incidentally. This is both a strength and a weakness of our study. On the one hand, it allows our study to assist in the selection of adjuvant therapy for patients with postoperative

proven advanced SCLC. On the other hand, it also affects the external veracity of this study to some extent and should be noted when generalizing the findings of this study. In addition, 34 patients in our cohort did not undergo any adjuvant therapy after surgery because of preference or the intolerance of side effects, and survival in this group might have been inherently poor, so there may be some bias. To try to address this issue, we further collated the patients' ECOG scores and compared them between groups receiving different adjuvant treatments and did not find any statistical difference among the groups (**Supplementary Table 1**). This may have avoided this bias to some extent. There were 11 patients with sublobectomy and 9 patients with residual tumor (R1), which may affect the results.

In conclusion, based on the analysis of 153 patients with SCLC after surgical resection, we investigated the effect of different adjuvant treatment modalities. There were survival benefits for adjuvant therapy, with a significant benefit in pT2-4 and pure SCLC patients compared to pT1 and combined SCLC patients, respectively. More importantly, for patients without pathologic lymph node metastasis, there was a survival benefit with adjuvant chemotherapy. However, for patients with pathologic lymph node metastasis, adjuvant chemoradiotherapy was required to achieve a survival benefit. Further prospective studies are needed to validate the results.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee and Institutional Review Board of Fudan University Shanghai Cancer Center. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

DL: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Visualization; Writing – original draft; Writing – review & editing. CD: Conceptualization; Data curation; Methodology; Software; Validation; Visualization; Writing – original draft; Writing – review & editing. QZ: Data curation; Investigation; Supervision; Validation; Writing – review & editing. FF: Conceptualization; Formal analysis; Methodology; Supervision; Validation; Writing – review & editing. SW: Investigation; Project administration; Supervision; Writing – review & editing. YL: Data curation; Investigation; Project administration; Supervision; Writing – review & editing. HC: Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing – review & editing. YZ: Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Visualization; Writing – review & editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.704517/full#supplementary-material

Supplementary Table 1 | ECOG scores of patients with non-adjuvant therapy, adjuvant chemotherapy and adjuvant chemoradiotherapy.

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Factors That Improve Chest Computed Tomography-Defined Sarcopenia Prognosis in Advanced Non-Small Cell Lung Cancer

Ming Yang[1,2,3*†], *Lingling Tan*[1†], *Lingling Xie*[4†], *Song Hu*[5], *Dan Liu*[6], *Jing Wang*[4] *and Weimin Li*[7,8,9*]

[1] The Center of Gerontology and Geriatrics, West China Hospital, Sichuan University, Chengdu, China, [2] Precision Medicine Research Center, West China Hospital, Sichuan University, Chengdu, China, [3] National Clinical Research Center for Geriatrics (WCH), West China Hospital, Sichuan University, Chengdu, China, [4] West China School of Nursing, West China Hospital, Sichuan University/ Department of Oncology, Shangjin Nanfu Hospital, Sichuan University, Chengdu, China, [5] Department of Radiology, Shangjin Nanfu Hospital, Sichuan University, Chengdu, China, [6] Population Health Sciences, German Center for Neurodegenerative Diseases (DZNE), Bonn, Germany, [7] Department of Respiratory and Critical Care Medicine, West China Hospital, Sichuan University, Chengdu, China, [8] Institute of Respiratory Health, Frontiers Science Center for Disease-related Molecular Network, West China Hospital, Sichuan University, Chengdu, China, [9] The Research Units of West China, Chinese Academy of Medical Sciences, Chengdu, China

Background: Whether muscle strength and physical performance should be components of sarcopenia remains controversial. This study evaluated the skeletal muscle index derived from computed tomography images at the 12th thoracic vertebra level (T12 SMI), handgrip strength, performance status, and their combination for predicting overall survival in patients with advanced non-small cell lung cancer.

Methods: Chest computed tomography, handgrip strength measurement, and bioelectrical impedance analysis were performed. Sarcopenia was defined based on the T12 SMI alone or the T12 SMI, handgrip, and/or physical performance (i.e. Asian Working Group for Sarcopenia [AWGS]-defined sarcopenia or severe sarcopenia).

Results: Overall, 639 participants were included; 488 (76.4%) died. At baseline, 160 (25.0%), 141 (22.1%), and 42 (6.6%) patients had computed tomography-defined sarcopenia, AWGS-defined sarcopenia, and AWGS-defined severe sarcopenia, respectively. Chest computed tomography-defined sarcopenia (hazard ratio [HR], 2.00; 95% confidence interval [CI], 1.65-2.43), AWGS-defined sarcopenia (HR, 2.00; 95% CI, 1.59-2.49), and AWGS-defined severe sarcopenia (HR, 3.01; 95% CI, 2.21-4.09) were more strongly associated with poor prognosis than a performance status score ≥2 (HR, 1.37; 95% CI, 1.10-1.73).

Conclusions: Adding handgrip strength and the performance status score to chest computed tomography-defined sarcopenia improved its prognostic ability. Oncological sarcopenia research should focus on muscle mass, strength, and function.

Keywords: muscle wasting, muscle depletion, image segmentation, lung cancer, prognosis

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***Correspondence:**
Weimin Li
weimin003@163.com
Ming Yang
yangmier@gmail.com

†These authors have contributed equally to this work

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INTRODUCTION

Sarcopenia is a skeletal muscle disorder characterized by a progressive and generalized loss of muscle quantity and quality (1). Sarcopenia is currently regarded as the hallmark of cancer cachexia (2) and has been confirmed to be a prognostic factor for poor outcomes in numerous malignancies (3–5). Although sarcopenia has become a major factor in the fields of oncological and geriatric research, several fundamental questions regarding sarcopenia remain unanswered (1).

The first and foremost question concerns the definition of sarcopenia (6). There currently exist two major opinions about the definition of sarcopenia (7). In the fields of oncology and surgery, most researchers use the term 'sarcopenia' to refer to low muscle mass without any measurement of muscle strength or function (7, 8). For instance, the recently published North American Expert Opinion Statement on Sarcopenia in Liver Transplantation recommends defining sarcopenia 'using only muscle mass' (9). Conversely, in the fields of geriatric and internal medicine, there is a consensus that sarcopenia should be defined based on low muscle mass, low muscle strength, and/or low physical performance (10, 11). Consequently, this knowledge gap regarding the definition of sarcopenia has hindered interdisciplinary cooperation (7).

Computed tomography (CT) represents one of the gold standard methods for skeletal muscle mass (SMM) measurement (12). The skeletal muscle area (SMA) on a single-slice CT image at the third lumbar vertebra (L3) level is the most widely used indicator in the literature (13) and is conventionally regarded as the *de facto* gold standard in CT body composition assessment (7, 12). Nevertheless, in clinical practice, abdominopelvic CT scans are less frequently performed than chest CT scans, particularly in patients with lung cancer. Furthermore, several studies on lung cancer had to exclude up to one-third of patients because of missing data from CT images at the L3 level (14, 15). Therefore, the identification of an SMM marker based on chest CT in patients with lung cancer would certainly benefit sarcopenia research (16, 17). SMA at the 12^{th} thoracic vertebra level (T12 SMA) has recently been shown to be a novel SMM marker representing whole-body SMM (18). Additionally, the T12 skeletal muscle index (T12 SMI) (i.e., T12 SMA/body height squared) and L3 SMI reportedly have a similar predictive value for 1-year mortality in older patients with trauma (19).

The present prospective cohort study aimed to answer the following three questions: (1) Is the T12 SMI a valid surrogate marker of whole-body SMM or trunk SMM in patients with advanced non-small cell lung cancer (NSCLC)? (2) Is chest CT-defined sarcopenia based on the T12 SMI associated with poor prognosis in this patient population? (3) Would the addition of handgrip strength and physical performance to chest CT-defined sarcopenia improve its predictive value for poor prognosis in this patient population?

MATERIALS AND METHODS

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki, and the study protocol was approved by the Biomedical Ethics Committee of West China Hospital, Sichuan University. All participants of this study provided signed informed consent.

Patients

Adult patients diagnosed with advanced NSCLC at the Department of Oncology of Shangjin Nanfu Hospital, Sichuan University from August 2017 to May 2019 were prospectively and consecutively recruited. Adult patients who met the following criteria were included in the study: (1) pathologically confirmed stage IIIB or IV NSCLC based on the Union for International Cancer Control's tumor–node–metastasis stage classification and (2) administration of first-line chemotherapy for the first time. However, patients (1) who received immune checkpoint inhibitor therapy, molecular targeted therapy, radiotherapy, or single-agent chemotherapy; (2) who had an implanted pacemaker; (3) who had a history of any other cancer type; (4) who had low-quality CT images or had any anatomical distortion (e.g. chest wall edema) or loss of any muscle mass area on CT images; and (5) who had visible edema were excluded from analysis.

Clinical and Anthropometric Variables

The following clinical variables were recorded by trained nurses within 48 hours upon first admission to our department: age, sex, smoking status (never smoker or ever smoker), histologic type (adenocarcinoma, squamous cell carcinoma, or large cell carcinoma), cancer stage (stage IIIB or IV), Eastern Cooperative Oncology Group (ECOG) performance status (PS) score, number of chemotherapy courses, and chemotherapy regimens. Additionally, serum creatinine, serum albumin, and hemoglobin levels were measured within 24 hours after admission. The updated version of the Charlson comorbidity index (CCI) was used to evaluate the number and severity of important comorbidities, including chronic obstructive pulmonary disease, renal disease, any malignancy, and cerebrovascular disease (20). Data on comorbidities were directly collected from original medical records. The total CCI score is 24 points, whereas the score for 'any malignancy' is 2 points (20). Hence, a CCI score ≥3 indicated that a patient not only had NSCLC but also at least one other important comorbidity. Body height and weight were measured using standard methods. Body mass index (BMI) was calculated as body weight (kg) divided by body height squared (m^2) and was categorized as follows: underweight, <20.0 kg/m^2; normal, 20.0-24.9 kg/m^2; and obese, ≥25 kg/m^2 (4).

CT Image Analysis

Chest CT scans were completed within 48 hours after admission for each participant using a 16-slice spiral CT scanner (Brilliance; Philips Healthcare, OH, USA) with a 5-mm slice thickness. Acquisition parameters were as follows: 100-140 kV, variable mA based on the patient's body size, and detector collimation of 0.75-1.5 mm. Unenhanced cross-sectional CT images at the T12 level were analyzed using a dedicated segmentation software (Mimics version 21.0; Materialise, Leuven, Belgium) to evaluate the T12 SMA.

On a single CT image, all visualized skeletal muscles with a threshold of −29 to +150 HU, including the erector spinae,

latissimus dorsi, rectus abdominis, obliquus externus, internus abdominis, and internal and external intercostal muscles, were segmented (12). The T12 SMI (cm^2/m^2) was calculated as the T12 SMA (cm^2) divided by the body height squared (m^2).

A trained observer (L.T.) who was blinded to patient outcomes during the analysis period segmented all CT images. To test the reliability of the T12 SMA determined by CT, a total of 30 participants were randomly selected from the cohort. To assess inter-observer reproducibility, another trained observer (S.H.) subsequently segmented the CT images again. Representative images are presented in **eFigure 1**.

Bioelectrical Impedance Analysis (BIA) of Body Composition

On the day of the CT scan, trained nurses utilized a segmental multifrequency BIA device (InBody 770; Biospace, Korea) to measure each participant's body composition, including the total lean body mass (LBM), trunk LBM, and appendicular LBM.

Handgrip Strength Measurement

On the day of the CT scan, trained nurses also measured the handgrip strength to the nearest 0.1 kg using a digital grip dynamometer (EH101; Xiangshan Inc., Guangdong, China) in accordance with the recommendation of the Chinese National Physical Fitness Evaluation Standard (21). Three readings were obtained for each hand, and the highest value was recorded for analysis. Handgrip weakness was defined as handgrip strength <28 kg for men and <18 kg for women (11).

Different Definitions of Sarcopenia

Sarcopenia was defined in the present study as CT-defined sarcopenia and Asian Working Group for Sarcopenia (AWGS)-defined sarcopenia.

(1) CT-defined sarcopenia (based on low SMM estimated by CT image analysis): As there currently exists no established cut-off value of the T12 SMI for determining low SMM, we set the optimal cut-off value for low SMM to predict overall survival (OS) as the diagnostic cut-off points for CT-defined sarcopenia using the maximally selected rank statistics method, as described by Lausen and Schumacher (22). This is a validated method for determining cut-off points that could optimally separate participants with respect to time to an event outcome (4, 23).

(2) AWGS-defined sarcopenia: According to the AWGS 2019 recommendation (11), patients with both low SMM and handgrip weakness (or low physical performance) are considered to have AWGS-defined sarcopenia, whereas patients with low SMM, handgrip weakness, and low physical performance are deemed to have AWGS-defined severe sarcopenia. The PS score is commonly used in routine clinical practice, with a PS score of 0-1 indicating good physical performance (24). In this study, low physical performance was defined as an ECOG PS score ≥2 points.

Measurement of OS

OS was defined as the number of months elapsed from the date of initial recruitment to the date of death or last follow-up for each patient. Patients were followed up until their death or up to the last week of August 2020, at which time they were confirmed to be alive *via* telephone review and were subsequently censored.

Statistical Analysis

Data analysis was conducted between October 3, 2020, and October 20, 2020. Histograms and the Shapiro–Wilk test were used to assess the distributions of continuous variables. All continuous variables were of normal distribution. Thus, continuous and categorical variables are expressed as mean (standard deviation) and number (percentage), respectively. Inter-observer validation of T12 SMI measurements was performed using interclass correlation coefficient analysis. Pearson's correlation coefficient (r) and scatterplots with a linear regression model were employed to assess the association of the T12 SMI with total LBM, trunk LBM, appendicular LBM, BMI, handgrip strength, and the T12 SMA. Hazard ratios (HRs) of the T12 SMI and handgrip strength for predicting OS were evaluated using a Cox proportional hazards regression model with a restricted cubic spline function with three knots for men and women. Subsequently, we determined the cut-off values of the T12 SMI using the maximally selected rank statistics method (22). Using these cut-off values and the diagnostic criteria mentioned above, we defined patients with or without CT-defined sarcopenia and patients with or without AWGS-defined sarcopenia. Group differences were analyzed using one-way analysis of variance and chi-square test (or Fisher's exact test), as appropriate.

OS curves for different groups were constructed using the Kaplan–Meier method and compared using the log-rank test. Additionally, univariate and multivariable analyses of OS were performed using Cox proportional hazards models, with the results presented as HRs with 95% confidence intervals (CI). Multivariable Cox Models 1, 2, and 3 evaluated the predictive value of CT-defined sarcopenia, AWGS-defined sarcopenia (as well as AWGS-defined severe sarcopenia), and physical performance, respectively, for predicting OS. In line with previous scientific literature (23, 25), the models were adjusted for age at baseline, sex, smoking status, histologic type, cancer stage, CCI, BMI groups, chemotherapy regimens, completion of at least four chemotherapy courses, and serum creatinine, serum albumin, and hemoglobin levels. Moreover, C-indexes were used to assess the discrimination performance of these models to predict OS (25), with c statistics of 0.5 indicating chance; 0.5-0.7, poor discrimination; 0.7-0.8, acceptable discrimination; 0.8-0.9, excellent discrimination; 0.9-0.99, outstanding discrimination; and 1.0, perfect prediction (23).

To assess the robustness of the results, a sensitivity analysis was conducted using the lowest quartile of the sex-specified T12 SMI to define low SMM, which was another widely employed method in previous studies (4). Subsequently, we accordingly redetermined CT-defined sarcopenia, AWGS-defined sarcopenia, and AWGS-defined severe sarcopenia, reperformed the univariate and multivariable analyses with the Cox proportional hazards models, and redrew the Kaplan–Meier curves.

Additionally, *a priori* subgroup analyses of multivariable Cox Models 1 and 2 were conducted according to age groups

(<60 or ≥60 years), sex, cancer stage, histologic type, physical performance (0-1 points or 2 points), and number of chemotherapy courses (1-3 courses or ≥4 courses).

Statistical analyses were performed using R software version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria) and SPSS software version 26.0 (IBM Corp., Armonk, NY, USA). *P* values <.05 were considered to indicate statistical significance.

RESULTS

Patient Characteristics

A total of 787 consecutive patients with advanced NSCLC were admitted to our department from August 2017 to May 2019. Of these patients, 82 refused to participate in this study. Furthermore, 15 patients with chest wall edema, 5 patients with low-quality CT images, 16 patients with missing data on handgrip strength, and 30 patients who received molecular targeted therapy were excluded. Accordingly, we included a total of 639 patients (410 men and 229 women) in the study. The median follow-up was 25 months (range: 15-36 months).

The mean age of the participants was 58.6 ± 8.9 years, with men being significantly older than women (mean age: 59.1 *vs*. 57.7 years, *P*=.049). The baseline characteristics of participants according to sex are summarized in **Table 1**. Overall, 294 (46%) and 345 (54.0%) patients had stage IIIB NSCLC and stage IV NSCLC, respectively. Because the histologic type, chemotherapy regimens, handgrip strength, and body composition variables were significantly different between men and women, we either adjusted for sex or split the data based on sex in the following analyses.

Association Between the T12 SMI, LBM, Handgrip Strength, and Physical Performance

T12 SMI measurements were highly reproducible between observers (**eFigure 2** in the **Supplement**). Considering that the T12 SMI is not a classical surrogate of muscle mass, we analyzed the association of the T12 SMI with BIA-derived LBM, handgrip strength, and physical performance. As shown in **eFigure 3** in the **Supplement**, the T12 SMI was highly correlated with trunk LBM and handgrip strength (*r*=0.78, *P*<.001 and *r*=0.70, *P*<.001, respectively) and was moderately correlated with total LBM (*r*=0.57, *P*<.001). Scatterplots with linear regression for these variables are presented in **eFigures 4A–E** in the **Supplement**. Moreover, both male and female patients with low physical performance (PS score ≥2) had a significantly lower T12 SMI (**eFigure 4F** in the **Supplement**).

Impact of the T12 SMI and Handgrip Strength on OS

The T12 SMI was a significant factor for OS in men (*P*<.001, **Figure 1A**) and women (*P*=.005, **Figure 1B**). Handgrip strength was also a significant factor for OS in men (*P*<.001, **Figure 1C**). Furthermore, lower handgrip strength exhibited a tendency toward poor prognosis in women (*P*=.068, **Figure 1D**).

Prevalence of Sarcopenia in Participants

Based on maximally selected rank statistics calculation, we set the cut-off values of the T12 SMI as 32.48 cm^2/m^2 for men and 27.82 cm^2/m^2 for women (**Figure 2**). At baseline, 160 (25.0%), 141 (22.1%), and 42 (6.6%) patients had CT-defined sarcopenia, AWGS-defined sarcopenia, and AWGS-defined severe sarcopenia, respectively. The prevalence of CT-defined sarcopenia, AWGS-defined sarcopenia, and AWGS-defined severe sarcopenia were not significantly different between men and women (**Table 1**). The clinicopathological characteristics of participants according to CT-defined sarcopenia or AWGS-defined sarcopenia are summarized in **eTable 1** in the **Supplement**.

Impact of CT-Defined Sarcopenia, Handgrip Weakness, Poor Physical Performance, and AWGS-Defined Sarcopenia on OS

A total of 488 (76.4%) patients died during the study period. Patients with CT-defined sarcopenia had a shorter OS than patients without CT-defined sarcopenia (**Figure 3A**, log-rank *P*<.001). Similarly, patients exhibiting handgrip weakness had a shorter OS than those with normal handgrip strength (**Figure 3B**, log-rank *P*<.001). Patients with low physical performance had a shorter OS than those with normal physical performance (**Figure 3C**, log-rank *P*<.001). Moreover, patients with AWGS-defined sarcopenia had a shorter OS than those without AWGS-defined sarcopenia, whereas patients with AWGS-defined severe sarcopenia had the worst prognosis (**Figure 3D**, log-rank *P*<.001).

Univariate and multivariable analyses with Cox proportional hazards models were performed to identify independent factors associated with OS (**Table 2**). After adjustment for the same confounders, CT-defined sarcopenia (Model 1: HR, 2.00; 95% CI, 1.65-2.43) and AWGS-defined sarcopenia (Model 2: HR, 2.00; 95% CI, 1.59-2.49) were associated with poor prognosis. AWGS-defined severe sarcopenia indicated a higher risk of poor prognosis (Model 2: HR, 3.01; 95% CI, 2.21-4.09). All these indicators were more strongly associated with poor prognosis than low physical performance (PS score ≥2) (Model 3: HR, 1.37; 95% CI, 1.10-1.73).

The c statistics of the multivariable Cox Models were compared (**Table 2**). The c statistics were 0.72 (95% CI, 0.69-0.74) for Model 1 and 0.76 (95% CI, 0.75-0.78) for Model 2, indicating moderate discrimination for OS. Model 2 was better than Model 1 in discriminating OS. The c statistic for Model 3 was 0.69 (95% CI, 0.67-0.72), indicating poor discrimination for OS.

Sensitivity Analysis

To examine the robustness of our results, we performed an *a priori* sensitivity analysis by defining low SMM as the lowest quartile of the sex-specified study population and subsequently redetermined the proportions of CT-defined sarcopenia, AWGS-defined sarcopenia, and AWGS-defined severe sarcopenia accordingly. Afterwards, we reperformed univariate and multivariable analyses with Cox proportional hazards models

TABLE 1 | Baseline characteristics of the study population according to sex.

Characteristics	Total (*n*=639)	Men (*n*=410)	Women (*n*=229)	*P* value[a]
Age, years, mean (SD)	58.6 (8.9)	59.1 (8.4)	57.7 (9.8)	.049
Age ≥60 years, *n* (%)	196 (30.7)	119 (29.0)	77 (33.6)	.227
Smoking status, *n* (%)				
Never smoker	311 (48.7)	97 (23.7)	214 (93.4)	<.001
Ever smoker	328 (51.3)	313 (76.3)	15 (6.6)	
Histologic type, *n* (%)				
Adenocarcinoma	394 (61.7)	213 (52.0)	181 (79.0)	<.001
Squamous cell carcinoma	201 (31.5)	173 (42.2)	28 (12.2)	
Large cell carcinoma	44 (6.9)	23 (5.9)	20 (8.7)	
Cancer stage, *n* (%)				
Stage IIIB	294 (46.0)	194 (47.3)	100 (43.7)	.375
Stage IV	345 (54.0)	216 (52.7)	129 (56.3)	
ECOG PS, *n* (%)				
0	385 (60.3)	249 (60.7)	126 (59.4)	.664
1	118 (18.5)	78 (19.0)	40 (17.5)	
≥ 2	136 (21.3)	93 (20.2)	53 (23.1)	
Body height, cm, mean (SD)	162.3 (7.7)	165.9 (6.0)	156.0 (6.3)	<.001
Body weight, kg, mean (SD)	60.9 (9.5)	63.4 (9.5)	56.6 (7.9)	<.001
BMI, kg/m^2, mean (SD)	23.1 (3.1)	23.0 (3.0)	23.3 (3.2)	.264
BMI groups, *n* (%)				
Underweight (BMI <20)	383 (59.9)	66 (16.1)	29 (12.7)	.460
Normal weight (BMI 20-24.9)	95 (14.9)	143 (62.4)	143 (62.4)	
Obese (BMI ≥25)	161 (25.2)	104 (25.4)	57 (24.9)	
Charlson comorbidity index ≥3, *n* (%)	196 (30.7)	124 (30.2)	72 (31.4)	.753
Chemotherapy regimen, *n* (%)				
Pemetrexed + carboplatin/cisplatin	239 (37.4)	138 (33.7)	101 (44.1)	<.001
Docetaxel + carboplatin/cisplatin	234 (36.6)	144 (35.1)	90 (39.3)	
Gemcitabine + carboplatin/cisplatin	30 (4.7)	21 (5.1)	9 (3.9)	
Paclitaxel + carboplatin/cisplatin	136 (21.3)	107 (26.1)	29 (12.7)	
Patients who completed at least four chemotherapy courses, *n* (%)	498 (77.9)	325 (79.3)	173 (75.5)	.277
Serum creatinine, μmol/L, mean (SD)	72.2 (16.4)	77.5 (16.0)	62.6 (12.6)	<.001
Serum albumin, g/L, mean (SD)	41.9 (2.5)	42.3 (2.5)	41.3 (2.5)	<.001
Hemoglobin, g/L, mean (SD)	125.4 (23.1)	127.9 (23.4)	120.8 (21.8)	<.001
Body composition variables, mean (SD)				
T12 SMA, cm^2	86.4 (18.2)	95.9 (14.4)	69.2 (9.8)	<.001
T12 SMI, cm^2/m^2	32.6 (5.9)	34.9 (5.4)	28.5 (4.4)	<.001
Total LBM, kg	26.5 (5.2)	28.5 (4.6)	23.0 (4.1)	<.001
Trunk LBM, kg	7.4 (1.9)	8.1 (2.0)	6.1 (0.3)	<.001
Appendicular LBM, kg	19.1 (4.2)	20.4 (3.8)	16.9 (3.9)	<.001
Handgrip strength, kg, mean (SD)	26.3 (6.9)	29.1 (5.8)	21.4 (5.9)	<.001
Handgrip weakness, *n* (%)	269 (42.1)	196 (47.8)	73 (31.9)	<.001
CT-defined sarcopenia, *n* (%)	160 (25.0)	102 (23.9)	58 (25.3)	.900
AWGS-defined sarcopenia, *n* (%)	141 (22.1)	97 (23.7)	44 (19.2)	.194
AWGS-defined severe sarcopenia, *n* (%)	42 (6.6)	30 (7.3)	12 (5.2)	.310

AWGS, Asian Working Group for Sarcopenia; BMI, body mass index; CT, computed tomography; ECOG PS, Eastern Cooperative Oncology Group performance status; LBM, lean body mass; SD, standard deviation; SMA, skeletal muscle cross-sectional area; SMI, skeletal muscle index (skeletal muscle area/height^2); T12, 12th thoracic vertebra. [a] *Group differences were analyzed using one-way analysis of variance and chi-square test (or Fisher's exact test), as appropriate.*

(**eTable 2** in the **Supplement**) and redrew the Kaplan–Meier curves (**eFigure 5** in the **Supplement**). The results remained almost identical.

Subgroup Analyses

Lastly, we performed *a priori* subgroup analyses of multivariable Cox Models 1 and 2. Most of these analyses revealed that CT-defined sarcopenia, AWGS-defined sarcopenia, and AWGS-defined severe sarcopenia were all significantly associated with poor prognosis (**Figure 4**). However, none of these conditions were significantly associated with poor prognosis in patients with large cell lung cancer.

DISCUSSION

The present study attempted to fill the knowledge gap between the fields of oncology and surgery and the fields of geriatric and internal medicine with respect to the definition of sarcopenia. To our knowledge, this is the first prospective cohort study that directly compared the prognostic values of CT-defined sarcopenia (i.e., low SMM) and AWGS-defined sarcopenia and severe sarcopenia (i.e., combination of low SMM, handgrip weakness, and/or low physical performance) in patients with lung cancer. We determined that CT-defined sarcopenia based on the T12 SMI derived from a single-slice chest CT image was a

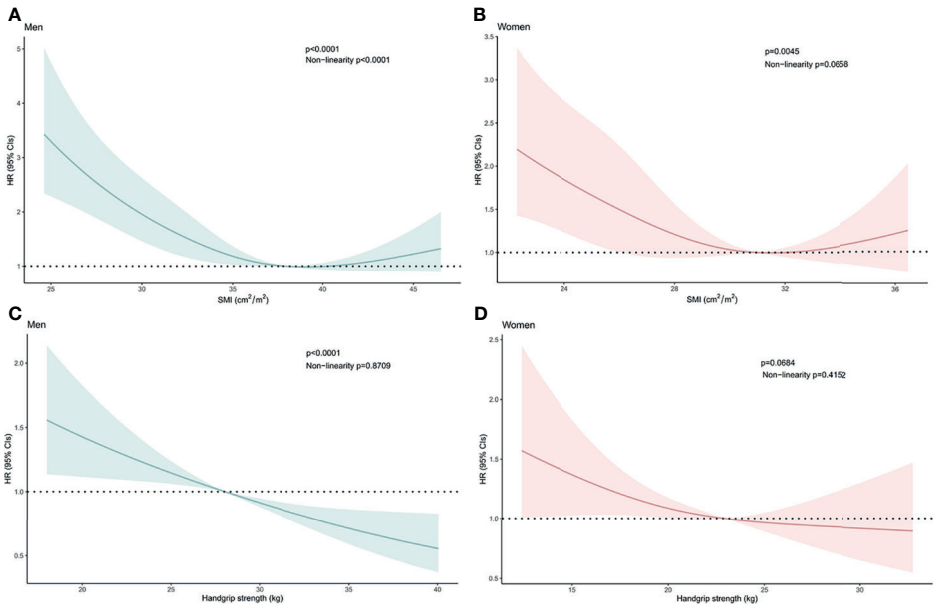

FIGURE 1 | Hazard Ratios of Overall Survival Related to **(A)** the Skeletal Mass Index at the 12th Thoracic Vertebra Level (T12 SMI) in Men, **(B)** the T12 SMI in Women, **(C)** Handgrip Strength in Men, and **(D)** Handgrip Strength in Women. Hazard ratios of the T12 SMI and handgrip strength were estimated as continuous data using a restricted cubic spline function with three knots. Bold lines represent the curves for the estimated hazard ratios related to the T12 SMI or handgrip strength in men and women. Shadowed areas indicate the corresponding 95% confidence intervals. CI, confidence interval; HR, hazard ratio.

better prognostic factor for OS than the conventional PS score. Furthermore, the addition of handgrip strength and the PS score to CT-defined sarcopenia could further improve OS discrimination in our study population.

In the present study, CT-defined sarcopenia based on the T12 SMI was associated with poor prognosis in patients with advanced NSCLC, even in those with normal PS scores. This finding highlights the crucial role of chest CT-defined sarcopenia in NSCLC. Numerous studies have proven that sarcopenia is a useful prognostic factor for predicting OS, disease-free survival, and adverse events of various treatments in patients with lung cancer; nevertheless, the majority of these studies had a retrospective design and were based on the L3 SMI or L3 psoas muscle mass derived from abdominal CT images (19, 26–28). We failed to retrieve any study addressing the T12 SMI and prognosis in lung cancer. However, we identified two small retrospective studies that examined the prognostic value of the change in the T12 SMA before and after surgery in patients with NSCLC (29, 30). Both studies were from the same research team, and their results confirmed that the postoperative decrease in the

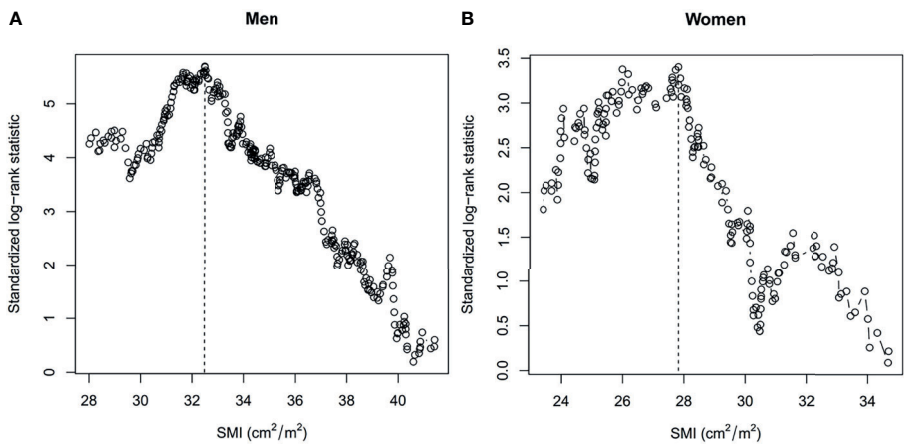

FIGURE 2 | Cut-off Values of the Skeletal Mass Index at the 12th Thoracic Vertebra Level (T12 SMI) in **(A)** Men and **(B)** Women Using Maximally Selected Rank Statistics. Dashed lines show the cut-off values. SMI, skeletal mass index.

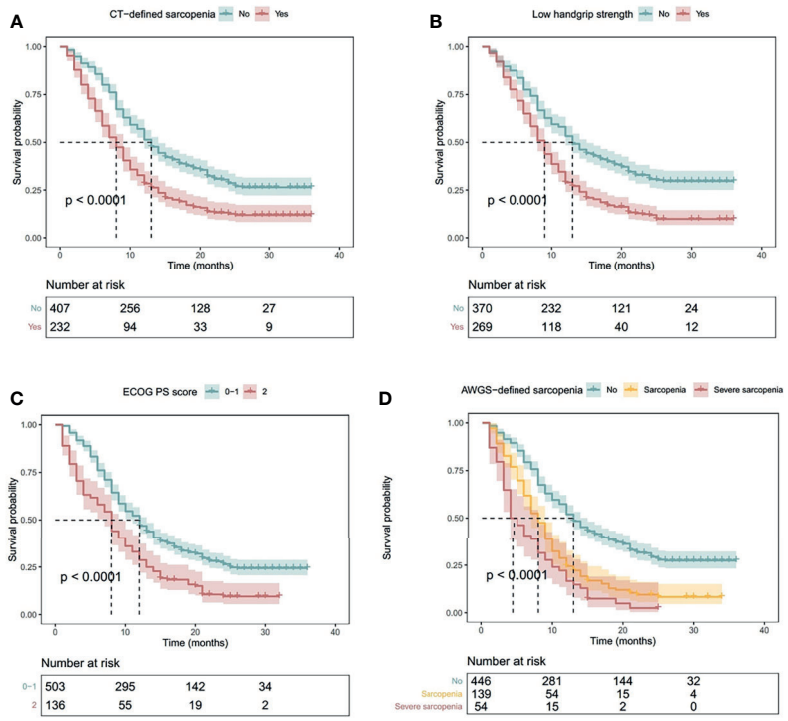

FIGURE 3 | Kaplan–Meier Curves Illustrating Overall Survival in Patients with **(A)** CT-Defined Sarcopenia, **(B)** Handgrip Weakness, **(C)** Low Physical Performance (ECOG PS Score ≥2), and **(D)** AWGS-Defined Sarcopenia or Severe Sarcopenia. *P* values indicate the results of the log-rank test. Shadowed areas indicate 95% confidence intervals. AWGS, Asian Working Group for Sarcopenia; CT, computed tomography; ECOG PS: Eastern Cooperative Oncology Group performance status.

T12 SMA was associated with poor prognosis (29, 30). Similarly, another retrospective study showed that the T12 SMA automatically derived from CT images was associated with all-cause mortality in a multicenter cohort of older community-dwelling men (31). These findings altogether suggest that further validation of the T12 SMI as a promising surrogate marker of whole-body SMM and a good prognostic factor is warranted in oncological and geriatric research.

Our study revealed that the combination of CT-defined sarcopenia, handgrip weakness, and/or low physical performance (i.e. AWGS-defined sarcopenia or AWGS-defined severe sarcopenia) could further improve OS discrimination. Few studies have addressed a similar issue in the literature. Burtin et al. recently published a prospective study that evaluated handgrip strength and fat-free mass (a surrogate indicator of BIA-derived SMM) for prognostic prediction in patients with stage I-II NSCLC treated with curative-intent radiotherapy (23). They concluded that handgrip weakness and low fat-free mass were independent prognostic factors for OS and that patients with both conditions exhibited worse prognosis (23). While their findings were in line with ours, they did not consider low physical performance as a component of sarcopenia. Our study employed a PS score ≥2 to define low physical performance, which is not recommended by either AWGS 2019 (11) or EWGSOP2 (10). Both guidelines recommend the use of the Short Physical Performance Battery, usual gait speed, or 5-time chair stand test for physical performance assessment. However, these tests are not routinely performed in clinical practice and are not only time-consuming but also labor-intensive. Our study indicated that a PS score ≥2, a convenient indicator, could be used to define low physical performance in patients with lung cancer.

Clinical Implications

Because chest CT scans are always available for patients with lung cancer, the clinical utility of chest CT scans, rather than abdominopelvic CT scans, is important in sarcopenia assessment. Furthermore, various organizations such as the US Preventive Services Task Force (32) and the Chinese Society of Clinical Oncology (33) have recommended low-dose chest CT scans for lung cancer screening in individuals with risk factors. The opportunistic utility of chest CT scans for screening lung cancer has been increasing to identify other diseases, such as chronic obstructive pulmonary disease and osteoporosis (34–36). Similarly, assessment of muscle health would be another opportunistic utility of these screening CT images.

The addition of handgrip strength and the PS score to chest CT-defined sarcopenia could further provide prognostic information on advanced NSCLC. Considering that the PS score is commonly obtained during routine oncological evaluation and that handgrip strength can be easily and repeatedly measured throughout cancer management without

TABLE 2 | Median survival and univariate and multivariable analyses for predictors of overall survival.

Variables	No. of Patients	No. of Deaths	Survival (Months)		Univariate Analysis			Multivariable Analysis Model 1			Multivariable Analysis Model 2			Multivariable Analysis Model 3		
			Median	95% CI	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value
CT-defined sarcopenia																
No	407	287	13.0	11.8-14.2	1	Reference		1	Reference		–	–	–	–	–	–
Yes	232	201	8.0	6.9-9.1	1.80	1.50-2.15	<.001	2.00	1.65-2.43	<.001	–	–	–	–	–	–
AWGS-defined sarcopenia																
No sarcopenia	446	311	13.0	11.7-14.3	1	Reference		–	–	–	1	Reference		–	–	–
Sarcopenia	139	125	8.0	6.9-9.1	2.00	1.62-2.46	<.001	–	–	–	2.00	1.59-2.49	<.001	–	–	–
Severe sarcopenia	54	52	4.0	2.3-5.7	2.99	2.22-4.03	<.001	–	–	–	3.01	2.21-4.09	<.001	–	–	–
ECOG PS																
0-1	503	367	12.0	10.8-13.2	1	Reference		–	–	–	–	–	–	1	Reference	
≥ 2	136	121	8.0	6.8-9.2	1.78	1.44-2.18	<.001	–	–	–	–	–	–	1.37	1.10-1.71	.006
Age per year	–	–	–	–	1.08	1.07-1.09	<.001	1.09	1.08-1.11	<.001	1.09	1.08-1.11	<.001	1.08	1.07-1.10	<.001
Sex																
Male	410	323	10.0	9.0-11.0	1	Reference		1	Reference		1	Reference		1	Reference	
Female	229	165	11.0	9.0-13.0	0.89	0.74-1.07	.208	0.77	0.59-1.02	.063	0.83	0.63-1.09	.184	0.80	0.61-1.05	.110
Smoking status																
Never smoker	311	238	11.0	9.7-12.3	1	Reference		1	Reference		1	Reference		1	Reference	
Ever smoker	328	250	11.0	9.6-12.4	1.05	0.88-1.26	.593	1.17	0.92-1.49	.226	1.22	0.96-1.55	.117	1.13	0.89-1.44	.306
Histologic type																
Adenocarcinoma	394	303	10.0	8.9-11.1	1	Reference		1	Reference		1	Reference		1	Reference	
Squamous cell carcinoma	201	146	13.0	11.5-14.5	0.87	0.71-1.07	.181	0.91	0.73-1.15	.494	0.92	.073-1.16	.485	0.89	0.70-1.12	.303
Large cell carcinoma	44	39	5.0	1.8-8.3	1.95	1.39-2.72	<.001	1.55	1.09-2.20	.023	1.42	1.01-2.02	.049	1.58	1.12-2.24	.010
Cancer stage																
Stage IIIB	294	203	17.0	14.5-19.5	1	Reference		1	Reference		1	Reference		1	Reference	
Stage IV	345	285	8.0	7.3-8.7	1.92	1.60-2.30	<.001	2.81	2.30-3.42	<.001	2.86	2.34-3.49	<.001	2.82	2.31-3.44	<.001
Charlson comorbidity index																
0	443	325	11.0	9.8-12.2	1	Reference		1	Reference		1	Reference		1	Reference	
≥1	196	163	10.0	8.5-11.5	1.23	1.02-1.48	.034	1.17	0.96-1.43	.187	1.18	0.97-1.44	.099	1.16	0.95-1.42	.144
BMI group																
Underweight	95	75	10.0	8.3-11.8	1.14	0.89-1.47	.303	0.92	0.70-1.21	.581	0.90	0.69-1.19	.472	1.05	0.80-1.37	.746
Normal	383	297	10.0	8.8-11.2	1	Reference		1	Reference		1	Reference		1	Reference	
Obese	161	116	12.0	10.6-13.4	0.80	0.65-0.99	.045	0.91	0.72-1.14	.444	0.84	0.67-1.05	.128	0.83	0.67-1.04	.098
Chemotherapy regimen																
Pemetrexed + carboplatin/cisplatin	239	184	10.0	8.8-11.2	1	Reference		1	Reference		1	Reference		1	Reference	

(Continued)

TABLE 2 | Continued

Variables	No. of Patients	No. of Deaths	Survival (Months)		Univariate Analysis			Multivariable Analysis Model 1			Multivariable Analysis Model 2			Multivariable Analysis Model 3		
			Median	95% CI	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Docetaxel + carboplatin/cisplatin	234	179	12.0	10.2-13.8	0.93	0.76-1.14	.476	1.05	0.84-1.32	.669	1.05	0.84-1.32	.656	1.07	0.86-1.34	.551
Gemcitabine + carboplatin/cisplatin	30	26	8.0	5.7-10.3	1.36	0.90-2.05	.142	0.97	0.64-1.48	.896	0.99	0.65-1.50	.949	1.12	0.74-1.71	.587
Paclitaxel + carboplatin/cisplatin	136	99	11.0	8.9-13.1	0.91	0.71-1.16	.451	1.02	0.78-1.31	.912	1.01	0.78-1.31	.945	1.00	0.78-1.30	.967
Patients who completed at least four chemotherapy courses																
No	141	120	9.0	8.1-9.9	1	Reference		1	Reference		1	Reference		1	Reference	
Yes	498	368	12.0	10.9-13.1	0.72	0.59-0.89	.002	0.78	0.59-0.97	.028	0.75	0.58-0.96	.021	0.82	0.64-1.05	.111
Serum creatinine per SD	–	–	–	–	1.09	1.00-1.18	.051	0.97	0.87-1.08	.569	0.98	0.88-1.09	.740	0.97	0.87-1.08	.606
Serum albumin per SD	–	–	–	–	1.06	0.97-1.16	.217	1.07	0.96-1.18	.305	1.07	0.96-1.18	.227	1.09	0.98-1.21	.119
Hemoglobin per SD	–	–	–	–	1.03	0.95-1.12	.501	1.03	0.93-1.13	.548	1.01	0.92-1.12	.797	1.00	0.91-1.10	.961
C statistic[a]								0.72	0.69-0.74		0.76	0.75-0.78		0.69	0.67-0.72	

AWGS, Asian Working Group for Sarcopenia; BMI, body mass index; CT, computed tomography; ECOG PS, Eastern Cooperative Oncology Group performance status; LBM, lean body mass; SD, standard deviation.

[a]A c statistic of 0.5 indicates chance; 0.5-0.7, poor discrimination; 0.7-0.8, acceptable discrimination; 0.8-0.9, excellent discrimination; 0.9-0.99, outstanding discrimination; and 1.0, perfect prediction.

increasing any burden to patients, it is reasonable to use the combination of low SMM (derived from chest CT), handgrip weakness, and low physical performance (PS score ≥2) to define sarcopenia in the oncological research field.

Limitations

Our study also has some limitations. First, our study was conducted at a single center; hence, the generalizability of our results seems to be limited. Second, in general, our study population might not be representative of patients with advanced NSCLC owing to potential referral bias. Third, we used a segmental multifrequency BIA device instead of dual-energy X-ray absorptiometry to estimate LBM. Nevertheless, according to a recent study, LBM measured by segmental multifrequency BIA has good agreement with dual-energy X-ray absorptiometry in ambulatory individuals (35). Fourth, we did not evaluate some important outcomes, including

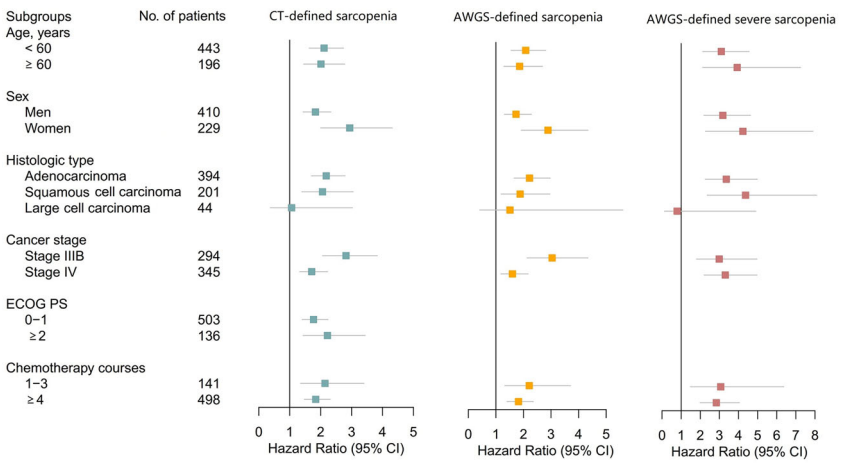

FIGURE 4 | Forest Plots Illustrating Subgroup Analyses According to Age, Sex, Histologic Type, Cancer Stage, ECOG PS, and Chemotherapy Courses. Subgroup analysis according to ECOG PS groups was not performed for AWGS-defined sarcopenia and severe sarcopenia because ECOG PS scores are a component of AWGS-defined sarcopenia and severe sarcopenia. AWGS, Asian Working Group for Sarcopenia; CI, confidence interval; CT, computed tomography; ECOG PS, Eastern Cooperative Oncology Group performance status.

disease-specific mortality, quality of life, functional decline, and the incidence of adverse events related to chemotherapy.

CONCLUSIONS

The SMI derived from a single-slice chest CT image at the T12 level was a valid surrogate marker of whole-body muscle mass. CT-defined sarcopenia based on the T12 SMI and a PS score ≥2 were independent prognostic factors for OS in patients with advanced NSCLC who received first-line chemotherapy. CT-defined sarcopenia was a better prognostic factor for OS than the conventional prognostic factor (PS score ≥2) in this patient population. The addition of handgrip strength and the PS score to chest CT-defined sarcopenia could further provide prognostic information on advanced NSCLC. Sarcopenia research in oncology should focus not only on muscle mass but also on muscle strength and function.

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 Biomedical Ethics Committee of West China Hospital, Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MY had full access to all data and takes responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design:MY and WL. Acquisition and analysis of CT images: SH and LT. Acquisition of clinical data: LX and JW. Data analysis and interpretation: MY, LT, and DL. Drafting of the manuscript: MY and LT. Critical revision of the manuscript for important intellectual content: All authors. Acquisition of funding: MY and WL. Administrative, technical, or material support: MY and WL. Supervision: MY and LX. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Diagnostic and Prognostic Significances of SOX9 in Thymic Epithelial Tumor

Xiaodong Yuan[1‡], Lei Huang[2†‡], Wenwu Luo[3†], Yufei Zhao[4], Björn Nashan[1], Fazhi Yu[5§] and Yun Liu[4*§]*

[1] Organ Transplant Center, Department of Hepatobiliary and Transplantation Surgery, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, China, [2] Department of Oncology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [3] Department of Pathology, Anhui Medical University, Hefei, China, [4] Department of Radiation Oncology, Anhui Provincial Cancer Hospital, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, China, [5] The First Affiliated Hospital of USTC, School of Life Sciences, University of Science and Technology of China, Hefei, China

Background: Thymic epithelial tumors (TETs) are rare tumors originating from the thymic epithelial cells. *SOX9*, a member of the family of *SOX* (SRY-related high-mobility group box) genes, has been considered as an oncogene and therapeutic target in various cancers. However, its role in TETs remains uncertain.

Methods: Using the immunohistochemistry method, the expression of SOX9 was analyzed in TETs tissues, including 34 thymoma (8 cases with type A, 6 with type AB, 6 with type B1, 9 with type B2, and 5 with type B3 thymomas) and 20 thymic cancer tissues and the clinicopathologic and prognostic significances were evaluated. Further bioinformatics analysis of gene expression profiles of thymomas with high and low SOX9 expressions and the corresponding survival analyses were based on the thymoma cases identified in The Cancer Genome Atlas (TCGA) database, with the median expression level of SOX9 selected as cutoff.

Results: Immunohistochemistry staining showed that SOX9 was highly expressed in the nuclei of the epithelial cells of the Hassall's corpuscles and of the TET tumor cells. SOX9 expression was significantly associated with histological type and high expression indicated unfavorable clinical outcomes of thymomas. Bioinformatics analysis revealed that genes positively associated with SOX9 expression were mapped in proteoglycans in cancer, cell adhesion molecules, and molecules involved in extracellular matrix-receptor interaction and the TGF-β signaling pathway, and that genes negatively associated with SOX9 expression were mapped in molecules involved in primary immunodeficiency, the T cell receptor signaling pathway, Th17 cell differentiation, PD-L1 expression, and the PD-1 checkpoint pathway in cancer. In addition, SOX9 expression was positively associated with POU2F3 and TRPM5 expressions, the master regulators of tuft cells, suggesting that high SOX9 expression might be associated with the tuft cell phenotype of thymomas.

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Junqi Liu,
The First Affiliated Hospital of Zhengzhou University, China
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Medical University of Vienna, Austria
Stefan Janik,
Medical University of Vienna, Austria

***Correspondence:**
Yun Liu
yunliu0608@126.com
Fazhi Yu
fzy1988@ustc.edu.cn

†ORCID:
Lei Huang
orcid.org/0000-0002-4225-9200

‡These authors have contributed equally to this work and share first authorship

§These authors have contributed equally to this work

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Moreover, high SOX9 expression was associated with immune dysregulation of thymoma, and M2 macrophage significantly dominated in the high SOX9 expression group.

Conclusion: SOX9 may serve as a diagnostic and prognostic marker for TETs. Notably, high SOX9 expression in TETs may indicate a tuft cell phenotype and an immune suppressive microenvironment of thymomas.

Keywords: SOX9, POU2F3, tuft cell, thymic epithelial tumor, tumor microenvironment

INTRODUCTION

Thymic epithelial tumors (TETs), including thymomas and thymic carcinomas, are rare tumors of the mediastinum and originate from the thymic epithelial cells (1, 2). Histologically, most of thymic tumors are composed of non-malignant-appearing thymic epithelial cells mixed with multiple proportions of lymphocytes, which makes it difficult to diagnose and predict the prognosis of thymic tumors (1). According to the World Health Organization (WHO) classification, thymic epithelial cells are categorized into six subtypes, including A, AB, B1, B2, B3, and C (also known as TCs) based on histological appearance (3–5). Nevertheless, TETs are regarded as malignant tumors regardless of subtype or histology. According to histological subtype, types A, AB, and B1 have an excellent overall survival rate of 90–95% at 10 years; The 5-year survival rates for types B2, B3, and C are 75%, 70%, and 48%, respectively (1). Surgery remains the main treatment followed by adjuvant radiation therapy for diseases invading surrounding tissues (6, 7). For patients with inoperable refractory or recurrent diseases, postoperative systemic chemotherapy is currently recommended (2). However, there is still a lack of standard treatment strategy after first-line failure. Hence, the exact pathologic diagnosis of TETs is essential for determining the treatment strategy and predicting the prognosis. Although some biomarkers have been identified for the diagnosis of TETs, more valuable markers for the diagnosis and prognosis prediction of TETs are urgently needed.

SOX9, a member of the family of *SOX* (SRY-related high-mobility group box) genes, exerts regulatory functions in multiple organs development, cell-fate decision, and differentiation (8). Accumulating studies have demonstrated that SOX9 is also involved in the development of multiple cancers, including gastrointestinal, breast, brain, urological, and lung cancers and others (9–13). Collectively, SOX9 plays critical roles in tumor development and progression, including tumor initiation, tumor microenvironment regulation, metastasis, and drug resistance (14, 15).

Abbreviations: BP, biological process; CC, cellular component; DEG, differentially expressed gene; GO, gene ontology; GSEA, Gene Set Enrichment Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function; OS, overall survival; PPI, Protein–Protein Interaction; SD, standard deviation; SOX9, SRY-related high-mobility group box 9; TCGA, The Cancer Genome Atlas; TETs, thymic epithelial tumors; TIMER, Tumor Immune Estimation Resource; TICs, Tumor-Infiltrating Immune Cells; TME, tumor microenvironment; WHO, World Health Organization.

Recently, using the immunohistochemistry method, we found that SOX9 was highly expressed in the epithelial component of thymus, especially in the epithelial cells of Hassall's corpuscles. Moreover, SOX9 was observed to be highly expressed in the nuclei of TET tumor cells and may serve as a diagnostic marker for thymomas. However, the molecular function of SOX9 in TETs has not been well documented yet. In this study, we first examined the expression of SOX9 in TETs to evaluate whether SOX9 could serve as a diagnostic marker for TETs. In addition, using bioinformatics methods, we further investigated the potential function of SOX9 in the development of TETs.

MATERIALS AND METHODS

Human Specimens and Ethics Approval

This study enrolled 34 thymoma (including 8 cases with type A, 6 with type AB, 6 with type B1 9 with type B2, and 5 with type B3 thymomas) and 20 thymic carcinoma tissues from the First Affiliated Hospital of Anhui Medical University (Hefei, China). This study was approved by the local ethics committees.

Immunohistochemistry and Staining Evaluation

Immunohistochemical staining was performed as previously described (16). Briefly, the sections were deparaffinized in serial ethanol dilutions and rehydrated. Heat-induced antigen retrieval was performed with 0.01 M sodium citrate buffer (pH=6.0) at 98°C for 10 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 10 min, followed by pre-incubation in 5% normal goat serum to block nonspecific staining for 30 min at room temperature to prevent unspecific binding of antibodies. The tissue sections were incubated with polyclonal rabbit anti-SOX9 antibody (AB5535; Sigma-Aldrich) at a dilution of 1:100 for 4 h at room temperature. The specimens were subsequently washed in phosphate buffered saline and incubated with anti-rabbit secondary antibody conjugated with horse radish peroxidase for 1 h at room temperature, and then detected with 3, 3'-diaminobenzidine for 8 min. After being counterstained with hematoxylin, all sections were dehydrated and mounted with malinol mounting medium. Immunostaining results for SOX9 were scored semi-quantitatively based on the intensity and proportion of positive tumor cell nuclei as previously described (16). In detail, the intensity score of nuclear SOX9 staining was classified into four grades: 0, negative; 1, weak with yellow color; 2, medium with brown color; 3, strong with black color. The proportion score of SOX9 positive cell nuclei was evaluated

under 200X magnification and was defined as 4 grades: 0, no positive cells; 1, positive cells: ≤ 30%; 2, 30% < positive cells ≤ 60%; 3, 60% < positive cells. The final immunostaining scores were evaluated by multiplying the intensity score and proportion score. The samples with final scores over 3 were identified as high SOX9 expression, and the others were identified as low SOX9 expression.

Database

Gene expression data and clinical features of TET samples (including 108 thymomas and 11 thymic carcinomas) were collected from the publicly available datasets of The Cancer Genome Atlas (TCGA) (https://tcga-data.nci.nih.gov/tcga/).

Identification of Differentially Expressed Genes (DEGs)

The R software (https://www.r-project.org/) and the *limma* package were utilized to identify DEGs by comparing cases with high and low SOX9 expressions. Gene expression with $|\log_2(\text{fold-change})| > 2$ and an adjusted $P < 0.05$ was considered as significant.

Gene Set Enrichment Analysis Heat-Maps, Volcano Plots, and Venn Diagrams

Heat-maps of the top 100 DEGs were constructed using the GSEA software (version 4.0.3) by the Broad Institute [Morpheus (broadinstitute.org)] (17) and the volcano plots of DEGs were generated using the GraphPad Prism software (version 5.03; GraphPad Software Inc.). To identify genes in DEGs regulated by transcription factor SOX9, the potential target genes of SOX9 were download from the ChIP Enrichment Analysis (CHEA) databases (https://maayanlab.cloud/Harmonizome/dataset/CHEA+Transcription+Factor+Targets) which was designed for the identification of target genes of transcription factors from published ChIP-chip, ChIP-seq, and other transcription factor binding site profiling studies (18). Then, the overlap of DEGs and potential target genes of SOX9 identified from the CHEA dataset were performed by Venn diagrams which was created using an online analysis tool (https://bioinfogp.cnb.csic.es/tools/venny/index.html) (18, 19).

Enrichment Analysis of Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) OF DEGs

The *clusterProfiler*, *org.Hs.eg.db*, *enrichplot*, and *ggplot2* packages in the R software were used to perform the GO and KEGG enrichment analyses of DEGs. GO categories, including biological processes (BPs), molecular functions (MFs), and cellular components (CCs), were analyzed. *P*- and q-values <0.05 were regarded to indicate significant enrichment.

Protein-Protein Interaction (PPI) Network Construction and Prediction of SOX9 Binding Sites Within the POU2F3 Promoter

PPI network was constructed by STRING database (http://string-db.org/) (20). Nodes with confidence of interactive relationship larger than 0.40 were used for building network. Disconnected nodes were hidden in the network. The JASPAR (http://jaspar.genereg.net) was used to predict the potential binding sites of *SOX9* within the promoter of POU2F3 by using the deposited *SOX9* binding site matrix profile MA0077.1 (21). According to the Ensembl deposited gene sequence, nucleotides have been analyzed from the 2000 upstream of the transcription starting site of POU2F3.

Tumor Immune Estimation Resource Database Analysis (TIMER) and Tumor-infiltrating Immune Cells Profile

TIMER is a comprehensive website for the automatic analysis and visualization of the associations between immune infiltration levels and a series of variables (https://cistrome.shinyapps.io/timer/) (22, 23). The cell-type identification by estimating relative subsets of RNA transcripts (CIBERSORT) is a computational method which is applied for estimating the TIC abundance profile in all thymoma tumor samples (24). Tumor immune and stromal scores as well as microenvironment scores were used to predict the level of infiltrating stromal and immune cells as well as tumor purity and evaluated by CIBERSORT online software (http://cibersort.stanford.edu/). The abundances of six types of immune cells (CD4+ T cells, CD8+ T cells, B cells, neutrophils, dendritic cells, and macrophages) were evaluated by the TIMER algorithm and the abundances of M1 and M2 macrophages were evaluated by the CIBERSORT algorithm based on gene expression data of thymomas from TCGA database.

Statistics Analyses

Continuous results were expressed as mean ± standard deviation (SD). Two-tailed unpaired Student *t*-test was used to compare continuous variables between groups. The chi-square test or Fisher's exact probability method were used to evaluate the correlation between SOX9 expression and clinical characteristics of patients. The associations of SOX9 expression with the expression of the indicated genes and the tumor microenvironment, stromal, and immuno-scores were analyzed using Parametric Correlation and the Pearson correlation (r) was calculated to evaluate the fitting strength for each correlation. All data analyses were conducted using the GraphPad Prism software (version 5.03; GraphPad Software Inc.) if not otherwise specified. Findings with *P* values less than 0.05 were considered significant.

The 108 thymoma cases from the TCGA thymoma database were categorized into high and low SOX9 expression groups using the median SOX9 expression level as threshold for survival analysis, where the Kaplan-Meier method was used and survival between groups were compared using the log-rank test with two-sided $P < 0.05$ indicating statistical significance.

RESULTS

Immunohistochemical Staining of SOX9 Expression in Thymic Tumors

To investigate the diagnostic significance of SOX9 in thymic tumors, immunohistochemistry staining of SOX9 expression was

performed in 34 thymomas (including 8 cases with type A, 6 with type AB, 6 with type B1, 9 with type B2, and 5 with type B3 thymomas) and 20 thymic carcinoma tissues. The representative staining patterns of SOX9 in the non-tumor area of TETs are shown in **Figure 1A**; SOX9 expression patterns in TET tumor tissues are shown in **Figure 1B**. SOX9 was observed to be expressed in the nuclei of thymic epithelial cells and tumor cells. SOX9 staining intensity in TET tissues is shown in **Figure 1C**. The ratios of strong SOX9 staining in different types of thymomas and thymic carcinomas are summarized in **Table 1**. Specifically, strong staining of SOX9 was observed in 6 of 8 (70%) cases with type A, 3 of 6 (50.00) of cases with type AB, 2 of 9 (22.22%) cases with type B2 thymomas, and 9 of 20 (45%) cases with thymic carcinomas. The ratios of strong SOX9 staining in the other types were all smaller than 33%.

SOX9 Expression Was Associated With Histological Types of Thymoma

We analyzed the association of SOX9 expression with clinical parameters of thymoma patients, including age, sex, and

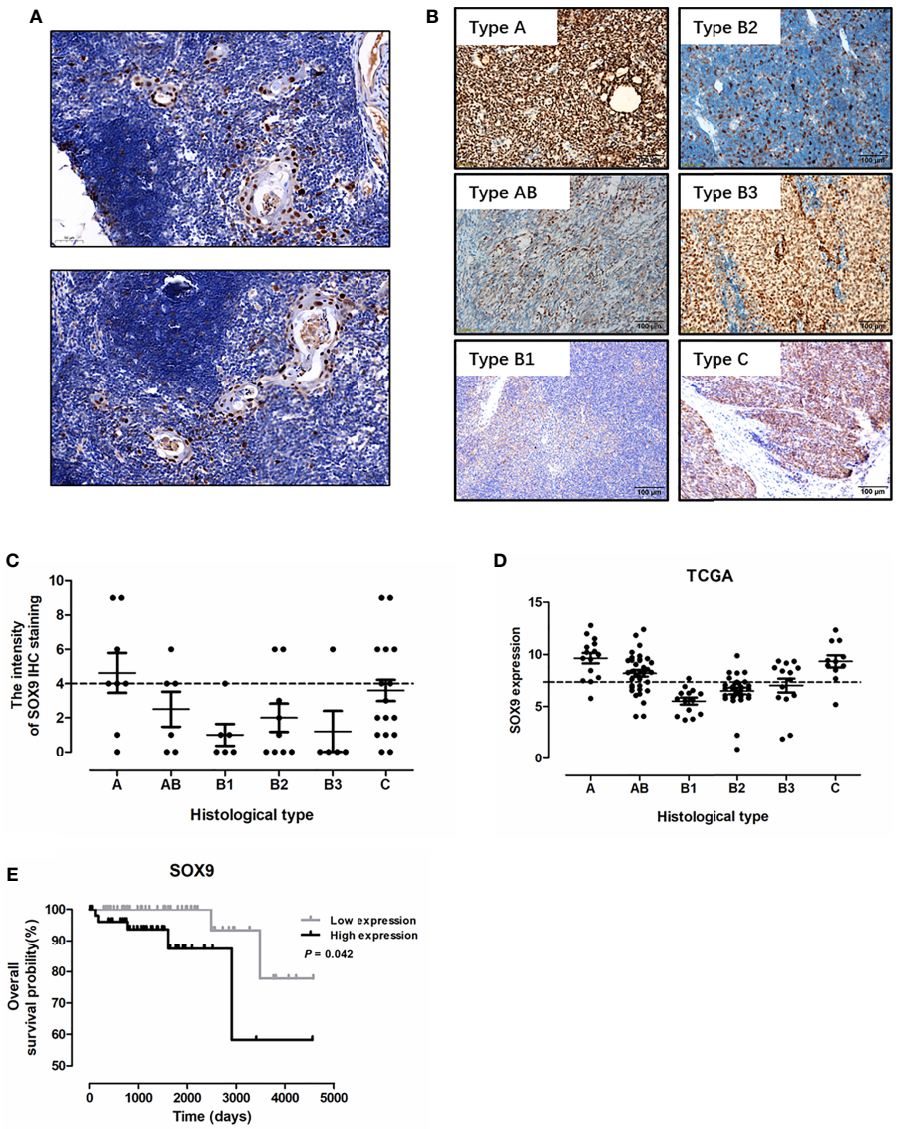

FIGURE 1 | SOX9 expression in thymic epithelial tumors and its prognostic significance. **(A)** Representative immunostaining images for SOX9 expression in the epithelial cells of Hassall's corpuscles in the non-tumor area of thymic epithelial tumors. **(B)** Representative immunostaining images for SOX9 in different histological types of thymomas and thymic carcinomas. **(C)** The distribution of SOX9 expression intensity quantified by IHC staining in thymomas and thymic carcinomas in our cohort. **(D)** The distribution of SOX9 RNA expression in thymomas and thymic carcinomas in the TCGA dataset. **(E)** Kaplan–Meier survival curves showing that thymoma patients from TCGA dataset with high SOX9 RNA expression had shorter overall survival time ($P < 0.05$), as determined by the log-rank test. Thymoma patients (n = 108) were categorized into high and low SOX9 expression groups with the median value of SOX9 mRNA expression level as cutoff.

TABLE 1 | Correlation between SOX9 protein expression level and clinicopathological parameters of patients with thymomas in our cohort.

Clinicopathological parameters	SOX9 staining		*P*
	low (n = 21)	high (n = 13)	
Age (years, mean ± SD)	52.29 ± 11.67	60.69 ± 13.87	0.0749
Sex			0.2793
Male	11 (52.38%)	10 (47.62%)	
Female	10 (76.92%)	3 (23.07%)	
Histological type			0.0131
A	2 (25.00%)	6 (75.00%)	
AB	3 (50.00%)	3 (50.00%)	
B1	5 (83.33%)	1 (16.67%)	
B2	7 (77.78%)	2 (22.22%)	
B3	4 (80.00%)	1 (20.00%)	

SD, standard deviation.

TABLE 2 | Correlation between SOX9 RNA expression and clinicopathological parameters of patients with thymomas in the TCGA cohort.

Clinicopathological parameters	SOX9		*P*
	Low (n = 54)	High (n = 54)	
Age (years, mean ± SD)	54.65 ± 12.78	59.89 ± 12.92	0.038
Sex			0.847
Male	29 (49.15%)	30 (50.85%)	
Female	25 (51.02%)	24 (48.98%)	
Histological type			<0.001
A	1 (6.67%)	14 (93.33%)	
AB	12 (33.33%)	24 (66.67%)	
B1	13 (92.86%)	1 (7.14%)	
B2	22 (75.86%)	7 (24.14%)	
B3	6 (42.86%)	8 (57.14%)	
History of myasthenia gravis			0.294
No	34 (47.89%)	37 (52.11%)	
Yes	20 (58.82%)	14 (41.18%)	
NA	0	3	
Masaoka stage			0.532
I-II	47 (52.22%)	43 (47.78%)	
III-IV	7 (43.75%)	9 (56.25%)	
NA	0	2	
Radiation therapy			0.012
No	29 (40.85%)	42 (59.15%)	
Yes	24 (66.67%)	12 (33.33%)	
NA	1	0	
New tumor event after initial treatment			0.221
No	46 (47.92%)	50 (52.08%)	
Yes	8 (66.67%)	4 (33.33%)	

TCGA, The Cancer Genome Atlas; SD, standard deviation; NA, not available.

histological type (**Table 1**), and found that SOX9 expression tended to correlate with the histological type of thymomas (**Table 1**, $P = 0.0131$). To further verify the clinical significance of SOX9 expression in TETs, we examined SOX9 expression using the RNA-seq data of thymomas in TCGA. **Figure 1D** shows the SOX9 mRNA expressions in different types of thymomas (n = 108) and thymic carcinomas (n = 11).

Then, SOX9 expression in thymomas was categorized into high and low expression groups, with the median level of SOX9 expression selected as the cutoff. High SOX9 expression was observed in 14 of 15 (93%) cases with type A, 24 of 36 (67%) cases with type AB, and 8 of 14 (57%) cases with type B3 thymomas. The ratios of high SOX9 expression in all the other types were smaller than 30%. In addition, SOX9 was also highly expressed in thymic carcinoma (**Figures 1B–D**). Then, we evaluated the association of SOX9 expression with the clinical and pathologic parameters of thymoma cases from TCGA dataset, including age, sex, histological type, history of myasthenia gravis, Masaoka stage, radiation therapy, and new tumor event after initial treatment, and found that SOX9 expression was correlated with the histological type of thymomas (**Table 2**, $P < 0.001$). In addition, the ratio of patient received radiation therapy after operation was higher in patients with low SOX9 expression group (**Table 2**, $P < 0.012$). Moreover, survival analysis revealed that patients with high SOX9 expression had shorter median overall survival time (**Figure 1E**). These results suggested that SOX9 expression was associated with the histological type of thymomas and might serve as an unfavorable prognostic marker for thymomas.

SOX9 Expression Was Associated With the Epithelial Cell Phenotype of Thymoma

To further explore the potential function of SOX9 in thymoma, we first performed gene set enrichment analysis (GSEA). The top 100 upregulated and downregulated genes identified by GSEA are shown in **Figure 2A**. GSEA results also showed that patients with high SOX9 expression had enrichment for the TGF-β signaling pathway and pathway in cancer (**Figure 2B**), while the low SOX9 expression group had enrichment for the primary immunodeficiency pathway and the T cell receptor signaling pathway (**Supplementary Figure 1**). DEGs related to SOX9 expression were further identified using the *limma* package of the R software ($|\log_2(\text{fold-change})| > 2$; adjusted $P < 0.05$). The results revealed that 291 genes were upregulated and 106 genes downregulated in patients with high SOX9 expression compared to those with low expression (**Supplementary Figure 2**). Then, the KEGG pathway and GO enrichment analyses were performed using the *clusterProfiler* R package to investigate the functions of these DEGs. The results from the KEGG pathway enrichment analysis indicated that these 291 upregulated DEGs were mapped to the proteoglycans in cancer, and molecules involved in axon guidance, cell adhesion, extracellular matrix-receptor interaction, and the TGF-β signaling pathway, and that these 106 downregulated DEGs were mapped to molecules involved in primary immunodeficiency, hematopoietic cell lineage, the T cell receptor signaling pathway, Th17 cell differentiation, PD-L1 expression, the PD-1 checkpoint pathway in cancer, Th1 and Th2 cells differentiation, et al. (**Supplementary Figure 3** and **Supplementary Tables 1, 2**). The DEGs related to these pathways are shown in **Figures 2C, D**. The results from the GO enrichment analysis indicated that the upregulated DEGs were mapped to molecules involved in the biological process of mesenchymal cell development, epithelial tube morphogenesis, extracellular matrix organization, stem cell development, et al.; and that the downregulated DEGs were mapped to molecules involved in the immune-related GO terms, such as the antigen

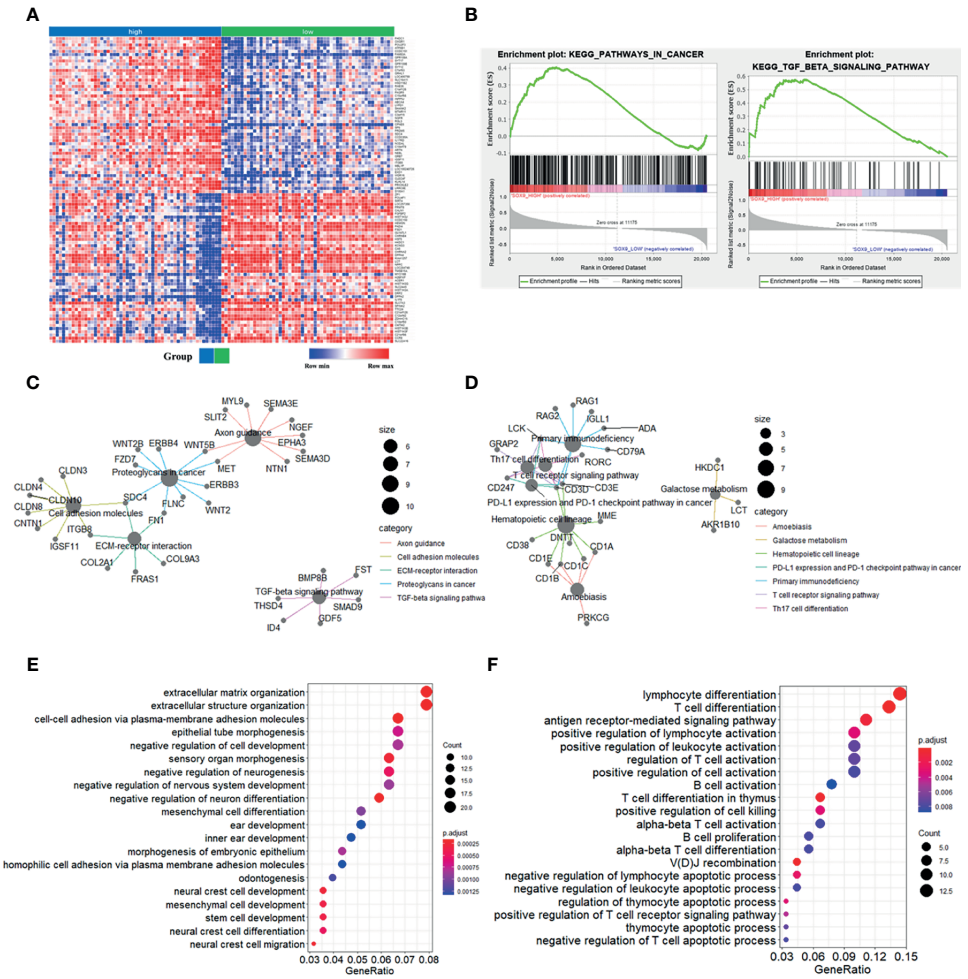

FIGURE 2 | Enrichment analyses of genes significantly correlated with SOX9 expression in thymomas. **(A)** Heat-map of the top 100 upregulated and downregulated genes determined by the Gene Set Enrichment Analysis (GSEA) analysis. **(B)** GSEA analysis revealed enrichment of the gene sets related to the "pathway in cancer and TGF-β signaling pathway" in patients with high SOX9 expression. **(C)** The cnetplot depicted the five enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways detected and their associated differentially-expressed genes in thymoma patients with high SOX9 expression. **(D)** The cnetplot depicts the seven enriched KEGG pathways detected and their associated differentially-expressed genes in thymoma patients with low SOX9 expression. **(E, F)** The dot-plot depicts the activity of the biological processes terms in thymoma patients with high and low SOX9 expressions, respectively.

receptor-mediated signaling pathway, T cell differentiation in thymus, positive regulation of lymphocyte activation, et al. (**Figures 2E, F** and **Supplementary Tables 3, 4**). Together, these results indicated that SOX9 expression was associated with the epithelial phenotype instead of immune phenotype of thymomas, and we proposed that SOX9 might be used as a potential marker for the epithelial components of thymomas.

SOX9 Was Correlated With Genes Associated With the Thymic Tuft Cells Phenotype in Thymoma

Hassall's corpuscles are known as cornified bodies within the medulla of human thymus. As a transcriptional factor, positive nuclear SOX9 staining was observed in the epithelial cells of Hassall's corpuscles (**Figure 1A**) where thymic tuft cells are located (25). SOX9 has been used as a marker for tuft cells in several tissues (26). These findings suggest a potential role of SOX9 in thymic medullary epithelial cells. Autoimmune regulator (AIRE) and homeodomain-interacting protein kinase 2 (HIPK2) are known as two critical transcriptional factors that play non-redundant roles in determining thymic tuft cells development and shaping thymic function (25, 27). We found that SOX9 expression was negatively correlated with AIRE expression but positively with HIPK2 expression (**Figure 3A**). By crossing the 291 DEGs positively associated with SOX9 expression with genes potentially regulated by SOX9 and identified from the CHEA dataset, 63 genes were identified (**Figure 3B**). The interactions between these 63 genes and SOX9 are shown in **Figure 3C**. The KEGG pathway enrichment analysis indicated that these 63 genes were mapped to

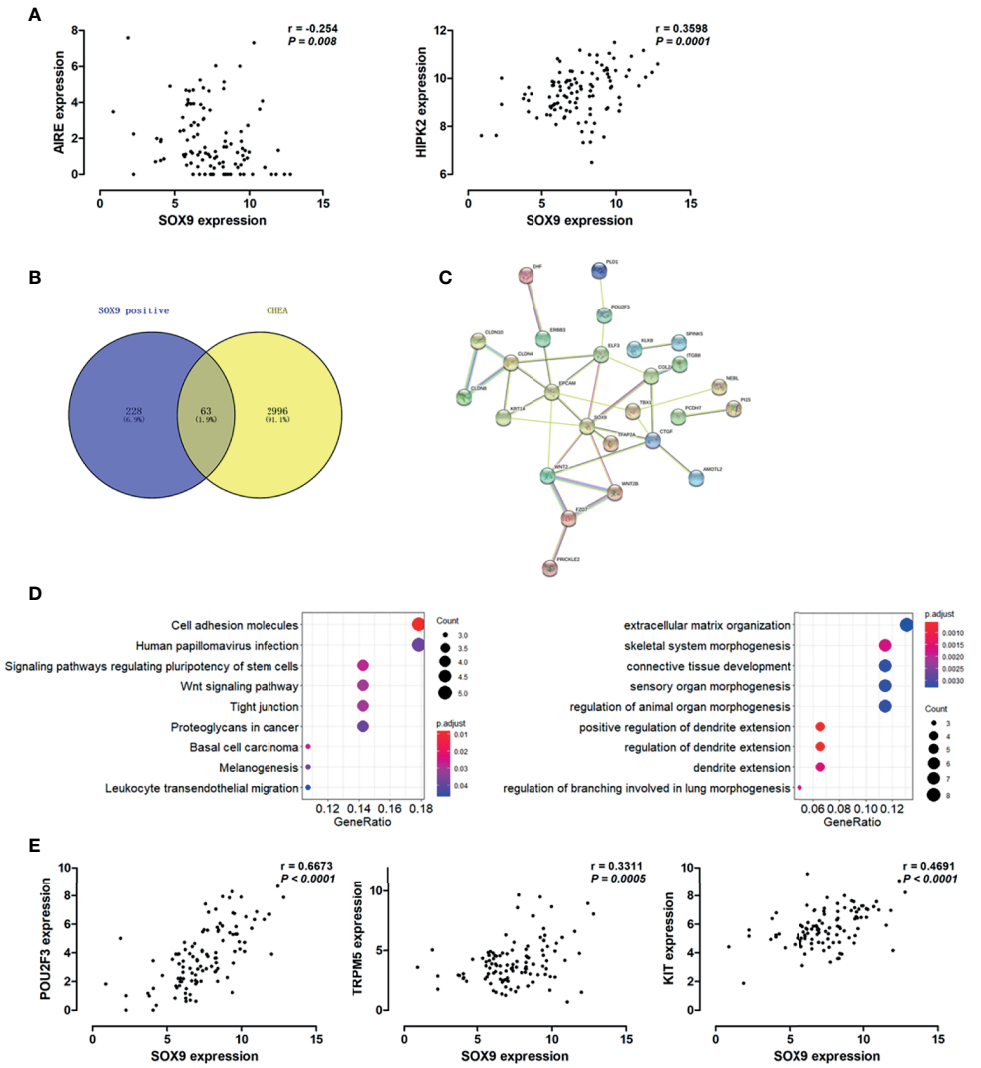

FIGURE 3 | SOX9 expression associated with the thymic tuft cells phenotype of thymomas. **(A)** Scatter plots illustrate the linear regression analyses for the associations between the expression of SOX9 and the expression of HIPK2 and AIRE in thymomas, respectively. **(B)** Venn diagram depicts the 63 common genes which are positively associated with SOX9 expression and regulated by SOX9. **(C)** Protein-protein interaction (PPI) network of these 63 common genes was constructed with the nodes with interaction confidence value > 0.95. Disconnected nodes were hidden in the network. **(D)** The dot-plot depicts the activity of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (right) and the biological processes terms (left) of these 63 genes. **(E)** Scatter plots illustrate the pearson correlation analysis for the associations between the expression of SOX9 and the expression of POU2F3, TRPM5, and KIT in thymomas, respectively.

molecules involved in the signaling pathway regulating the pluripotency of stem cells, the Wnt signaling pathway, tight junction, et al. (**Figure 3D** and **Supplementary Table 5**). In addition, the GO enrichment analysis revealed that these 63 genes were involved in extracellular matrix organization and sensory organ morphogenesis (**Figure 3D** and **Supplementary Table 6**). Among these genes, *POU2F3*, a transcriptional factor for thymic tuft cells development (25, 28), was shown to be positively correlated with SOX9 expression (**Figure 3E**, P <0.001, R^2 = 0.445). JASPAR analysis revealed that *POU2F3* carries 6 putative SOX9-binding sites along its DNA transcriptional regulatory region, with a homology higher than 80% (**Supplementary Table 7**). In addition, SOX9 expression was positively associated with the expressions of TRPM5, which is required for the function of thymic tuft cells, and KIT, which is frequently expressed in thymic carcinomas (**Figure 3E**) (25, 28). Moreover, we observed that SOX9 was correlated with almost all members of the TAS2R family (**Figure 4**) which are overexpressed in thymic tuft cells (25). Taken together, these data further supported the notion that SOX9 might be used as a marker for thymic epithelial cells and explained the association of its expression with the histological type of TETs, especially the one representing a tuft cell phenotype of thymomas.

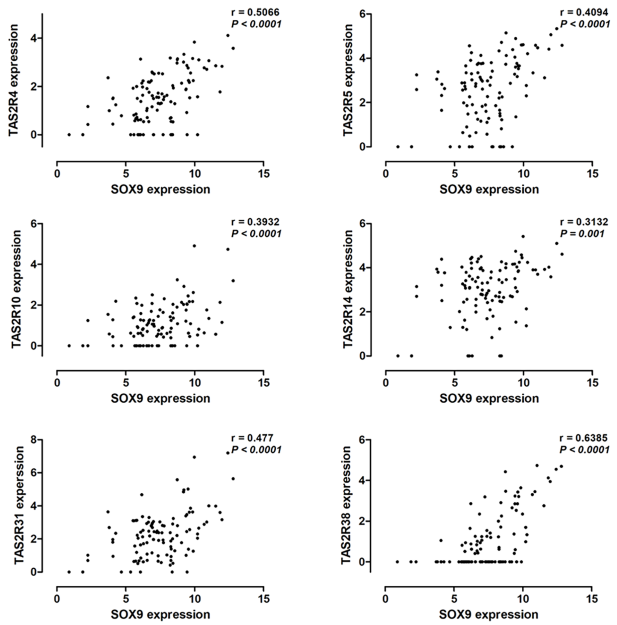

FIGURE 4 | Pearson correlation analysis to explore the correlation between SOX9 expression and the expression of the TAS2R family members.

SOX9 Expression Was Associated With Immune Suppressive Microenvironment of Thymoma

Bioinformatics analysis revealed that SOX9 expression was associated with genes related to the extracellular matrix-receptor interaction pathway and extracellular matrix structure organization, indicating a potential role of SOX9 in regulating tumor microenvironment. We further investigated the role of SOX9 in tumor microenvironment; the microenvironment score, tumor stromal score, and immune score, as well as the infiltration of immune cells including B cells, CD4+T cells, and CD8+T cells in thymoma tumor tissues were analyzed using the TIMER estimation application. In the TIMER estimation, the xCELL and CIBERSORT methods were selected to digitally portray the cellular heterogeneity landscape in tumor tissues. Then, we analyzed the association of SOX9 expression with these scores and immune cells infiltrating levels, and found that SOX9 expression was positively correlated with stromal score but negatively with immune score and microenvironment score (**Figure 5A**). In addition, we found that thymoma patients with higher SOX9 expression had less infiltration of B cells, CD4+ T cells, and CD8+ T cells, but higher infiltration of macrophages. Notably, patients with high SOX9 expression had a significantly higher infiltration of M1 and M2 macrophages compared to the low SOX9 expression group (**Figure 5C**). However, M2 macrophage significantly dominated in the high SOX9 expression group (**Figure 5D**). Of note, we observed that SOX9 expression was negatively associated with the expressions of LCK and RORC, which are involved in the development, function, and differentiation of T and Th17 cells, respectively (**Figure 5E**). Survival analysis revealed that thymoma patients with high LCK or RORC expressions had favorable clinical outcomes (**Figure 5F**). Taken together, we proposed that SOX9 expression might be associated with an immune suppression tumor microenvironment of thymomas.

DISCUSSION

As a transcription factor, SOX9 has been implicated in the initiation, development, and progression of tumors in multiple organs (15). However, the role of SOX9 in TETs has not been reported yet. In this study, we found that SOX9 was expressed in the nuclei of the epithelial cells of Hassall's corpuscles and in the epithelial component of TET cells in almost all cases. Of note, SOX9 expression was significantly correlated with the histological type of thymomas and might serve as a negative prognostic marker for thymomas. In addition, bioinformatics analysis further revealed that SOX9 was positively associated with genes regulating epithelial tube morphogenesis and extracellular matrix, and negatively associated with genes related to immune cell differentiation, PD-L1 expression, and the PD-1 checkpoint pathway in cancer. Taken together, these findings suggest that SOX9 could be used as a marker for thymic epithelial cells and a diagnostic and prognostic marker for TETs.

Tuft cells are chemosensory epithelial cells with a unique "tuft" of long and thick microvilli on their apical side (26). Tuft cell-like medullary thymic epithelial cells were identified in murine thymus (25). It has been proposed that thymic tuft cells are immunologically important, highly differentiated epithelial cells in the thymic medulla (25, 28, 29). Yamada et al. reported that a tuft cell-like signature was highly prevalent in thymic squamous cell carcinoma (28). In line with these findings, our results showed that positive nucleus staining of SOX9 was observed in almost all TET tissues. In thymomas, we found that SOX9 expression was positively correlated with the expression of HIPK2, which is a critical transcriptional factor determining thymic tuft cells development and shaping thymic function (25). POU2F3, which is required for the development and function of thymic tuft cells, was found to be highly expressed in thymic squamous cell carcinomas (25, 28). By JASPAR analysis, we found that *POU2F3* might be a target of SOX9. Among the 63 genes potentially regulated by SOX9, 6 genes are involved in sensory organ morphogenesis, including *COL2A1*, *FBN2*, *TBX1*, *TFAP2A*, *WNT2*, and *WNT2B*. In addition, SOX9 was correlated with almost all members of the TAS2R family which is a key component of the canonical taste transduction pathway and may be coordinated in the chemosensory specificities of thymic tuft cells (25). Taken together, our results indicated that SOX9 expression might be associated with the tuft cell phenotype of thymoma.

In this study, we observed that SOX9 expression was associated with the tumor microenvironment (TME) of thymoma, with SOX9 expression positively correlated with the tumor stromal scores while negatively with the tumor immune scores. Bioinformatics analysis revealed that genes positively associated with SOX9 expression in thymomas were enriched

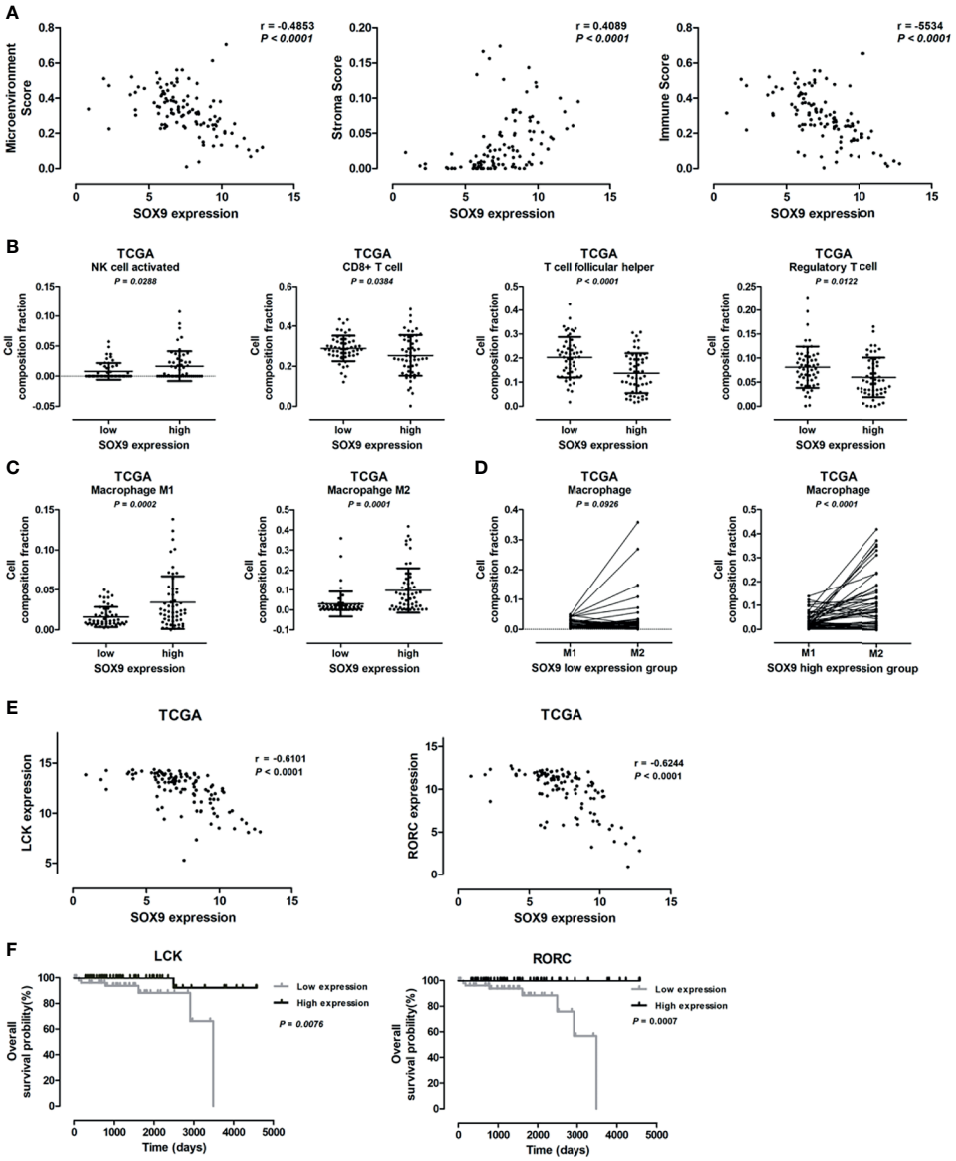

FIGURE 5 | Correlation between SOX9 expression and immune cells infiltration level. **(A)** Scatter plots illustrating the pearson correlation analysis for the associations of SOX9 expression with tumor microenvironment score, stroma score, and immune scores in thymomas, respectively. **(B)** Dot plots representing the infiltration of immune cells, including activated natural killer cell, CD8+ T cell, follicular T cell, and regulatory T cell, between patients with high and low SOX9 expressions. **(C)** Dot plots representing M1 and M2 cells between patients with high and low SOX9 expressions. **(D)** Dot plots representing the infiltration of M1 and M2 cells in patients with high or low SOX9 expression. **(E)** Scatter plots illustrating the pearson correlation analysis for the associations of SOX9 expression with LCK and RORC expression in thymomas, respectively. **(F)** Kaplan–Meier survival curves showing that thymoma patients from TCGA dataset with low LCK or RORC RNA expression had shorter overall survival time ($P < 0.05$), as determined by the log-rank test. Thymoma patients (n = 108) were categorized into high and low LCK or RORC expression groups with the median value of LCK or RORC mRNA expression level as cutoff.

in the extracellular matrix-receptor interaction pathway and the TGF-β signaling pathway, the latter of which plays important roles in regulating stromal cells and has potent immunosuppressive effects on both innate and adaptive immune cells in the tumor microenvironment (30). It has been known that immune cells are important constituents of the tumor microenvironment and critically participate in the development and progression of various tumors. Increasing evidence highlights that adaptive immune cells, including T and B cells, and innate immune cells, such as macrophages and natural killer cells, contribute to tumor progression when present in the tumor stroma (31). We observed differential activation of tumor associated macrophages, with patients with high SOX9 expression had enrichment of M2 macrophages. The M2 macrophages, which secrete anti-inflammatory cytokines such as IL-10, IL-13, and IL-4 and which express abundant

arginase-1, mannose receptor (MR, CD206), and scavenger receptor, tend to show an immune suppressive phenotype (31, 32). Preciously, it has been revealed that M2-macrophages can suppress the antitumor activity of cytotoxic CD8+ T cells within tumors (33). In line with the previous work, we observed that the number of CD8+ T cells decreased in the tumor samples of patients with high SOX9 expression, while the M2 macrophage abundance increased. Moreover, SOX9 was negatively correlated with genes associated with T or Th17 cell development, such as *LCK* and *RORC*. Survival analysis revealed that thymoma patients with high LCK or RORC had favorable clinical outcomes. Together, these findings suggested that SOX9 expression might indicate a competitive interaction between M2 macrophages and CD8+ T cells, and an immune suppressive microenvironment of thymomas, which consequently led to enhanced pro-tumorigenic activity.

To the best of our knowledge, this is the first study proposing the potential roles of SOX9 in thymoma. However, the precise mechanism of SOX9 in the initiation and progression of TETs were not well investigated in this study, due to a lack of thymic tumor cell lines and the unavailability of animal models of thymic tumors. The bioinformatics findings need to be further validated by both *in vitro* and *in vivo* experiments.

In conclusion, we comprehensively analyzed the expression profile and the diagnostic values of SOX9 in TETs based on immunohistochemistry examination and bioinformatics analysis. Our findings provided evidence that SOX9 could serve as a marker for thymic epithelial cells and as a diagnostic and prognostic marker for TETs. Notably, SOX9 expression in TETs might indicate a tuft cell phenotype and an immune suppressive microenvironment of thymomas. However, the specific role and the precise mechanism of SOX9 in the initiation and progression of TETs need to be further extensively investigated.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The study involving human participants were reviewed and approved by Anhui Medical University and the First Affiliated Hospital of USTC. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conception, design, and hypothesis: XY, LH, and YL. Clinical design and organization: XY, YL, WL, and YZ. Patients sampling and pathological experiments: WL, XY, and YL. Drafting the article: XY, LH, YL, and FY. Data discussion, reviewing and editing the article critically: XY, YL, LH, BN, and YZ. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.708735/full#supplementary-material

Supplementary Figure 1 | GSEA analysis revealed the enrichment of the gene sets related to the "pathway in cancer and TGF-β signaling pathway" in patients with high SOX9 expression.

Supplementary Figure 2 | Volcano plot showing the differentially up- and down-regulated genes in thymoma patients with high SOX9 expression compared to those with low SOX9 expression. The median value of SOX9 expression level was selected as cutoff. Differentially expressed genes (DEGs) satisfying the criteria of $|\log_2(\text{fold-change})| > 2$ with an adjusted $P < 0.05$ was considered as significant. DEGs positively and negatively correlated with SOX9 expression are represented as red and green dots, respectively.

Supplementary Figure 3 | The dot-plot depicts the activity of the KEGG pathways in thymoma patients with high and low SOX9 expressions, respectively.

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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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Evaluation Expression of miR-146a and miR-155 in Non-Small-Cell Lung Cancer Patients

*Neda K. Dezfuli[1,2], Shamila D. Alipoor[3], Neda Dalil Roofchayee[1,4], Sharareh Seyfi[5], Babak Salimi[5], Ian M. Adcock[6] and Esmaeil Mortaz[1,4]**

[1] Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran, [2] Department of Immunology and Laboratory Sciences, School of Allied Medical Sciences, Dezful University of Medical Sciences, Dezful, Iran, [3] Molecular Medicine Department, Institute of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran, [4] Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [5] Chronic Respiratory Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran, [6] Airways Disease Section, National Heart and Lung Institute, Imperial College London, London, United Kingdom

Background: Non–small-cell lung cancer (NSCLC) is the major type of lung cancer. MicroRNAs (miRNAs) are novel markers and targets in cancer therapy and can act as both tumor suppressors and oncogenes and affect immune function. The aim of this study was to investigate the expression of miR146a and miR155 in linked to blood immune cell phenotypes and serum cytokines in NSCLC patients.

Methods: Thirty-three NSCLC patients and 30 healthy subjects were enrolled in this study. The allele frequencies of potential DNA polymorphisms were studied using polymerase chain reaction (PCR)–restriction fragment length polymorphism (PCR-RFLP) analysis in peripheral blood samples. Quantitative reverse transcription PCR (qRT-PCR) was used to measure the expression of miR-146a and miR-155 in peripheral blood mononuclear cells (PBMCs). Serum cytokine (IL-1β, IL-6, TNF-α, TGF-β, IL-4, IFN-γ) levels were determined by ELISA. The frequency of circulating CD3+CTLA-4+ and CD4+CD25+ FOXP3+ (T regulatory cells/Treg) expression was measured by flow cytometry.

Results: miR-146a was significantly downregulated in PBMC of NSCLC patients ($P \leq 0.001$). Moreover, IL-6 and TGF-β levels were elevated in NSCLC patients ($P \leq 0.001$, $P \leq 0.018$, respectively). CD3+ CTLA-4+ and Treg cells frequencies were higher in patients than in control subjects ($P \leq 0.0001$, $P \leq 0.0001$, respectively). There was a positive correlation between miR-155 and IL-1β levels ($r=0.567$, $p \leq 0.001$) and a negative correlation between miR-146a and TGF-β levels ($r=-0.376$, $P \leq 0.031$) in NSCLC patients. No significant differences were found in the relative expression of miR-146a and miR-155, cytokine levels or immune cell numbers according to miR-146a and miR-155 (GG/GC/CC, TT/AT/AA) genotypes. However, there was a positive correlation between miR-146a and IL-1β levels ($r=0.74$, $P \leq 0.009$) in GG subjects and a positive correlation between miR-146a expression and CD3+CTLA4+ cell frequency ($r=0.79$, $P \leq 0.01$) in CC genotyped subjects. Conversely, a negative correlation between miR-146a

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***Correspondence:**
Esmaeil Mortaz
emortaz@gmail.com

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expression and Treg cell frequency (r=–0.87, P ≤ 0.05) was observed with the GG genotype. A positive correlation between miR-155 and IL-1β expression (r=0.58, p ≤ 0.009) in the TT genotype and between miR-155 expression and CD3+CTLA-4 cell frequency (r=0.75, P ≤ 0.01) was observed in the AT genotype.

Conclusions: The current data suggest that the miR-146a expression in PBMC and serum TGF-β and IL-1β levels may act as blood markers in NSCLC patients. Further study is needed to elucidate the link between immune cells and serum miR146 at early disease stages.

Keywords: cytokines, immune system, NSCLC, miR-155, miR-146a

INTRODUCTION

Lung cancer is one of the leading causes of cancer mortality worldwide, accounting for more than 1.4 million deaths per year (1). The two major types of lung cancer are non-small-cell lung cancer (NSCLC) (responsible for 85% of all lung cancers) and small-cell lung cancer (about 15% of all lung cancer) (2). Despite improvements in early diagnosis and new therapeutic strategies, 5-year survival rates remain at 10–20% (1). The poor prognosis is due to various factors including diagnosis at advanced disease stages, tumor heterogeneity, and relatively limited understanding of lung cancer biology (3). In the last decade, immune checkpoint antibodies against markers of exhausted T cells such as PD-1 (programmed death protein)/PD-L1 (programmed death protein ligand) and CTLA-4 (cytotoxic T-cell lymphocyte antigen 4) have been successful in treating multiple solid tumor malignancies including lung cancer (4, 5).

Surgical resection is the most common treatment for early-stage tumors and is used in combination with chemotherapeutic agents for advanced lung cancer patients. In addition, chemotherapy treatment is required for metastatic disease (6–8). Recent studies have shown large increases in the survival of lung cancer patients since the introduction of targeted and immune-based therapies.

Early detection of lung cancer is critical (9), and the immune system is a key player in the development and progression of cancers (10). Tumors often arise at the sites of chronic inflammation linked to the presence of distinct immune cells in the tumor milieu. The immune system plays a critical role in the progression of cancer by releasing pro- or antitumorigenic factors (11). Non-coding microRNAs (mRNAs) are novel mediators of the immune response associated with inflammation and cancer development (12). miRNA expression is important in tumor cell function, and they indicate disease progression and response to therapy (13). Dysregulated miRNA production has been reported in several chronic inflammatory diseases (14) where they modulate immune responses. In particular, miR-146 (146a and 146b) and miR-155 have been reported as being essential in regulating the immune system (15).

Previously, we have shown that miR-146a rs2910164 and miR-155 rs767649 polymorphisms may act as genetic risk factors for the susceptibility to Iranian NSCLC patients (16). Studies show an anti-inflammatory function of miR-146a, whereas miR-155 has an inflammatory function (15, 17, 18). Thus, understanding the pattern expression of these miRNAs could be useful in following cancer pathogenesis and progression (19). We hypothesize that relative levels of peripheral blood miR-146a and miR-155 expression may be used to diagnose NSCLC. We, therefore, evaluated miR-146a and miR-155 expression in PBMC and correlated this with blood Treg, CD3+CTLA-4+ cell, and serum cytokine levels in NSCLC patients.

MATERIAL AND METHODS

Study Participants

Thirty-three patients with newly diagnosed NSCLC (57.9 ± 9.5 years old, mean ± SD) were recruited at the Masih Daneshvari Hospital (Tehran, Iran) between April 2017 and September 2019. Histology and clinical parameters confirmed the presence of lung cancer, and patients were not on any treatment and had no history of other cancers or inflammatory diseases. Age- and sex-matched controls (n=30) were also recruited following a general health check and a negative history of cancer and inflammatory diseases (**Table 1**). The Ethics Committee of Shahid Beheshti University of Medical Sciences approved the study, and all subjects gave written informed consent (Ethics committee approval number: IR.SBMU.MSP.REC.1397.525).

Sampling Procedure

Ten ml whole blood samples from healthy control and patients groups was collected into separate tubes containing blood clot activating gel for obtaining serum (cytokine assay), heparin tubes for PBMC isolation, and EDTA tubes for flow cytometry.

Genotyping of miR-146a and miR-155 for Possible SNP

Genomic DNA was isolated from peripheral blood cells using a DNA extraction kit (High Pure PCR template preparation kit, Roche, Germany, cat. No.11796828001) according to the manufacturer's instructions. The concentration and quality of

TABLE 1 | Demographic information of participants.

Parameters	Lung cancer, n = 33	Control, n = 30
Age (Years, Mean ± SD)	57.9 ± 9.5	53.3 ± 7.7
Gender (n, %)		
Female	9	9
Male	24	21
Smoking status (n, %)		
Smoker	16	13
Non-smoker	17	17
Histological subtype (n, %)		
ADC	27	
LCC	1	
SCC	5	
Stage (n, %)		
I	1	
II	3	
III	8	
IV	21	
MiR-146a Genotype		
GG	11	10
GC	14	14
CC	8	6
MiR-155 Genotype		
TT	19	17
AT	10	9
AA	4	4

ADC, adenocarcinoma; LCC, large-cell carcinoma; SCC, squamous cell carcinoma.

DNA was measured by Nanodrop 2000 (Thermo Fisher Scientific, USA). Specific SNPs (Rs2910164 and Rs767649) were genotyped using PCR-Restriction Fragment Length Polymorphism (RFLP). PCR reactions were performed using super PCR master mix (YEKTA TAJHIZ AZMA, Tehran, Iran, Cat No: YT1553-YT1554) using a Thermal Cycler (Bio-Rad, CA, USA). The purity of the samples was assessed using 260/280 nm and 260/230 nm ratios, and the concentrations of isolated DNA from healthy subjects and patients are presented in **Supplementary Tables 1, 2**. The primer sequences for each PCR reaction are shown in **Table 2**. The cycle parameters for the PCR analysis were as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. To identify the miR-146 C/G polymorphism, the PCR product was digested with the restriction enzyme mnlI (Thermo Fisher, USA, Cat No. ER1071) at 37°C for 4 h. The PCR product (miR-155 T/A polymorphism) was incubated at 37°C for 4 h with the restriction enzyme TSP45I (Thermo Fisher, USA, Cat No. ER1511) and the digestion products detected by 3% agarose gel electrophoresis.

PBMCs Isolation

Whole blood (5 ml) was collected in heparin-containing tubes, and peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation. Briefly, the blood was diluted with equal volume of PBS buffer and then slowly added to 5 ml lymphocyte separation medium (Ficoll Paque, BAG Health Care GmbH, Germany, Cat No: 70125). After centrifuging at 278 × g at room temperature (RT) for 30 min, the supernatant was removed and the cell pellet washed with cold PBS. After one more wash and centrifugation, the supernatant was removed and 1 ml TRIzol (Invitrogen, CA, USA) was added to the cell pellet and stored at −80°C for isolation of RNA as described below.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from isolated PBMCs as described earlier (20). Briefly, cells containing TRIzol were treated by chloroform (Merck, Germany), and after isopropanol (Merck, Germany) sedimentation and ethanol washing, total RNA was diluted in sterile DEPC-treated water. The concentration and purity of RNA was determined by Nanodrop 2000 spectrophotometer (**Supplementary Tables 1, 2**). Extracted RNA was reverse transcribed using the miRCURY LNA Universal RT microRNA cDNA Synthesis Kit (miRCURY LNA RT Kit-QIAGEN, MD, USA) according to the manufacturer's instructions.

Quantitative RT-PCR Analysis

miR146a and miR-155 were detected by real-time PCR assays by using the SYBR Green Master Mix kit (QIAGEN, MD, USA). miRNA primers were purchased from QIAGEN (has-miR-146A-5p, Cat. No YP00204688; has-miR-155-5p, Cat no. YP00204308), and quantitative PCR was performed using Real-time PCR (Roche, Manheim, Germany). The real-time PCR program included the following steps: an initial denaturation step at 95°C for 10 min; 45 amplification cycles that consisted of a denaturation step (10 s at 95°C) and an annealing step (60 s at 60°C). Expression levels of miRNAs were normalized to the level of miR-16 (QIAGEN, MD, USA) as a control miRNA using the $2^{-\Delta\Delta Ct}$ method.

Flow Cytometry Assay

Two ml whole blood containing EDTA was collected from participants. To determine the immunophenotyping of T cells, surface staining of CD4 and CD25 markers was performed using mouse antihuman CD25-FITC (Biolegend, San Diego, CA, USA)

TABLE 2 | PCR primer sequences used and expected fragment sizes.

Polymorphism	Primer sequence	Restriction enzyme	Product size (bp)
Rs2910164	F: 5'-AGAACTGAATTCCATGGGTTG-3' R: 5'-TGCTTAGCATAGAATTCAAGTC-3'	mnlI	Uncut product: 248 G Allele: 171 + 77 C Allele: 171 + 45+32
Rs767649	F: 5'-CCT GTA TGA CAA GGT TGT GTT TG-3' R: 5'-GCT GGC ATA CTA TTC TAC CCA TAA-3'	TSP451	Uncut product: 294 A Allele: 252 + 42 T Allele: 158 + 94+42

and CD4-PE (Immunostep, Salamanca, Spain) for 30 min at 4°C. Cells were then washed and incubated with fixation and permeabilization solution buffer (BD Biosciences, San Diego, CA, USA) for 15 min at 4°C. Subsequently, cells were washed with cold PBS and intracellular staining performed using a FOXP3-APC antibody (eBioscience, CA, USA) for 30 min in 4°C. Isotype-matched antibodies were used as controls for all the samples. Separate cells were incubated with CTLA-4-PE (Biolegend, San Diego, CA, USA) and CD3-APC (Biolegend, San Diego, CA, USA), for 30 min at 4°C with isotype-matched antibodies used as controls. Ten thousand events were evaluated by FACS Calibur (BD Biosciences). Data were processed using Flow Jo software version 8.

Cytokine Analysis

Three ml whole blood in tubes without anticoagulants was harvested and after isolation of serum stored at −80°C. The levels of cytokines including IL-1β (R&D, Minneapolis, USA), IL-6 (R&D, Minneapolis, USA), TNF-α (R&D), IL-4 (Invitrogen, Vienna, Austria), IFN-γ (Invitrogen), and TGF-β1 (eBioscience, CA, USA) were measured in the serum of all participants according to the manufacturer's instructions. The optical density was read by an ELISA plate-reader at a wavelength of 450 nm with a reference wavelength of 545 nm. All the assays were performed in duplicate on the same plate to be able to compare the groups.

Statistical Analysis

Results were presented as the mean ± standard deviation (SD). Comparisons between two groups were analyzed using a Student's *t*-test for the variables with a normal distribution and by the non-parametric Mann-Whitney U test for non-normally distributed data. Differences among multiple groups were compared using one-way analysis of variance (ANOVA) followed by a *post-hoc* Dunnett's test. Receiver operating characteristic curve (ROC) analysis was applied to evaluate the potential of miRNA levels as diagnostic markers. Pearson correlation analysis was applied to measure the linear correlation between two sets of data. All statistical tests were carried out using SPSS-25 software (SPSS, Inc.). Graph Pad Prism software was used for drawing graphs. A P value ≤0.05 was considered statistically significant.

RESULTS

Genotyping

Genotyping of patients and controls was performed by PCR-RFLP. The number of patients possessing miR-146a (GG/GC/CC) and miR-155 (TT/AT/AA) genotypes is shown together with their demographics in **Table 1**.

Expression of miR-146a and miR-155 in PBMCs of NSCLC Patients

miR-146a was significantly downregulated in PBMCs from NSCLC patients (P ≤ 0.001, **Figure 1A**), whereas miR-155 expression was not significantly different between NSCLC patients and the healthy control group (**Figure 1A**). ROC analysis of miR146 expression gave an AUC=0.859 (P ≤ 0.0001, Sensitivity: 81.82%, Specificity: 90%), whereas the criterion was >0.405 in NSCLC patients (**Figure 1B**). No significant differences were seen with the ROC

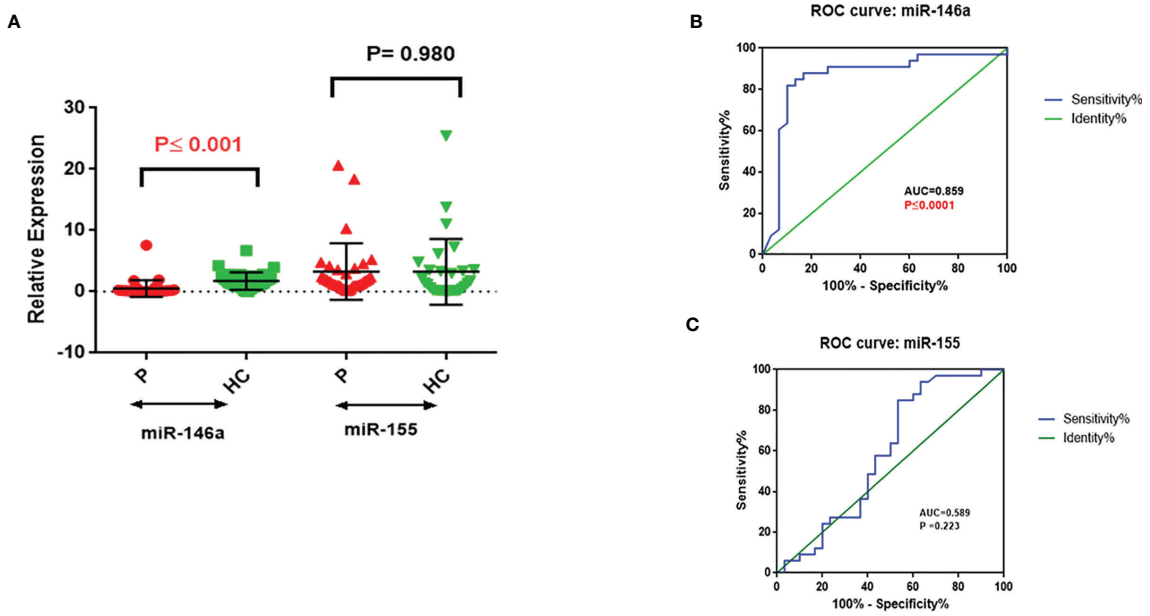

FIGURE 1 | Relative expression of miR-146a and miR-155 in PBMC with ROC correlation in NSCLC and healthy control. **(A)** miR-146a and miR-155 relative expression; miR-146a significantly downregulated in NSCLC PBMCs. **(B)** miR-146a and **(C)** miR-155 expression level ROC curve; miR-146a identification of NSCLC patients from healthy controls.

analysis of miR-155 as a predictor of NSCLC (AUC=0.589, P=0.223) (**Figure 1C**).

Serum Cytokines

IL-6 and TGF-β levels in NSCLC patients were significantly higher than in healthy controls (P ≤ 0.001 and P ≤ 0.018, respectively). There were no differences in TNF-α, IL-1β, IFN-γ, and IL-4 levels between control subjects and NSCLC patients (**Table 3** and **Figure 2**).

Immunophenotyping of Treg and CD3+ CTLA-4+ Lymphocytes

The immunophenotyping gating strategy for Treg (CD4+ CD25+ FOXP3+) and CD3+ CTLA-4+ T cells is depicted in representative samples from participants in **Figure 3A** (Upper panel for Treg cells and lower panel for CD3+CTLA-4+ cells). The frequency of Treg cells in PBMCs from NSCLC patients was five-fold greater than in healthy controls (10.3 *vs* 2.1%, P≤0.0001, **Figure 3B** left panel), whereas that for CD3+CTLA4+bearing lymphocytes was 10-fold greater in NSCLC patients compared to healthy controls (49.3 *vs* 4.8%, P≤0.0001, **Figure 3B** right panel) (**Table 4**).

Correlation Analysis

No correlation was seen between any of the variables analyzed with the type and stages of NSCLC except for significantly reduced IL-6 levels among patients with Stage III disease (**Table 5**). There was a negative correlation between miR-146a and TGF-β expression (r=−0.376, P ≤ 0.031, **Table 6** and **Figure 4A**) and a positive correlation between miR-155 and IL-1β levels (r=0.567, p ≤ 0.001, **Table 6** and **Figure 4B**).

TABLE 3 | Cytokine results in patients and control groups.

Cytokine	Groups				P value (2-tailed)
	Patients (33)		Controls (30)		
	Mean (SD) pg/ml	Range (Min–Max) pg/ml	Mean (SD) pg/ml	Range (Min–Max) pg/ml	
IL-1β	4.5 (11.8)	0.01–19.3	0.73 (3.5)	0.01–62.7	0.089
IL-6	37.2 (41.2)	2.1–170.6	8.9 (11.0)	1.5–41.2	0.001
TNF-α	0.97 (5.4)	0.01–31.2	2.4 (7.9)	0.01–37.9	0.395
IFN-γ	8.1 (35.5)	0.01–202.4	5.9 (18.1)	0.01–100.3	0.759
IL-4	1.8 (10.3)	0.01–59.7	1.3 (3.2)	0.01–12.3	0.809
TGF-β	809.6 (349.9)	150.4–1665.7	623.7 (243.8)	0.00–1258.7	0.018

Comparisons between the groups were performed using Student's t-test for the variables with a normal distribution and non-parametric Mann-Whitney U test for non-normally distributed data. The values in red font are significant.

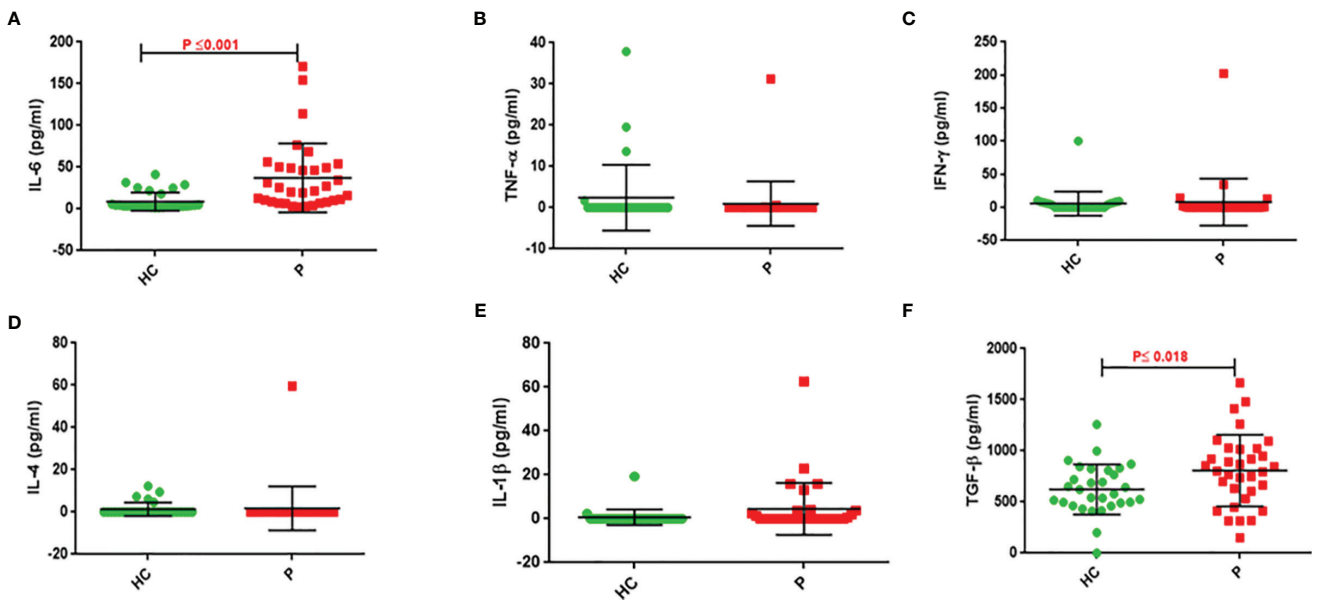

FIGURE 2 | Serum cytokine levels of patients compared to control group. Serum IL-6 **(A)**, TNF-α **(B)**, IFN-γ **(C)**, IL-4 **(D)**, IL-1β **(E)**, and TGF-β **(F)** concentrations in serum of NSCLC patients and healthy subjects. Serum levels of IL-6 and TGF-β in NSCLC patients are significantly higher than that of controls (P ≤0.001 and 0 ≤ 0.018, respectively).

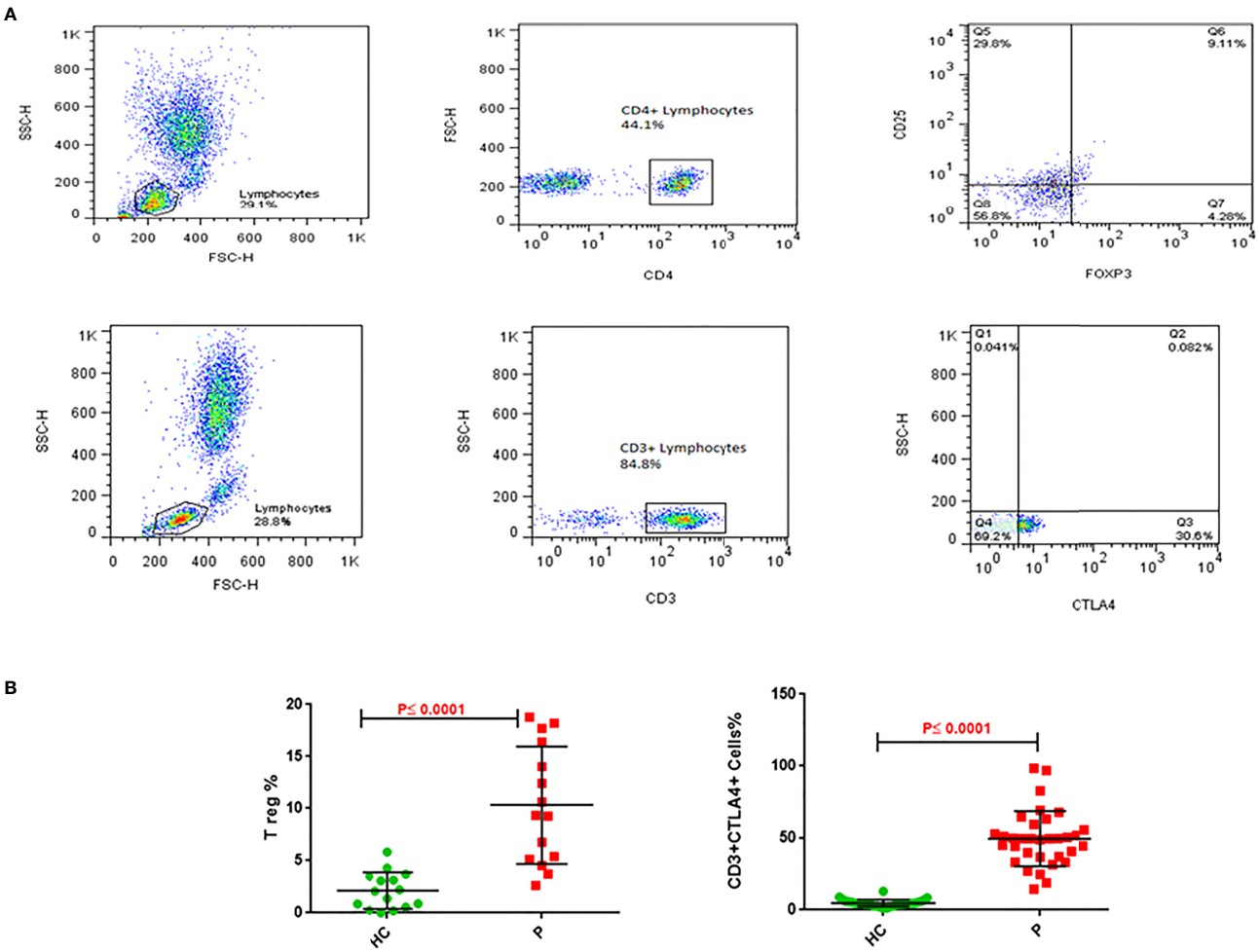

FIGURE 3 | Treg and CD3/CTLA-4 cells in blood of NSCLC patients. (A) Upper panel: Gating strategy for Treg cells frequency; analysis of a blood sample for CD25, as well as FoxP3 expression in the CD4+ lymphocyte gate. **(A)** Lower panel: Gating strategy for CD3+CTLA4+ cells percentage. Analysis of a blood sample for CTLA4 expression in the CD3+ lymphocyte gate. **(B)** Flow cytometry analysis of frequencies Treg and CD3/CTLA-4 in blood cells stained with related antibodies of NSCLC and healthy control subjects, which was calculated statistically and plotted to the graph (P ≤ 0.0001). The dots indicate the means, and the error bars indicate the standard deviations of all patients and healthy control measurements.

TABLE 4 | Flow cytometry results of Treg and CD3/CTLA4 in patients and control.

	Groups	N	Mean (Min–Max)	SD	P value (2-tailed)
CD4+CD25+Foxp3+ lymphocytes	Patients	15	10.3% (2.6–18.8%)	5.6	≤0.0001
	Control	15	2.1% (0.00–5.8%)	1.7	
CD3+CTLA4+ lymphocytes	Patients	33	49.3% (14.5–98.2%)	19.1	≤0.0001
	Control	30	4.8% (1.1–12.9%)	2.4	

Comparisons between the groups were performed using Student's t-test for the variables with a normal distribution and non-parametric Mann-Whitney U test for non-normally distributed data. N; numbers.
The values in red font are significant.

There was no significant effect of the Rs2910164 genotypes (GG/GC/CC) on miR-146a expression or on the levels of cytokines and Treg and CD3+CTLA-4+ cells in NSCLC patients and control subjects (**Table 7**). Furthermore, there was no effect of the Rs767649 genotypes (TT/AT/AA) on miR-155 expression levels or on the levels of cytokines, Treg, and CD3/CTLA-4 cells in the patient and control groups (**Table 8**).

In contrast, there was a positive correlation between miR-146a and IL-1β levels (r=0.74, P ≤ 0.009) with the GG

TABLE 5 | Data analysis on correlation of NSCLC patients with type and stages of diseases with studied parameters.

Parameters	NSCLC type	N	Mean ± SD	P value ADC&SCC	NSCLC Stage	N	Mean ± SD	P value II&III	P value II&IV	P value III&IV
miR-146a expression	ADC	27	0.29 ± 0.49	0.33	II	3	0.25 ± 0.37	0.50	0.95	0.27
					III	8	1.33 ± 2.59			
	SCC	5	1.69 ± 3.3		IV	21	0.23 ± 0.43			
miR-155 expression	ADC	27	3.56 ± 5.06	0.49	II	3	2.07 ± 1.36	0.98	0.64	0.44
					III	8	2.04 ± 1.67			
	SCC	5	1.95 ± 1.6		IV	21	3.58 ± 5.46			
IL-1β (pg/ml)	ADC	27	4.59 ± 12.5	0.94	II	3	0.01 ± 0.0	0.50	0.48	0.63
					III	8	3.2 ± 7.96			
	SCC	5	4.99 ± 10		IV	21	5.84 ± 14.0			
IL-6 (pg/ml)	ADC	27	37.2 ± 41.8	0.80	II	3	71.6 ± 37.1	0.03	0.25	0.41
					III	8	24.5 ± 23.7			
	SCC	5	42.4 ± 45.3		IV	21	38.6 ± 45.8			
TNF-α (pg/ml)	ADC	27	1.17 ± 6.0	0.70	II	3	0.01 ± 0.0	1.0	0.70	0.53
					III	8	0.01 ± 0.0			
	SCC	5	0.12 ± 0.25		IV	21	1.53 ± 6.80			
IFN-γ (pg/ml)	ADC	27	9.91 ± 39.1	0.58	II	3	0.01 ± 0.0	1.0	0.62	0.42
					III	8	0.01 ± 0.0			
	SCC	5	0.01 ± 0.01		IV	21	12.7 ± 44.2			
IL-4 (pg/ml)	ADC	27	2.22 ± 11.4	0.67	II	3	0.01 ± 0.0	1.0	0.71	0.54
					III	8	0.01 ± 0.0			
	SCC	5	0.01 ± 0.0		IV	21	2.85 ± 13.0			
TGF-β (pg/ml)	ADC	27	839.4 ± 345.3	0.24	II	3	819.2 ± 185.3	0.94	0.93	0.99
					III	8	800.1 ± 478.0			
	SCC	5	637.0 ± 400.7		IV	21	802.0 ± 332.1			
Treg Cells (%)	ADC	12	10.42 ± 6.0	0.89	II	3	9.08 ± 5.83	0.38	0.90	0.26
					III	3	13.98 ± 6.37			
	SCC	3	9.90 ± 4.4		IV	9	9.51 ± 5.53			
CD3+CTLA4+ Cells (%)	ADC	27	47.4 ± 18.4	0.12	II	3	67.1 ± 26.9	0.36	0.07	0.33
					III	8	53.1 ± 19.5			
	SCC	5	62.0 ± 21.0		IV	21	45.2 ± 17.5			

Comparisons between NSCLC types and stages were performed using Student's t-test for the variables with a normal distribution and non-parametric Mann-Whitney U test for non-normally distributed data.
The values in red font are significant.

genotype and with the frequency of CD3+CTLA4+ cells (r=0.79, P ≤ 0.01) in CC genotype. Finally, there was a negative correlation between miR-146a expression and Treg cell frequency (r=−0.87, P ≤ 0.05) in patients with the GG genotype; a positive correlation between miR-155 and IL-1β (r=0.58, p ≤ 0.009) in the TT genotype and with the frequency of CD3+CTLA4+ cells (r=0.75, P ≤ 0.01) in patients with the AT genotype (**Table 9**).

DISCUSSION

We found that miR-146a expression in PBMCs is downregulated in NSCLC patients compared to healthy control subjects. In contrast, serum IL-6 and TGF-β levels as well as CD3+ CTLA4+ and Treg cell frequencies in blood were elevated in NSCLC patients. In addition, the expression of miR-146a is negatively associated with a high serum level of TGF-β. No

TABLE 6 | Association between miR-146a and miR-155 expression with cytokines and Treg and CD3/CTALA-4 in patients.

Parameters	N	MiR-146a Expression		MiR-155 Expression	
		Pearson correlation (r)	P value (2-tailed)	Pearson correlation (r)	P value (2-tailed)
IL-1β (pg/ml)	33	0.224	0.209	0.567	0.001
IL-6 (pg/ml)	33	−0.243	0.173	−0.150	0.403
TNF-α (pg/ml)	33	−0.055	0.761	−0.016	0.931
IFN–γ (pg/ml)	33	−0.085	0.639	−0.089	0.624
IL-4 (pg/ml)	33	0.191	0.288	−0.078	0.666
TGF-β (pg/ml)	33	−0.376	0.031	0.030	0.869
CD4+CD25+Foxp3+ lymphocytes (%)	15	−0.296	0.283	0.415	0.124
CD3+CTLA4+ Lymphocytes (%)	33	−0.056	0.757	0.024	0.894

Data are analyzed using linear regression, and r values are from Pearson's correlation coefficient test.
The values in red font are significant.

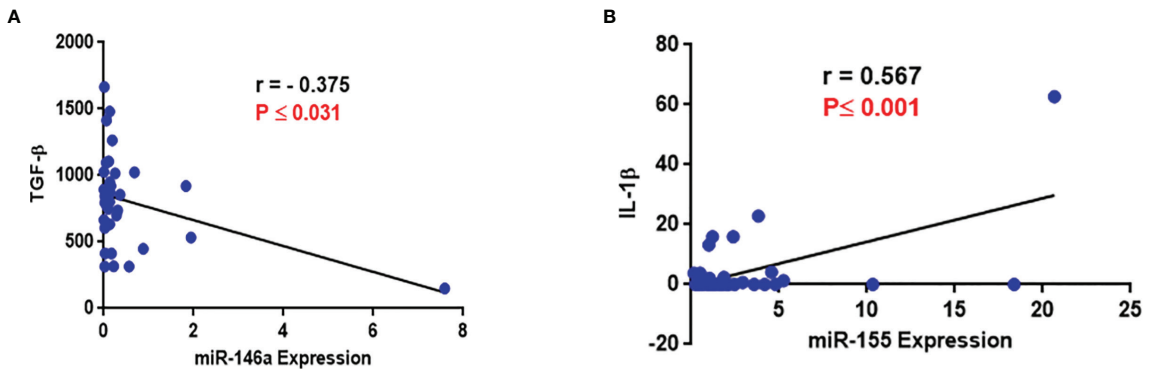

FIGURE 4 | Evaluation of correlation of miR-146a and miR-155 with cytokines **(A)** Correlation between miR-146a and TGF-β level; a negative correlation between miR-146a and TGF-β level was observed. **(B)** Correlation between miR-155 and IL-1β level; a positive correlation between miR-155 and IL-1β level was observed.

differences in the expression of miR-155 between patients and controls was found, although miR-155 expression positively associated with higher serum levels of IL-1β. In addition, there was a positive correlation between miR-146a expression and IL-1β levels in patients with a GG genotype and with CD3+CTLA4+ cells frequency in the CC genotype. There was a negative correlation between miR-146a and Treg cells frequency in patients with a GG genotype. Finally, there was a positive correlation between miR-155 expression and IL-1β in the TT genotype and CD3+CTLA4+ cells frequency in the AT genotype.

To our knowledge this is the first study showing miR-146a and miR-155 expression levels in PBMC of NSCLC patients. The importance of miR-146a downregulation and higher levels of serum cytokines, Treg, and CTLA4+ cells in relation to the biological and clinical aspects of NSCLC needs to be further examined. miR-146a is involved in the development of various cancers and in suppressing inflammation through the modulation of the innate immune response (21, 22). Larger, multicentered studies should investigate whether this correlation between TGF-β and IL-1β with the expression of miR-146a and miR-155 is validated and has a clinical impact in the pathophysiology of NSCLC patients.

miR-146a and miR-146b are members of the miR-146 family, which are found on chromosomes 5 and 10. miR-146a and miR-146b have similar structure but a different mature sequence (23). miR-146a has an important role in cell signaling and regulation of toll like receptor (TLR) pathways (24). Knockout of miR-146a leads to excessive production of inflammatory cytokines such as TNF-α and IL-6, which, in turn, induces chronic inflammation and increases susceptibility to cancer and loss of Treg cell function (25). Indeed, miR-146a suppresses the growth and migration and induces apoptosis of NSCLC cells (26). miR-146a also induces G0/G1 cell cycle, which may suppress the proliferation of lung cancer cells (27). Interestingly, miR-146a expression is significantly lower in lung cancer tissue, which suggests that it acts as a tumor suppressor *via* targeting EGFR expression (26). EGFR-tyrosine kinase inhibitors (EGFR-TKIs) are used successfully as targeted therapies in lungs cancer (26, 28).

High levels of miR-146a-5p are seen in the serum and tissue of NSCLC patients (29). miRNA146a-5P has effects on the survival and proliferation of NSCLC cells through binding and suppression of TRAF-6 (29). Interestingly, the expression of serum miR-146a, but not miR-155-5p, is increased in patients progressing from stage I to stage II NSCLC (30). Indeed, elevated miR-146a levels in serum exosomes have been proposed as a diagnostic marker in the early stages of NSCLC (30). However, poor prognosis has shown related to the lower serum levels and tissue expression of miR146a (31). In the current study we did not find any significant changes in the expression of miR-146 or

TABLE 7 | Effect of the Rs2910164 genotypes on miR-146a expression, cytokines, and Treg, CD3+CTLA-4+ cells.

Parameters	Genotype	Patients				Controls			
		N	Mean	SD	P	N	Mean	SD	P
miR-146a expression	GG	11	1.22	2.22	0.092	10	2.45	1.90	0.081
	GC	14	0.12	0.14		14	1.62	1.05	
	CC	8	0.16	0.22		6	0.87	0.46	
IL-1β (pg/ml)	GG	11	3.59	7.97	0.593	10	2.17	6.07	0.29
	GC	14	3.05	5.11		14	0.01	0.00	
	CC	8	8.32	22.02		6	0.01	0.00	
IL-6 (pg/ml)	GG	11	16.67	21.93	0.067	10	3.18	1.04	0.061
	GC	14	54.74	53.32		14	9.89	12.02	
	CC	8	34.93	23.26		6	16.18	13.99	
TNF-α (pg/ml)	GG	11	2.84	9.41	0.388	10	0.01	0.00	0.207
	GC	14	0.02	0.03		14	5.20	11.19	
	CC	8	0.08	0.19		6	0.01	0.00	
IFN-γ (pg/ml)	GG	11	1.25	3.91	0.401	10	2.10	3.46	0.650
	GC	14	18.04	53.93		14	9.16	26.47	
	CC	8	0.28	0.69		6	4.71	4.11	
IL-4 (pg/ml)	GG	11	5.44	18.00	0.380	10	1.81	2.97	0.596
	GC	14	0.01	0.00		14	0.68	2.53	
	CC	8	0.01	0.00		6	2.06	5.03	
TGF-β (pg/ml)	GG	11	715.81	340.98	0.112	10	558.30	135.77	0.218
	GC	14	756.33	289.71		14	707.10	251.46	
	CC	8	103.19	402.48		6	538.13	332.41	
CD4+CD25+Foxp3+ lymphocytes (%)	GG	5	11.42	6.47	0.845	5	1.34	1.45	0.324
	GC	5	9.20	6.07		5	3.03	2.33	
	CC	5	10.34	5.42		5	2.01	1.11	
CD3+CTLA4+ Lymphocytes (%)	GG	11	44.23	13.44	0.548	10	4.80	3.24	0.726
	GC	14	50.95	21.69		14	1.83	0.48	
	CC	8	53.48	21.72		6	2.37	0.96	

Differences among three groups were compared using one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test.

miR-155 in type and stages of NSCLC patients, indicating that progression through disease stages did not affect the expression of these miRNAs.

miR-155 expression is increased in the lung tissue of NSCLC patients and correlates with disease progression (32). miR-155 increases the survival of Treg cells by increasing sensitivity of these cells to IL-2 *via* attenuating suppressor of cytokine signaling 1 (SOCS1) pathways (15). Moreover, miR-155 positively feedbacks on NF-κB signaling by inhibiting SH-2 containing inositol 5' polyphosphatase 1 (SHIP-1) and SOCS1 (33). miR-155 also acts as a positive regulator of cytokine production in macrophages (34). Upregulation of miR-155 expression in lung cancer tissues, plasma, and sputum is associated with an increased risk of NSCLC where there are no current good diagnostic markers (35–37). Moreover, miR-125a-3p, miR-125b-5p, miR-155-5p are reported to be increased in stage I of lung adenocarcinoma (38). In the current study, no significant difference in the expression of miR-155 in the PBMC of NSCLC and healthy controls was seen, although miR-155 is significantly upregulated in several NSCLC cell lines (SPC-A-1, A549, H2170) (39).

We show that CD3/CTLA4 frequency was markedly higher in NSCLC patients and that this positively correlated with miR146a in patients with a CC genotype. CTLA-4 is a T cell-restricted immune checkpoint that inhibits the T-cell response when it attaches to B7 on antigen-presenting cells (APCs). Moreover, CTLA-4 suppresses IL-2 production and thereby blocks cell-cycle progression, leading to induction and maintenance of T-cell tolerance. Under physiological conditions, CTLA-4 decreased the T-cell response to foreign antigens as well as to autoantigens. T-cell expression of CTLA-4 is elevated by TGF-β, a suppressive cytokine secreted by the tumor cells (40–42). We report here a negative correlation of miR-146 expression with TGF-β. This suggests that using miR-146a may be able to be used as a prediction of the clinical response of advanced NSCLC patients to immune checkpoint inhibitors (ICI), and this may be considered a limitation of the study and should be investigated in future studies. miR-146 tightly regulates cytokines such as TNF-α and IL-1β through different signaling pathways including NF-κB and MEK-1/2 and JNK-1/2 (43). miR-146a has a major effect on programmed cell death, and its overexpression suppresses cell migration and proliferation in NSCLC cell lines and has potential as a tumor-suppressive and anti-inflammatory agent. In addition, mir-155 is involved in the crosstalk between cancer and inflammation and additional research in this (44).

TGF-β is linked with cancer progression and is associated with poor prognosis of NSCLC patients (45, 46). We show elevated serum TGF-β levels in NSCLC patients that negatively correlated with the expression of miR-146a in PBMC. Interestingly, patients with high serum levels of miR-146a achieved a higher overall response rate to therapies and a longer survival time (31).

IL-6 plays an important role in early stages of lung cancer and potentiates immune responses resulting in cell proliferation and expansion of the tumor mass (47). Higher serum IL-6 levels in NSCLC patients in the current study was not correlated with miR-146a or miR-155 expression, although a positive correlation

TABLE 8 | Effect of the Rs767649 genotypes on miR-155 expression, cytokines, and Treg, CD3+CTLA-4+ cells.

Parameters	Genotype	Patients				Controls			
		N	Mean	SD	P	N	Mean	SD	P
miR-155 Expression	TT	19	4.69	5.75	0.124	17	5.11	6.53	0.084
	AT	10	1.36	0.46		9	1.10	1.14	
	AA	4	1.35	0.69		4	0.14	0.06	
IL-1β (pg/ml)	TT	19	6.18	14.98	0.597	17	0.01	0.00	0.239
	AT	10	3.15	6.08		9	2.41	6.39	
	AA	4	0.01	0.00		4	0.01	0.00	
IL-6 (pg/ml)	TT	19	37.76	40.21	0.810	17	8.14	9.62	0.399
	AT	10	41.18	50.96		9	12.60	15.02	
	AA	4	24.97	20.77		4	3.92	1.54	
TNF-α (pg/ml)	TT	19	1.66	7.16	0.714	17	4.28	10.29	0.356
	AT	10	0.01	0.00		9	0.01	0.00	
	AA	4	0.15	0.28		4	0.01	0.00	
IFN-γ (pg/ml)	TT	19	1.60	4.30	0.257	17	8.93	23.87	0.596
	AT	10	23.73	63.71		9	1.63	3.37	
	AA	4	0.22	0.42		4	2.73	3.39	
IL-4 (pg/ml)	TT	19	0.01	0.00	0.327	17	1.43	3.46	0.673
	AT	10	5.98	18.88		9	1.74	3.54	
	AA	4	0.01	0.00		4	0.01	0.00	
TGF-β (pg/ml)	TT	19	747.51	362.40	0.342	17	674.65	297.33	0.437
	AT	10	946.76	346.64		9	552.42	118.36	
	AA	4	761.86	260.50		4	567.59	178.42	
CD4+CD25+Foxp3+ lymphocytes (%)	TT	6	12.30	4.78	0.470	6	3.10	1.82	0.175
	AT	6	8.14	6.26		6	1.75	1.62	
	AA	3	10.71	6.40		3	0.94	0.99	
CD3+CTLA4+ Lymphocytes (%)	TT	19	53.06	15.39	0.432	17	4.90	1.81	0.447
	AT	10	43.69	26.72		9	5.23	3.53	
	AA	4	45.67	10.96		4	3.39	1.67	

Difference among three groups was compared using one–way analysis of variance (ANOVA) followed by post-hoc Dunnett's test.

TABLE 9 | Correlation between miR-146a and miR-155 expression with the level of cytokines, Treg, and CD3+CTLA-4+ cells in patients based on genotypes.

Parameter	Genotype	N	miR-146a Expression		Genotype	N	miR-155 Expression	
			Pearson correlation(r)	P value(2-tailed)			Pearson correlation(r)	P value(2-tailed)
IL-1β (pg/ml)	GG	11	0.74	0.009	TT	19	0.58	0.009
	GC	14	–0.30	0.29	AT	10	–0.25	0.48
	CC	8	–0.08	0.84	AA	4	–0.22	0.77
IL-6 (pg/ml)	GG	11	–0.34	0.30	TT	19	–0.21	0.38
	GC	14	–0.27	0.35	AT	10	–0.25	0.48
	CC	8	0.22	0.59	AA	4	–0.54	0.45
TNF-α (pg/ml)	GG	11	–0.16	0.62	TT	19	–0.7	0.76
	GC	14	–0.21	0.46	AT	10	*	*
	CC	8	–0.29	0.47	AA	4	–0.89	0.10
IFN-γ (pg/ml)	GG	11	–0.17	0.60	TT	19	0.01	0.96
	GC	14	–0.28	0.31	AT	10	–0.22	0.53
	CC	8	–0.09	0.82	AA	4	0.68	0.31
IL-4 (pg/ml)	GG	11	0.10	0.75	TT	19	*	*
	GC	14	*	*	AT	10	–0.07	0.83
	CC	8	*	*	AA	4	*	*
TGF-β (pg/ml)	GG	11	–0.57	0.06	TT	19	0.15	0.52
	GC	14	–0.26	0.35	AT	10	–0.16	0.64
	CC	8	–0.21	0.60	AA	4	–0.31	0.68
Treg Cells (%)	GG	5	–0.87	0.05	TT	6	0.56	0.24
	GC	5	0.61	0.26	AT	6	0.21	0.68
	CC	5	–0.02	0.96	AA	3	0.60	0.58
CD3+CTLA4+ Cells (%)	GG	11	–0.05	0.86	TT	19	–0.15	0.53
	GC	14	0.09	0.73	AT	10	0.75	0.01
	CC	8	0.79	0.01	AA	4	–0.54	0.45

Data are analyzed using linear regression, and r values are from Pearson's correlation coefficient test.
**Cannot be computed because at least one of the variables is constant.*
The values in red font are significant.

between miR-146a and IL-1β was observed especially in patients with a GG genotype. miR-146a regulates IL-1β expression (48–50), and IL-1β plays an important role in tumor progression by enhancing angiogenesis, amplifying myeloid-derived suppressive cells (MDSCs) and shifting macrophages towards an M2 phenotype (51, 52).

miR-146a and miR-155 are expressed in Treg cells (53), and there was a negative correlation between miR-146a and the frequency of Treg cells in NSCLC patients with a GG genotype. Furthermore, there was a positive correlation between miR-155 and CTLA4+ T-cell frequency in patients with an AT genotype. Overall, these changes (low miR146 with high Treg and high CD3/CTLA-4) may suggest a damping of the immune system response with poor disease prognosis. Among the 33 NSCLC patients, only eight patients had a CC genotype, and these had a positive correlation between miR-146a expression and the frequency of CD3+CTLA4+ lymphocytes.

In conclusion, the current study shows a connection between downregulation of miR-146 with increased serum levels of TGF-β.Thus, blocking TGF-β using monoclonal antibodies may potentiate the effects of ICIs such as CTLA4 blockers, resulting in an immune brake, enhancing T-cell cytotoxicity and enhancing cancer cell killing. The data presented here have clinical implications for NSCLC; however, measuring miR-146 in parallel with serum cytokine levels may provide a better evaluation of the immune response and outcome of disease.

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 National Research Institute Tuberculosis and Lung Disease. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ND and EM did experiments. ND and NR did statistical analysis. SS and BS as oncologists helped in patient's approval and confirmed patient's status. ND and EM wrote the first and final draft of the paper. SDA revised the paper and comments for discussion. IMA as a native writer edited the article and advised last edition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.715677/full#supplementary-material

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REVIEW
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Preclinical Models and Resources to Facilitate Basic Science Research on Malignant Mesothelioma – A Review

Ben William Johnson, Ken Takahashi and Yuen Yee Cheng*

Asbestos Diseases Research Institute, Sydney, NSW, Australia

Malignant mesothelioma is an aggressive cancer with poor prognosis, predominantly caused by human occupational exposure to asbestos. The global incidence of mesothelioma is predicted to increase as a consequence of continued exposure to asbestos from a variety of sources, including construction material produced in the past in developed countries, as well as those currently being produced in developing countries. Mesothelioma typically develops after a long latency period and consequently it is often diagnosed in the clinic at an advanced stage, at which point standard care of treatment, such as chemo- and radio-therapy, are largely ineffective. Much of our current understanding of mesothelioma biology, particularly in relation to disease pathogenesis, diagnosis and treatment, can be attributed to decades of preclinical basic science research. Given the postulated rising incidence in mesothelioma cases and the limitations of current diagnostic and treatment options, continued preclinical research into mesothelioma is urgently needed. The ever-evolving landscape of preclinical models and laboratory technology available to researchers have made it possible to study human disease with greater precision and at an accelerated rate. In this review article we provide an overview of the various resources that can be exploited to facilitate an enhanced understanding of mesothelioma biology and their applications to research aimed to improve the diagnosis and treatment of mesothelioma. These resources include cell lines, animal models, mesothelioma-specific biobanks and modern laboratory techniques/technologies. Given that different preclinical models and laboratory technologies have varying limitations and applications, they must be selected carefully with respect to the intended objectives of the experiments. This review therefore aims to provide a comprehensive overview of the various preclinical models and technologies with respect to their advantages and limitations. Finally, we will detail about a highly valuable preclinical laboratory resource to curate high quality mesothelioma biospecimens for research; the biobank. Collectively, these resources are essential to the continued advancement of precision medicine to curtail the increasing health burden caused by malignant mesothelioma.

Keywords: mesothelioma, research resources, preclinical models, facility, biobank

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Meng Xu Welliver,
The Ohio State University,
United States

Reviewed by:
Antonio Caputi,
University of Bari Aldo Moro, Italy
Carolyn Presley,
The Ohio State University,
United States

****Correspondence:***
Ben William Johnson
ben.johnson@adri.org.au

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INTRODUCTION

Malignant mesothelioma (MM) is an incurable and highly aggressive form of cancer associated with occupational or environmental exposure to asbestos; a long-established human carcinogen (1). The global incidence of MM cases, approximated by the number of deaths, has increased significantly. The most recent Global Burden of Disease (GBD) study estimated 29,000 mesothelioma deaths (2), while other researchers estimated 38,000 mesothelioma deaths each year as a consequence of the augmented and widespread use of asbestos over the last century (3). The cancer develops most commonly within the mesothelial tissue of the pleura, accounting for approximately 80% of all MM cases, and in rarer cases; the peritoneum, pericardium, and the tunica vaginalis (4). Most cases of MM develop after a long latency period; on average 40 years (ranging between 30 to 60 years following asbestos exposure, with patients being diagnosed at a mean age of 74 years (5). With few available biomarkers and treatment options, the median survival of MM patients after diagnosis is 12-18 months following first-line standard chemotherapy with cisplatin plus pemetrexed (6, 7). To address this issue, substantial research efforts have been conducted over the past years, having provided valuable insights into the carcinogenic properties of asbestos fibres and their associated molecular alterations; as well as significant preclinical studies that have provided the foundation for the development of innovative diagnostic and treatment strategies. Despite these research efforts, the diagnosis and treatment of MM remains ineffective. It is not always practical/feasible for researchers to investigate MM biology and novel diagnostic/therapeutic strategies in MM patients directly; primarily because: 1) MM is a rare cancer, meaning that few patients can be enlisted for randomised clinical trials, and 2) invasive surgical procedures are required for sampling tumour tissue, which is often not possible to perform in MM patients with deteriorating health (8). Hence, further basic science research and development of improved MM-specific biological models are needed to address the ongoing asbestos burden and current clinical limitations associated with the diagnosis and treatment of MM.

The objective of this review article is to summarise and evaluate the effectiveness of current preclinical biological models and technologies that are currently available to researchers investigating MM. Furthermore, this review will provide an overview of some of the most valuable and extensive MM-specific biobanks that are available to researchers worldwide.

RESOURCES FOR RESEARCH

High quality research into asbestos-related disease requires a well-established laboratory that is equipped with an extensive range of resources and highly trained researchers. Typical resources that are essential to an asbestos-related disease research laboratory include a repository of high quality biospecimens, known as a biobank; as well as modern laboratory facilities (e.g. biological safety cabinets for *in vitro* cell culture experiments and animal housing for *in vivo* rodent experiments), technology (e.g. next generation sequencing platforms) and established laboratory techniques (e.g. three-dimensional cell culture). These factors combined are what provide an effective foundation to support basic science research that has strong potential for translation into clinical trials and ultimately into clinical practice in order to provide improved standards of diagnosis and treatment to individuals affected by MM.

The highly aggressive asbestos-related cancer, MM, is associated with poor prognosis and is notoriously resistant to conventional cancer-based therapies. Therefore, an understanding of the biological characteristics and associated molecular pathways that drive the development and progression of MM tumours is greatly warranted. The use of a variety of preclinical models, such as cell lines, mouse models and human-derived clinical samples, are highly advantageous to research that aims to elucidate the biological mechanisms of MM. These models are also very useful for the identification of novel prognostic and diagnostic biomarkers, and for the testing of novel therapeutic strategies. The different types of biological models, techniques and technologies available to MM researchers are discussed in detail below.

Cell Lines

Cell lines that have been established from primary human or animal cells can be propagated repeatedly under controlled conditions outside of their natural environment. They are an invaluable resource for research into disease and have led to multiple important medical-related discoveries and developments. MM cell lines have been widely utilised as an *in vitro* preclinical model by researchers to study the pathogenesis and molecular mechanisms of MM, particularly to facilitate an assessment of cellular response to novel anti-cancer agents (e.g. platinum-based chemotherapy drugs), cytokine production, response of immune effector cells, and to define various genetic and phenotypic characteristics (9).

The first human MM cell lines were established in 1982 from the abdominal fluid of a patient (10) and the first malignant pleural-derived MM cell line, H-Meso-1, was established by Reale et al. in 1987 (11). Since that study, a variety of MM cell lines have been established and characterised with over 400 currently listed in Cellosaurus (https://web.expasy.org/cgi-bin/cellosaurus/search). Stable MM cell lines have an almost unlimited growth potential and are frequently used as a preclinical tool for research due to their easy handling, manipulation and capacity to generate high-throughput data. Constant characterisation of the cell lines *via* the analysis of typical MM markers (e.g. mesothelin, calretinin, 5T4, podoplanin, cytokeratins, and $HBME_1$), karyotyping and/or short tandem repeat/single-nucleotide polymorphism analysis is important to confirm that they maintain properties consistent with the original tumour subtype (8). Whilst a range of MM cell lines are commercially available, it should be noted that primary MM cells represent a better *in vitro* model given that they more closely resemble molecular and histological characteristics to

those of the original tumour (12, 13). For instance, commercial MM cell lines have been reported to exhibit significant molecular and karyotypic differences in comparison to primary MM cell lines, due to the greater number of divisions associated with the continuous culture of established commercial MM cell lines (13). It has been suggested that these molecular and karyotypic discrepancies can be attributed to the generation of highly selected clonal tumour cell populations that only partially represent those comprising the original tumour (9). Hence, it has been proposed that established MM cell lines are better suited for preliminary screening studies, followed by subsequent confirmation of the experimental findings using primary cancer cells sourced from patients (14). The applications, advantages and disadvantages of both established MM cell lines and primary MM cells are summarised in **Table 1**.

A number of murine MM cell lines, such as AB1, AB12, AB22, 40, 40L, AE17, and AK7, have been generated from spontaneously arising MM tumours in wild-type mice exposed to asbestos (15, 16). These cell lines display similar phenotypical and functional characteristics akin to human MM and have been widely used by researchers for *in vitro* assays or for implantation in immunocompetent mice of the same genotype for *in vivo* studies (8). Furthermore, a whole exome sequencing analysis of 15 murine MM cell lines demonstrated that murine MM has a similar mutation rate to human MM (17). This finding establishes relevance to human-based MM basic science research and justifies their continued use.

Animal Models

Animal models are an *in vivo* preclinical model that are highly valuable in facilitating the understanding of the pathogenesis, biology and progression of MM in a living system. Additionally, animal models are useful for the development and preclinical testing of novel therapeutic drugs. The introduction of genetic mutations in rodents often results in the development of tumours that closely resemble the human disease. Hence, animal models are not only a highly valuable resource, but an important requirement for research aimed to translate novel intervention, diagnostic or treatment strategies into the clinical setting. Here we describe the applications, advantages and disadvantages of eight different types of rodent models that can be utilised for MM-based research, as also summarised in **Table 1**. These include asbestos exposure, inhalation, injection, xenograft, syngeneic subcutaneous, orthotopic, genetic predisposition and the transgenic MexTAg mouse models.

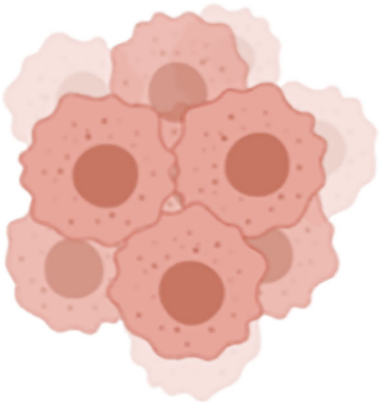
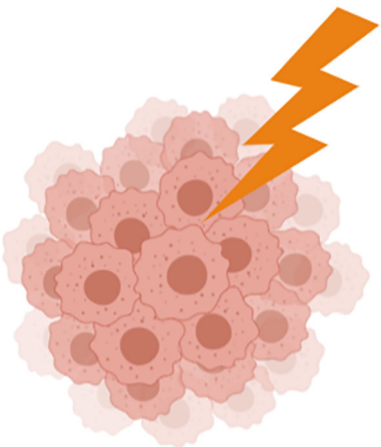
Asbestos Exposure, Injection and Inhalation Models

A number of studies have successfully induced MM tumour development in mice and rats *via* means of inhalation or injection of the asbestos fibres, or in hamsters through exposure to the Simian Virus 40 (SV40) (18, 19). The first asbestos exposure studies on laboratory rat models were conducted in the 1960's, showing successful MM tumour development after intrapleural or intrathoracic (IT) injection of different forms of asbestos fibres (20). A subsequent study also conducted IT-based inoculations with amphibole and serpentine asbestos fibres in mice, however fibrosis and granulomas were more frequently observed (21). Intraperitoneal (IP) injection of asbestos fibres was therefore favoured by subsequent carcinogenicity studies in mice, which resulted in the development of MM malignancies in greater than 20% of wild type mice (22). Although peritoneal MM accounts for roughly 10% of all human MM cases, it shares similar pathogenetic mechanisms and poor drug sensitivity of the more common pleural MM (8). Furthermore, MM tumours of IP injection models were found to possess all possible morphological traits as observed in human MM (23). In contrast to injection-based MM animal models, inhalation-based models are more representative of human exposure to asbestos on the basis that they precisely emulate the human inhalation conditions, which is particularly advantageous to preclinical studies aiming to simulate the initial disease pathogenesis and/or assess the carcinogenicity of varying types of asbestos (24). The practicality of inhalation-based models is hampered by a number of factors however, including the complexity and cost of setting up exposure chambers and difficulty to control the amount of inhaled asbestos fibres. Consequently, inhalation-based animal studies require specialised safety equipment and facilities that are not widely accessible or affordable to perform in many research laboratories (8, 9). Furthermore, several studies have demonstrated a discordance in cytogenetic, gene expression and gene inactivation in inhalation-based MM rat models compared to the human MM counterpart (19, 25–27). This indicates that whilst inhalation models may closely mimic human exposure to asbestos, the biological mechanisms leading to disease pathogenesis may not necessarily reflect that of human MM. Therefore, the type of model utilised by researchers should be carefully selected depending on the objective(s) of the study. If the potential carcinogenicity of various types of airborne asbestos fibres is being investigated, then an inhalation model is probably the most appropriate model; conversely, if the various biological processes that occur post-exposure are being investigated, then an injection model would be a suitable alternative.

Xenograft Models

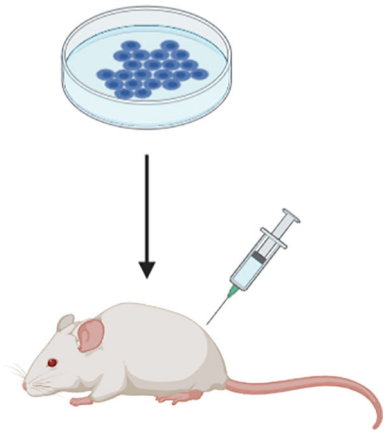
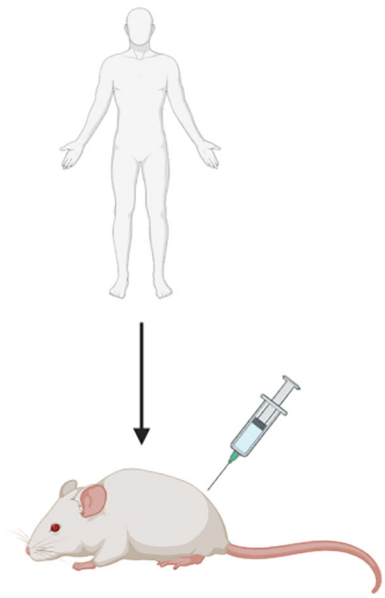
Xenograft models of MM constitute the transplantation of human solid MM tumours or cell lines into mice and are highly useful for studying molecular mechanisms that drive tumour growth and drug toxicity. Patient-derived xenograft (PDX) models are mouse models that consist of tumour biopsies or tumour cells sourced from patient pleural effusions. It has been shown that a PDX model of MM closely resemble both the histological and molecular characteristics of the primary tumour (28). All xenograft models of MM typically require the use of immunocompromised mice (i.e. mice lacking an intact immune system) so as to avoid rejection of the foreign tumour tissue or cells. This includes the hairless 'nude', severe combined immunodeficient (SCID) and recombination-activating gene (RAG) knockout mice; which lack T cells, both T and B cells, and adaptive immune cells, respectively (9). The main disadvantages of xenograft models is that they don't reflect the complex tumour-immune interactions that occur in humans and therefore cannot be used for studies aiming to explore the role of

TABLE 1 | Summary of the types of *in vitro* and *in vivo* preclinical models of MM, their applications to research and their main advantages and disadvantages.

Preclinical model	Model Type	Application to MM research	Advantages	Disadvantages
Primary MM cells 	*In vitro*	*Investigating the genetic and phenotypic characteristics of MM. *Determining cellular response to novel therapeutic agents. *Identifying and/or validation of diagnostic and prognostic/predictive biomarkers.	*Cost-effective. *Easy to manipulate and handle. *Same genotypic and histological characteristics in comparison to the original MM tumour. *Absolute control of physical environment.	*Limited lifespan in culture *Very prone to contamination *Lack of 3D structure; limited cell-cell interactions; unnatural substrate
Established MM cell lines 	*In vitro*	*Same applications as for primary MM cells.	*Cost-effective. *Easy to manipulate and handle. *Absolute control of physical environment. *Easy to maintain. *Unlimited lifespan in culture. *High-throughput capacity.	*Cells change over time in culture (i.e. genotypic and phenotypic drifting) = reduced genotypic and histological similarities compared to the original tumour. *Lack of 3D structure; limited cell-cell interactions; unnatural substrate.
Asbestos injection 	*In vivo*	*Determining pathogenic mechanisms of MM development. *Identifying early biomarkers of MM.	*Exhibits similar pathogenetic, drug sensitivity and morphological characteristics to human MM.	*Not representative of human exposure to asbestos (i.e. concentrations of asbestos fibres reaching mesothelial cells are much higher than would be expected for real-world human exposure). *Low incidence and long latency of tumour development.

(Continued)

TABLE 1 | Continued

Preclinical model	Model Type	Application to MM research	Advantages	Disadvantages
Asbestos inhalation 	*In vivo*	*Investigating the carcinogenicity of airborne asbestos fibres. *Identifying early biomarkers of MM.	*More representative model of human exposure to asbestos.	*Requires expensive safety equipment, PPE and facilities. *Poses a greater hazard risk to staff and surrounding environment. *Not always feasible to regulate the quantity of inhaled asbestos fibres. *Molecular mechanisms/genetic traits do not always resemble that of human MM. *Low incidence and long latency of tumour development.
Cell line-derived xenografts 	*In vivo*	*Investigating the molecular mechanisms that mediate MM tumour growth and tumour response to drug treatment. *Identification of predictive biomarkers.	*Reproducible tumour growth.	*Lack of an intact immune system means that TME does not accurately reflect that of human MM. *Not suitable for studies aiming to explore the role of immune cell populations in regards to tumour clearance and response to immunochemotherapy. *Tumours formed from cell lines do not reflect intra-tumour heterogeneity typical of human MM tumours. *TME is gradually replaced by murine cells over generations.
Patient-derived xenografts 	*In vivo*	*Same applications as for cell line-derived xenografts.	*Maintain the main histological features of human MM, including the stromal component. *The heterogeneity of the original tumour is at least partially preserved	*Lack of an intact immune system means that TME does not accurately reflect that of human MM. *Not suitable for studies aiming to explore the role of immune cell populations in regards to tumour clearance and response to immunochemotherapy. *TME is gradually replaced by murine cells over generations.

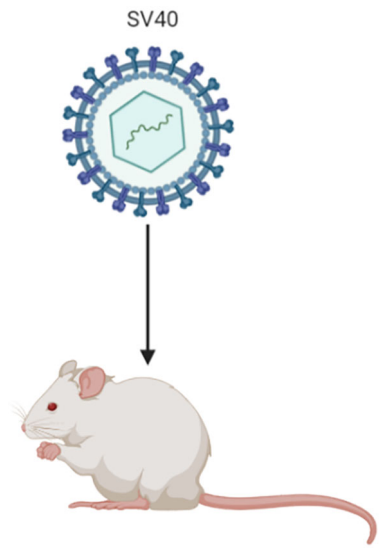
(Continued)

TABLE 1 | Continued

Preclinical model	Model Type	Application to MM research	Advantages	Disadvantages
Syngeneic subcutaneous 	*In vivo*	*Analysing tumour growth in response to novel therapeutic agents (e.g. pharmacological studies).	*Tumour retains many histological features comparable to human MM solid tumours. *Tumour growth is generally rapid. *Tumour growth can be directly observed and measured.	*Tumour develops in an atomically irrelevant site, therefore the TME is not reflective of the human MM TME.
Orthotopic 	*In vivo*	*Same applications as for subcutaneous.	*Tumour develops in an anatomically relevant site. *Tumour generally grows more rapidly and invasively than the subcutaneous model. *Tumour development is influenced by the host tissue and relevant host factors such as immune system, TME, vasculature and metabolites. *Intraperitoneal models conserve similar pathological, histological, progression and response to treatment as pleural mesothelioma.	*Advanced level of technical skill/training required for intrapleural injection. *Tumour growth cannot be directly observed or measured.

(Continued)

TABLE 1 | Continued

Preclinical model	Model Type	Application to MM research	Advantages	Disadvantages
Genetic predisposition 	*In vivo*	*Determining the pathogenic mechanisms of MM tumour development. *Studying genetic traits that drive MM tumour development.	*Molecular characteristics of the tumour are comparable to human MM. *Higher incidence of MM development and more rapid tumour growth compared to wild type mice.	*High tendency to develop spontaneous unrelated tumours, rendering this model unsuitable for pharmacological studies. **P53* KO mice do not accurately reflect a gene mutation typically seen in human MM. *Tumour growth cannot be directly observed or measured.
MexTAg 	*In vivo*	*Determining the pathogenic mechanisms of MM tumour development. *Studying genetic traits that drive MM tumour development. *Analysing tumour growth in response to novel therapeutic agents (e.g. pharmacological studies).	*Guaranteed 100% incidence of MM tumour development. *Rapid, uniform and predictable disease development upon exposure to asbestos. *Exhibits similar disease pathology and response to treatment as seen in human MM. *Low incidence of unrelated tumour development.	*Tumour growth cannot be directly observed or measured. *Tumours of this model are mostly of the sarcomatoid type, which is not an accurate reflection of the more common epithelioid type seen in human MM tumours.

the immune system in relation to tumour clearance and immunochemotherapy response (29, 30). This concept is particularly relevant to the recent open-label, randomised, phase 3 clinical study, CheckMate 743, which demonstrated a significant improvement to the overall survival of MM patients treated with the combinational immunotherapeutic treatment regimen; ipilimumab plus nivolumab. Patients subjected to this novel treatment regimen exhibited a median overall survival of up to 18 months compared to 12 months for the conventional cisplatin-pemetrexed chemotherapy treatment regimen (7, 31), and as a result ipilimumab-nivolumab was approved by the Food and Drug Administration (FDA) as a first-line combination treatment regimen for patients with unresectable MM. Ipilimumab and nivolumab are both antibodies that elicit an immune-mediated anti-tumour response upon binding to components of the immune system; specifically the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) receptor, respectively (32). Xenograft models are deficient in these T cell proteins. Hence, the use of an immunocompromised xenograft model of MM, such as the SCID and RAG knockout mice, would be unsuitable for use in prospective preclinical studies aiming to explore and develop this treatment regimen further. Furthermore, the transplantation of tumour cell lines to induce tumour formation in these models

does not accurately reflect the intra-tumour heterogeneity of human MM tumours (14). Even in instances where human tumour tissue is transplanted, the tumour microenvironment (TME) is gradually replaced by murine cells over generations. It has therefore been suggested that the use of a humanised mouse model is a more suitable alternative for studies focused on anti-tumour immune response, whereby the mouse immune system is substituted with a human one (14). NOD SCID gamma (NSG) mice, which lack the interleukin 2 receptor gamma subunit (IL-2RG) that is involved in differentiation and function of numerous haematopoietic stem cells, are commonly utilised for this type of research (33). Whilst this model is useful for assessing anti-tumour immune response in MM, there is an associated risk of incomplete differentiation of the haematopoietic stem cells, high cost and the longer time required to attain NSG mice harbouring a human immune system that should carefully be considered by researchers wishing to utilise this model.

Syngeneic Subcutaneous Models

Syngeneic subcutaneous murine tumour models involve the injection of inbred mouse-derived MM tumour cells directly under the skin surface of immunocompetent mice of the same in-bred strain, which then develop into subcutaneous solid tumours. The key advantages of these models is that the MM tumours develop in the presence of an intact immune system, established tumours retain many histological features akin to human solid tumours, tumour growth is rapid, and tumour growth in response to novel therapeutics can easily be visualised and measured during the course of the experiment (34, 35). Furthermore, the tumour growth rate is highly reproducible when a controlled number of cells are inoculated (36). The main disadvantage of using this model however, is that the tumour develops in an anatomically irrelevant site and that the rapid tumour growth may impede normal stromal development and immune cell invasion (9, 35). Despite this limitation, there are chemo- and immuno-based therapies that have been successfully translated into the clinical setting using this type of model (37). It has therefore been suggested that the syngeneic subcutaneous model remains a useful tool for the purpose of studying therapeutic interventions for MM, such as immunotherapy-based assessment, as long as results are replicated using other anatomically relevant tumour-bearing models (35).

Orthotopic Models

Orthotopic models represent a more human-like disease model; the tumour develops in an anatomically relevant site and are usually more rapid growing and invasive than the subcutaneous model. This type of model closely resembles human MM, given that the tumour cells grow along the serosal surfaces, form nodules in the peritoneum, develop metastases, and form ascitic fluid in some cases (38, 39). Most importantly, the tumour develops with respect to the host tissue and its growth and development is influenced by relevant host factors such as the immune system, vasculature, metabolites and TME (9). Advanced technical skill is required for intrapleural orthotopic models as there is an associated risk of inducing a hemothorax and/or pneumothorax during the intrapleural injection procedure (35). Intraperitoneal models of MM are relatively easier to perform by less skilled researchers and conserve similar pathological, histological, progression and response to treatment as pleural mesothelioma (40, 41); therefore the intraperitoneal model is more commonly preferred over the intrapleural model. The main limitation associated with orthotopic models is that tumour growth cannot be directly observed or measured, however, this can be overcome *via* the use of fluorescence-based small animal imaging techniques. For example, the proliferation of cancer cells expressing the luciferin gene, that converts a substrate to emit light, can be measured to provide a reliable indicator of tumour growth (9, 35).

Given that the orthotopic model and syngeneic model possess an intact immune system and that tumour response to treatment can be monitored *in situ*, these models are particularly beneficial to researchers aiming to monitor the *in situ* progressive MM tumour regression in response to novel drug treatments; particularly immunotherapeutic agents such as the aforementioned ipilimumab and nivolumab.

Genetic Predisposition Models

Genetic predisposition mouse models have been developed in accordance with characteristic gene losses typically seen in human MM; primarily in the *NF2*, *BAP1* and *CDKN2a/ARF* gene loci. Such models have been established by genetically modifying them so that they no longer express these genes, either individually or in combination, commonly referred to as gene 'knockout' models. Although mutations of the *p53* tumour suppressor gene have only been reported in a few cases of MM and is not believed to play a role in driving MM tumour development, *p53*-deficient mice have been developed and have exhibited a higher incidence and more rapid tumour progression than wild type mice; particularly following asbestos exposure in the peritoneum (42–44). An alternative model, a heterozygous *Nf2* mouse, was first reported by Altomare et al. Upon repeated exposure of the heterozygous *Nf2* mice to asbestos, they found that these mice were notably more susceptible to MM development compared to their homozygous *Nf2* counterparts, with a reported incidence of 85% and 59%, respectively (44). Furthermore, the molecular features of the tumours were found to resemble that of human MM tumours, including activation of *Akt*; homozygous deletion of tumour suppressor genes *p16 (Ink4A), p14 (ARF)/p19(Arf)*, and *p15(Ink4B)*; and loss of the *Nf2* protein, Merlin (44). Other researchers have induced heterozygous *BAP1* mutations in mice in order to investigate the incidence of MM in humans carrying *BAP1* germline mutations, even with no known history of exposure to asbestos, as was the case for four members of a European family (45). Overall, the mutant *BAP1* mice exhibited increased susceptibility to MM following peritoneal injection of asbestos, as well as some without injection, with incidence of MM being double and median survival shorter for the *BAP1* mutant mice compared to the wild type controls (46). Thus, this model effectively demonstrated *BAP1* loss to be a key genetic driver of

MM development, as well as being translatable to the *BAP1*-impaired human MM cases. Whilst these genetically modified mouse models have facilitated our growing knowledge of MM pathogenesis and associated molecular biology, unfortunately the *p53*, *Nf2* and *Bap1* heterozygous knockout mice have a high tendency to frequently develop spontaneous tumours, such as lymphomas, sarcomas and adenocarcinomas. Hence, these models have been deemed unsuitable for pharmacological studies (i.e. novel drug testing) of MM (35). To overcome this problem, Robinson et al. established a transgenic mouse model; a model highly susceptible to MM tumour development, but with a low associated incidence of other tumour types; the MexTAg mouse (47).

Transgenic MexTAg Mouse Model

The MexTAg transgenic mouse model of MM was developed by Robinson et al. through the engineering of mesothelial cells to express the oncogenic SV40 virus large T antigen (SV40 Tag), and has been utilised to highlight co-carcinogenicity between asbestos and SV40 (48). Whilst SV40 alone does not induce MM development in this model, its oncogenic potential facilitates a guaranteed 100% incidence of disease, rapid, uniform and predictable disease development upon exposure to asbestos (9). The MexTAg mice develop MM tumours that exhibit similar disease pathology and treatment responses to human MM (47). Another notable advantage of the MexTAg mouse model is that it has a lower chance of developing unrelated tumours in comparison to wild-type mice or the heterozygous and conditional mesothelioma knockout mouse models (35). It has been proposed that the Tag transgene does not influence the overall molecular mechanism of MM development in this model. Rather it phenocopies p16 loss, which induces the characteristic accelerated disease progression in this model following asbestos exposure (49). Furthermore, it has been suggested that the MexTAg model is a functional equivalent to human MM being that it similarly exhibits a loss of tumour suppressor genes such as *CDKN2A* ($P16^{INK4a}/p14^{Arf}$), *NF2, BAP1* and *p53* (9). The suitability of the MexTAg mice for preclinical studies was assessed by Robinson et al., upon subjecting this mouse model to treatment with gemcitabine; a cytotoxic drug proven to exhibit some efficacy in human MM (47). The results of this study showed that the MexTAg mice treated with gemcitabine had a median survival of 48 weeks compared to 33 weeks for the untreated vehicle control. Given the strong concordance of MM response to gemcitabine of the MexTAg model to that of human MM, this study effectively demonstrated the translatability of the model to the clinical setting. It should be noted however, that most MM tumours that develop in this model are of the sarcomatoid type, which is different from the more common epithelioid type seen in humans (48). As with the orthotopic model, fluorescence-based small animal imaging techniques are required in order to monitor tumour growth in the MexTAg model.

Human Biospecimens

Well characterised human biospecimens are an invaluable resource required for the advancement of translational research aimed to improve the diagnosis and treatment of MM. Types of human MM biospecimens include pleural, pericardial and peritoneal tumour tissue biopsy samples; as well as matched whole blood, plasma, serum, pleural effusion specimens and lymphocytes. In addition to their usefulness for the generation of primary cell cultures and transplantation into mouse models, human biospecimens are highly useful for biomarker validation research aimed to identify novel biomarkers to facilitate an understanding of cancer aetiology. Such knowledge can then be applied to the design and development of improved MM-specific diagnostic techniques and targeted therapies to provide an accurate diagnosis and improved prognosis for patients with MM. The diagnosis of MM in the clinical setting is particularly challenging due to a lack of effective diagnostic biomarkers and the requirement of an invasive percutaneous needle biopsy procedure or video-assisted thoracoscopic surgery (VATS) required to attain a definitive diagnosis (50). These procedures are not always feasible to perform on MM patients with significantly declining health and are dependent on the availability of services (e.g. trained staff and medical resources) (50). To date, a number of less-invasive blood-based biomarkers have been investigated for MM, such osteopontin and fibulin-3, however a poor associated specificity and/or sensitivity have rendered them unsuitable for clinical implementation as diagnostic and/or prognostic biomarkers of MM (51, 52). Continued use of human-derived biospecimens to identify and validate novel less-invasive biomarkers that are highly sensitive and specific for MM is greatly warranted and would represent a significant advancement for the diagnosis and treatment of MM. The use of large collections of well preserved biospecimens have proven to be particularly beneficial to the success of preclinical studies aiming to identify and validate novel less-invasive biomarkers for an accurate and/or early detection of MM. For example, a study conducted by Creaney et al. utilised pleural effusion samples collected from 1,331 MM patients, whereby it was established that effusion-derived mesothelin exhibits a 95% specificity for MM; justifying the clinical utility of pleural effusion-derived mesothelin as a biomarker to facilitate a definitive diagnosis of MM (53). Human biospecimens intended for use in downstream research applications are typically stored under strictly controlled conditions in a biobank facility, usually in a -80°C freezer or liquid nitrogen tank, to ensure sample integrity is maintained for subsequent histological, proteomic, genomic or transcriptomic analyses at a later date.

Laboratory Techniques and Technology

The inability of early laboratory techniques and technologies to adequately reproduce the complex heterogeneity and/or tumour microenvironment (TME) of MM tumours is a major contributing factor to limiting our understanding of MM tumour biology and the non-concordant results obtained from previous preclinical studies and those from clinical studies. Promisingly, laboratory technologies and techniques are constantly evolving. It is therefore of vital importance that researchers select and apply the most up-to-date and clinically-relevant techniques and technology in order to produce data that

best represents the clinical behaviour of MM as possible and provide a more comprehensive understanding of MM biology. Some of the useful modern techniques and technologies currently available to researchers include three-dimensional (3D) cell culture techniques and next generation sequencing (NGS) technologies, as described in detail below and summarised in **Table 2**.

2D vs 3D Cell Culture

Cell monolayer culture, otherwise known as two-dimensional (2D) cell culture, is commonly utilised by researchers for large-scale drug testing as cells grown in this manner are easy to handle and are cost effective, however drug sensitivity data obtained from this *in vitro* model has frequently been shown to differ to their *in vivo*/clinical counterparts. MM is typically resistant to a range of chemotherapeutic drugs tested on patients in the clinical setting, however this trend is not always accurately modelled by 2D cell culture. Furthermore, drug sensitivity data derived from 2D cell culture has led to false expectations upon the subsequent testing of drugs in human clinical trials, as well as resulting in a waste of time and expenses. For instance, the proteasome inhibitor, bortezomib, was found to be highly effective in monolayer malignant pleural mesothelioma (MPM) cell line cultures (54–56), however follow-up phase II studies produced disappointing results (57, 58). To rectify this issue, recent research has led to the development and testing of 3D cell culture techniques, which more closely mimic solid tumours and their associated TME compared to 2D cell culture. There are three types of 3D models that have been developed, which includes spheroids, tumour fragment spheroids (TFS) and organ-on-a-chip.

Spheroids involve the seeding of established cell line or primary cell suspensions on 3D structures composed of an artificial matrix (i.e., polyHEMA). It has been demonstrated that spheroids acquire multicellular resistance to a variety of treatments, which more closely resembles the chemoresistance effect frequently seen in MM patients (59, 60); a trend not seen for monolayer cultures. This can most likely be attributed to the fact that some genes that mediate resistance to cell death are differentially expressed in a 3D organisation of cells compared to 2D culture (61, 62). The main limitation of this 3D model however, is the absence of other cell populations from the TME (14).

TFS constitutes an *ex vivo* model of living tumour tissue. These differ from cell-based spheroids on the basis that small fragments of the original tumour tissue are grown into 3D structures. This technique does not require an artificial matrix; rather it relies on the tumour cells' ability to generate and self-organise complex extracellular matrix (ECM) and cell to cell interactions. TFS are highly reliable and can be utilised for many different and/or repeat experiments given that they can contain viable tumour cells for weeks to months (8). Furthermore, it has been reported that TFS retain multiple characteristics of the original tumour for up to 3 months, including the presence of viable mesothelioma cells, macrophages and a collagen-rich stroma (63).

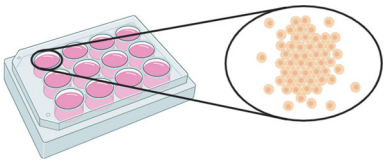
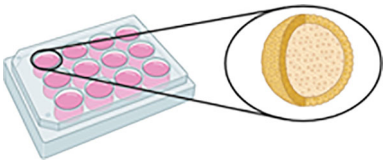
Organ-on-a-chip is a relatively novel technology that incorporates the integration of bioengineering with microfluidics to better mimic the *in vivo* TME. Multiple tissues can be seeded within one chip, which therefore enables researchers to explore the interactions between MM cells/tissues and other host cells/tissues within a single experiment. MM tumour organoids have been developed to facilitate the screening and prediction of suitable therapeutic options that are specifically tailored to individual patients (i.e., personalised therapeutics). This was effectively demonstrated in a study by Mazzocchi et al., which showed that the MM tumour grown on a chip responded to chemotherapy that mimicked the chemotherapy-induced tumour response of the associated patient. It also demonstrated the efficacy of using the organ-on-a-chip platform to predict the effectiveness of a chemotherapy drug based on a targetable mutation specific to the tumour genotype of individual MM patients (64).

Next-Generation Sequencing (NGS) and Quantitative Polymerase Chain Reaction (PCR)

Various "-omics" technologies, particularly genomics and transcriptomics, have significantly improved our understanding of MM-specific gene alterations and aberrant molecular signalling. The technology enabling whole genome and transcriptome constitutes an amalgamation of discoveries and innovations in molecular biology. The introduction of the polymerase chain reaction (PCR) in 1988 enabled researchers to make numerous gene-related discoveries, until the entirety of the human genome was sequenced in 2004 (65). Since then a number of technologies, collectively called "next-generation sequencing" (NGS), have become available and increasingly accessible to researchers conducting genome-wide studies.

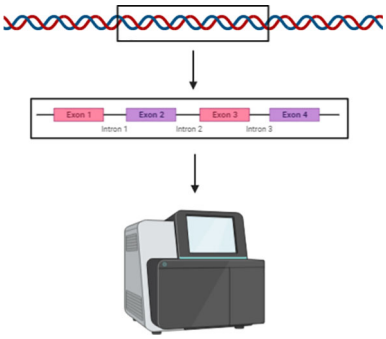
Massively parallel sequencing (MPS), a form of NGS, is a term used to refer to a grouping of high-throughput DNA sequencing methodologies that enable the simultaneous generation of millions of sequence reads. Such techniques are typically applied to perform whole genome sequencing, whole transcriptome sequencing, and targeted sequencing. Whole genome sequencing enables the determination of the complete human DNA sequence, and is therefore a highly useful technique for discovering a wide range of genetic variation. Transcriptome sequencing enables researchers to study the presence and quantity of RNA transcripts in a particular tissue sample at a specific timepoint, therefore, differences in gene expression and alternatively spliced gene transcripts can be identified. Targeted sequencing refers to the sequencing of a specific region of the genome (e.g. the exome) or subset of genes (66). All three of these approaches have been applied to MM, producing data that is highly useful in regards to identifying aberrant genetic variants associated with MM development and potential therapeutic targets. Examples of MPS technology/platforms that have been utilised for previous MM-based studies include the Roche/454-pyrosequencer, Illumina Genome Analyzer 2, Illumina HiSeq, Ion Torrent Personal Genome Machine, and SOLiD 5500 (66–72). The Ion Torrent platform in particular was utilised in a study by Sneddon et al. to perform whole exome and

TABLE 2 | Summary of the applications of *in vitro* 2D and 3D cell culture methods for MM research and their main advantages and disadvantages.

Method	Application to MM research	Advantages	Disadvantages
2D cell culture 	*Large scale drug testing *Identification and/or validation of novel biomarkers. *Investigating the role of genes in MM progression.	*Cost-effective *Easy handling. *Easy to maintain. *High throughput capacity.	*Drug sensitivity data generated from this method does not always reflect that of the *in vivo*/clinical counterpart. * Lack of 3D structure; limited cell-cell interactions; unnatural substrate. *Lack of cellular heterogeneity/complexity compared to the original tumour. * Gene expression less similar to *in vivo* tumours.
3D cell culture (includes spheroids, TFS and organ-on-a-chip) 	*Studying therapeutic efficacy of novel drugs. *Identification and/or validation of novel biomarkers. *Studying cell-to-cell and cell-to-extracellular matrix signaling.	*More representative of the *in vivo* tumour structure/complexity. *Gene expression more similar to *in vivo* tumours. * Drug response better reflects *in vivo*/clinical drug response. *Increased cell-to-cell and cell-to-extracellular matrix signalling.	*TFS and organ-on-a-chip require access to fresh surgical MM tumour samples = low throughput capacity. *Complex handling. *Less cost-effective.
Whole genome sequencing   	*Studying all types of MM-specific genetic variation across the entire genome.	*Detects coding, non-coding and structural variants across the entire genome.	*High associated cost. *Large volume of data to process and store. *Numerous variants of unknown significance can be detected. I.e. limited knowledge to fully understand / appreciate the significance of detected unknown variants.
Transcriptome sequencing   	*Studying all types of aberrant MM-specific mRNA / transcript variation.	*Rapid, precise, quantitative measurement of gene expression. *High sensitivity enables detection of low-abundance transcripts. *DNA sequences can be unambiguously mapped to unique regions of the genome instead of relying on existing genome sequence data. *Useful for the discovery of single-nucleotide polymorphisms and rare mutations. *More affordable compared to whole genome sequencing.	*Transcript quantitation can be affected by biases introduced during cDNA library construction and sequence alignment. *Accurate sequence annotation and data interpretation can be computationally challenging in the absence of pre-existing reference genome(s).

(Continued)

TABLE 2 | Continued

Method	Application to MM research	Advantages	Disadvantages
Targeted sequencing 	*Studying unique MM-specific alterations at the sites of specific regions of the genome (i.e. exosomes) or subset of genes.	*Significantly less time-consuming and more cost-effective than whole genome sequencing. *Specific areas of the genome can be sequenced at a greater depth than whole genome sequencing. *Reduced volume of data to process and store than whole genome sequencing.	*Only focuses on limited regions of the genome, meaning it does not take into account any other genetic variants outside of the focus/target gene panel.
Droplet digital PCR 	*Studying unique MM-specific gene copy number variations, DNA mutations or deletions. *Detection and validation of MM-specific biomarkers.	*Provides an absolute and independent quantification of DNA without the need for a standard curve. *Generated data is more accurate and reproducible than conventional qPCR. *Capable of detecting very low concentrated target molecules from variably contaminated samples.	*Equipment and reaction running costs are more expensive than conventional qPCR. *Requires advanced skill and handling compared to conventional qPCR.

transcriptome sequencing on DNA and RNA harvested from tumour cell cultures derived from human pleural effusion samples. This study effectively determined that *BAP1*, *CDKN2A* and *NF2* alterations occur in pleural effusion-derived tumour cells at a higher frequency than what is typically seen in MM tumour samples, as well as identifying high frequency alterations for the *TRAF7* and *LATS2* genes. Furthermore, this study identified previously unreported alterations in the *FGFR3* gene and chromosome regions 19p13.3, 8p23.1 and 1p36.32; thus highlighting novel mutations of MM that warrant further investigation in terms of their suitability as diagnostic and/or treatment response monitoring biomarkers of MM (73). Additional novel chromosome alterations have been detected by Serio et al., whereby a high-resolution array-comparative genomic hybridisation (a-CGH) performed on peritoneal MM patient samples revealed deletions at regions 8p23.1 and 1q21; both of which were found to be co-deleted in the majority of the tested patient samples (74). Hmeljak et al. recently carried out a comprehensive analysis of 74 MM tumours as a contribution to the The Cancer Genome Atlas (TCGA), which produced valuable genomic, epigenomic, and transcriptomic data using high-throughput array and NGS technology (75). Additionally, a recent study conducted by Oey et al. utilised whole genome sequencing to effectively characterise mutations and structural alterations using DNA derived from human primary tumours and matched cultured cells (12). This study was able to establish that the majority of genetic drivers of MM are associated with structural alterations, as opposed to point mutations.

The advent of quantitative PCR (qPCR), or real-time PCR, has significantly revolutionised the way researchers quantify gene expression in biological samples. The main benefits to using qPCR over other conventional semi-quantitative PCR techniques is that they are capable of generating quantitative data at a 10,000- to 100,000-fold higher sensitivity than RNase protection assays; are able to detect a single copy of a specific transcript; can reliably detect gene expression differences as low as 23% between samples; can differentiate between different messenger RNAs (mRNAs) with nearly identical sequences; do not require post-amplification sample manipulation; and are relatively more high-throughput (76–79). The main disadvantage is that qPCR equipment and reagent running costs are considerably more expensive than standard PCR methods (79). Droplet digital PCR (ddPCR) is the most modern version of qPCR, which was made commercially available in 2011 (80, 81). As with non-digital qPCR, the ddPCR technology involves Taq polymerase in a standard PCR

reaction to amplify a target DNA segment from a complex biological sample using pre-validated primer/probe assays (82). Unlike non-digital qPCR however, the ddPCR partitions the PCR reaction into thousands of individual reaction vessels prior to amplification and the data is acquired at the reaction end point. The advantage of using ddPCR over non-digital qPCR is that it provides an absolute and independent quantification of DNA without the need for a standard curve, thereby yielding more precise and reproducible data than non-digital qPCR (82, 83). Furthermore, the ddPCR can be applied to detect extremely low concentrated target molecules from variably contaminated samples, whereby the sample dilution requirements to ensure a consistent reaction efficiency, primer annealing and quantification cycle (Cq) values for non-digital qPCR would likely result in undetectable target levels (84, 85).

Most recently, we applied the ddPCR technique to a collection of serum samples obtained from MM patients, whereby the assay was optimised for the purpose of detecting circulating methylated microRNA (miR-34b/c) (86). Its degree of methylation in circulating DNA was previously reported to be associated with the development of MM (87). This study therefore effectively demonstrated that miR-34b/c is a promising biomarker candidate for predicting disease progression in patients with MM, as well as demonstrating the feasibility of ddPCR technology to detect circulating biomarkers in MM patient-derived biospecimens. We further demonstrated the utility of the ddPCR technique for MM biospecimen-derived biomarker detection using a large cohort of MM tissue samples, whereby co-deletion of the cyclin-dependent kinase inhibitor 2A (CDKN2A) and methylthioadenosine phosphorylase (MTAP) genes were detected *via* ddPCR. The homozygous loss of CDKN2A detection *via* ddPCR yielded a concordance rate of 92% with the gold standard fluorescence *in situ* hybridisation (FISH) diagnostic technique (88). Collectively these studies have highlighted that the ddPCR technique is highly reliable for MM-based research aimed to detect and validate novel biomarkers of MM, and demonstrated the potential utility of the ddPCR technique to replace or be used as an alternative to the current biopsy-reliant FISH diagnostic method.

THE BIOBANK

A biobank is widely defined as a facility for the collection, preservation, storage and supply of biological samples and associated data, which follows standardised operating procedures and provides material for scientific and clinical use (89). These biospecimens and data are highly valuable to scientists conducting research aimed to provide new insights into human diseases, their causes and associated molecular biology, to develop better preventative measures, and to develop improved diagnostic tests and therapies. Biobanking is usually carried out by a designated Biobank Officer; a process which is typically initiated by the Biobank Officer making contact with the patient or donor, followed by the transferal of the biospecimens and associated data to an institution that hosts the biobank. Biobanks have been established in a variety of institutions, such as medical research institutions, and pharmaceutical and biotechnology companies; as well as independent companies (both for profit and non-profit) that provide biobanking services and sample access to the research community. Increasingly, patients are allowed access to their data (90). There are three main types of human biobanks that exist and are often designed according to the intended research goal. These include population biobanks for the purpose of obtaining biomarkers of population identity and susceptibility, which contain DNA collected from a large cohort of representative healthy donors of a country/region/ethnic group; epidemiological disease-oriented biobanks for research focused on biomarkers of exposure, typically comprising a large collection of biospecimens derived from a healthy exposed cohort/case-control design for the purpose of studying germline DNA or serum markers and large quantities of collected data; and disease-oriented general biobanks (e.g. tumour banks) for research focused on biomarkers of disease, which consist of human biospecimens and their derivatives (e.g. DNA), as well as accompanying clinical data (90).

Properties of the Biobank

A biobank stores human biospecimens, such as tissue, blood, other body fluids, cells and associated derivatives (e.g. DNA, RNA and protein) collected for a specific (sometimes general) research purpose. These samples are typically stored in low temperature (-80°C) freezers and/or ultralow temperature (-150°C) liquid nitrogen vapour phase tanks for long-term storage, as the low temperatures preserve the quality and integrity of the DNA, RNA, proteins and cellular components. Different sample collection methods and processing conditions are important factors to consider for the purpose of preserving the quality of the sample and are dependent on the type of biospecimen being collected.

Human tissues are usually obtained from surgeries or autopsies immediately following histopathological examination by a pathologist. Processing the collected tissue specimen in neutral-buffered formalin is the most widely accepted clinical practice for the preservation of tissue specimens, such as for the preparation of formalin-fixed paraffin embedded (FFPE) tissue. The "next generation" era has revealed several limitations regarding the use of FFPE samples for molecular, genetics and protein-based studies; with fresh or frozen tissue being a more reliable alternative, particularly for downstream investigations involving whole-genome amplification, whole-genome sequencing, and complementary DNA (cDNA) microarray analysis (91, 92).

Blood is also a common biospecimen that is biobanked for research purposes and is collected in tubes containing preservatives and additives. The type of tube or additive used for collection is dependent on the required blood fraction (e.g. plasma, serum, white blood cells and red blood cells) and the intended downstream research application(s). For instance, ethylenediaminetetraacetic acid (EDTA)-coated collection tubes are generally preferred for DNA- and protein-based assays, whereas Heparin tubes are more suitable for

metabolomic studies (91, 93, 94). Furthermore, the optimal storage temperature is dependent on the stability of the specific blood-based biomolecule(s) being investigated, however both -20°C and -80°C storage temperatures are generally considered to be optimal for maintaining the integrity and stability of every blood component type (91, 95, 96).

DNA and RNA derivatives, that have been extracted from the parent tissue and/or blood biospecimens, are also commonly stored in a biobank. The success of downstream molecular analyses and quality of generated data is highly dependent on the integrity of the stored DNA and RNA samples. RNA is particularly more prone to degradation than DNA and the associated yield and quality is influenced by the type of sample it is derived from. For instance, FFPE tissue-derived RNA yield and quality is generally poor in comparison to fresh frozen tissue-derived RNA on account of the cross-linking of nucleic acids that is induced by formalin and the lengthy time interval between tissue resection and fixation (91, 97, 98). To ensure that DNA and RNA quality is maintained, they are typically stored at -80°C without repeated freeze-thaw cycles.

Overall, the quality and success of research aimed to advance health care practices is highly reliant on the correct processing and storage conditions of the biobanked human biospecimens. It is therefore of crucial importance that researchers process and store the biospecimens at the conditions that are most optimal for the intended aims of the research investigation and the type of analyte being measured.

MM-Specific Biobanks

Some of the most notable MM-specific biobanks worldwide, that consist of an extensive collection of annotated MM patient-derived specimens, include the MesobanK, Cambridgeshire, UK; Cancer of Respiratory Tract (CREST) biorepository, National Cancer Research Institute, Genoa, Italy; the National Centre for Asbestos Related Diseases (NCARD), Perth, Australia; and the Asbestos Diseases Research Institute (ADRI) biobank, Sydney, Australia.

The MesobanK UK in particular, offers an extensive collection of centrally located patient-derived biospecimens. The main objectives of the MesobanK UK is to provide a framework for the systematic collection, curation and quality assurance of well-annotated MM biospecimens that will facilitate high quality basic science, translational and clinical research based on mesothelioma (99). Upon its completion the MesobanK is expected to be comprised of 750 patient tissue microarrays, 300 matched blood and pleural fluid samples, and associated annotated clinical data, as well as 26 newly developed cell lines that can be readily accessed by researchers worldwide upon request (99). It is currently the only MM-specific biobank that offers such a service.

The CREST biorepository was established to investigate the molecular mechanisms and to develop tools and strategies for the primary and secondary prevention of respiratory-tract cancers, which includes both MM and lung cancer. The main goal of the CREST biorepository is to provide a comprehensive resource of respiratory cancer-related biospecimens along with annotated details of corresponding epidemiologic and clinical data in order to facilitate high quality molecular epidemiological and translational studies of respiratory tract cancers, but with particular emphasis on MM (100). The CREST biorepository is particularly beneficial to epidemiological studies focusing on exposure to airborne carcinogens, the identification of subgroups of affected individuals and to estimate cancer risk associated with early molecular events (100). Dating from January 2011, the CREST biorepository was reported to have obtained biospecimens from a total of 1,857 subjects; comprised of 454 lung cancer, 245 MM, 130 other cancer types, and 1,028 healthy controls (101). The biobanked samples sourced from these subjects include tissue biopsies, pleural fluid, saliva, whole blood, plasma, serum and lymphocytes (101).

The NCARD biobank was established to facilitate research focused on the development and implementation of improved clinical outcomes relating to the diagnosis and treatment of asbestos-related diseases, including mesothelioma, for people of Western Australia and worldwide. Since its establishment in 1994, the biobank has obtained samples from 3000 Western Australian subjects, which has enabled the generation of approximately 10,000 biospecimens, such as tissue, blood, pleural fluid and urine; as well as 80 cancer cell lines. External research investigators can obtain biospecimens from the NCARD biobank for use in approved research projects upon approval of a formal application, which is reviewed by the biobank management committee.

The ADRI biobank comprises an extensive collection of MM biospecimens sourced from patients of the Sydney and Greater Western Sydney region. Specifically, these biospecimens are obtained from six different hospital sites, which includes Strathfield Private, Royal Prince Alfred, Concord Repatriation General, Westmead and Sydney Adventist hospitals. The main objective of the ADRI biobank is to provide researchers with high quality biospecimens and annotated data to facilitate research aiming to improve the diagnosis and treatment of MM, and to develop effective preventative measures. The ADRI biobank contains over 2,000 MM patient-derived biospecimens, which includes fresh frozen tissue, FFPE tissue, pleural fluid, blood, primary cells and cell lines; as well as over 12,000 derivatives, which includes tumour DNA, tumour RNA, plasma, buffy coat, serum and red blood cells. The biobank is intended primarily as an in-house resource to be used by ADRI research staff, however external requests for access to samples may be granted for Ethics approved projects in some cases.

Collectively, these biobanks constitute a valuable source of high quality biospecimens and associated clinical data that are critically important for researchers undertaking MM-related investigations. Collaboration across MM biobanks at both national and international levels should be encouraged to promote the sharing of biospecimens and clinical data. Although MM is globally increasing it is still rare in comparison to other cancers and carcinomas which poses a challenge to the collection of biospecimens. Research directed at genetic differences in relation to the causality, progression and response to treatment, has not been adequately addressed so far

but can hugely benefit from a collaborative scheme. A more global and interactive MM-specific biobanking network would be particularly beneficial to researchers investigating epidemiological-related factors influencing the disease mechanisms, diagnosis and treatment of MM. From a global perspective, we advocate the establishment of MM biobanks in the many developing countries that continue to use asbestos and which have recently started to diagnose mesothelioma. To this end, we have been engaged in providing international training workshops to improve the recognition and diagnosis of MM (102).

EXPERT COMMENTARY AND CONCLUSIONS

Mesothelioma continues to represent a significant burden on public health worldwide and its incidence is unlikely to decrease in the coming years given the long latency associated with its pathogenesis in asbestos-exposed individuals, combined with continued human exposure to asbestos fibres in the environment. Despite the previous substantial preclinical research efforts that have been devoted to improving our understanding of MM biology with respect to the development of improved diagnostic and therapeutic strategies, clinical practice involving the diagnosis and treatment of MM has remained relatively unchanged over the past few decades and consequently patient prognosis has not improved significantly. Hence, continued basic science research using preclinical models of MM is greatly needed in order to further expand our knowledge of MM biology and to investigate improved diagnostic and treatment strategies. With further investigation of the developmental biology of MM using *in vitro* and *in vivo* models, it will become possible to identify and characterise additional MM-specific molecular targets that can potentially be pursued for the testing and development of improved biomarkers and therapeutic strategies.

As we have summarised, there are a variety of useful preclinical models available to researchers studying MM. Different models, whether they be cell-based or animal-based, have their own intrinsic advantages and disadvantages; no model is perfect. The accuracy and reliability of the generated experimental data is highly dependent on the type of model selected and its suitability to the specific aims or criteria being addressed in the study. Ultimately, MM-based studies that employ accurate preclinical models will stand a better chance at progressing through to clinical trials; particularly studies that are able to reproduce the experimental data using multiple model types. For example, studies that are investigating the efficacy of novel immunotherapeutic agents for MM would only produce clinically relevant and reliable data by utilising a syngeneic subcutaneous and/or orthotopic model, given that they both possess an intact immune system.

Laboratory technology/techniques are constantly evolving, with significant technological advancements having been attained in regards to 3D cell culture, NGS technology and qPCR. These techniques/technology are fundamental to research aiming to explore MM tumour cell response to novel drug treatments, and the identification of novel biomarker candidates that possess valuable diagnostic and/or prognostic qualities. It is crucially important that researchers utilise the most current techniques and technology where possible. For instance, a study examining chemotherapy drug cytotoxicity in a 3D MM cell culture system will likely generate preclinical data that more accurately mimics chemotherapy drug behaviour in the clinical setting compared to the same experiment conducted in a 2D cell culture system. The ddPCR technique was given particular emphasis in this review given that it is a highly reliable and precise PCR technique that has shown emerging potential for the detection and validation of MM-specific biomarkers in recent years. Given the superior sensitivity of this modern PCR technique, it would be highly beneficial to prospective studies aiming to detect and validate novel circulating MM-specific biomarkers that would not normally be detected by other conventional qPCR platforms. Less invasive blood-based biomarkers are particularly lacking for MM and invasive biopsy procedures are still required to attain a definitive diagnosis (103, 104). Hence, prospective research studies aiming to validate and develop a blood-based biomarker panel for MM, through the application of the ddPCR technique, would constitute a significant advancement in the field of MM clinical diagnostics.

Given that mesothelioma is a relatively rare cancer in comparison to other disease types, access to patient biospecimens is somewhat limited and therefore collaborations between expert mesothelioma research centres worldwide should be strongly encouraged to overcome this limitation. Such multi-centre collaborations would enable the sharing of biobanked research specimens and associated data, which would facilitate the development of projects using a large and diverse sample cohort. In turn, these studies would be likely to produce statistically powered data to support the efficacy/validity of novel biomarkers and treatment strategies that would have strong potential to progress through to clinical trials. Furthermore, such multi-centre collaborative studies would enable researchers to more easily afford the high costs typically associated with modern laboratory technologies, such as NGS.

AUTHOR CONTRIBUTIONS

YC and BJ conceived the paper, where BJ and YC wrote the paper and all three authors edited the final draft. All authors contributed to the article and approved the submitted version.

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REVIEW
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Role of Immune Checkpoint Inhibitor Therapy in Advanced *EGFR*-Mutant Non-Small Cell Lung Cancer

Nathaniel Wiest[1], *Umair Majeed*[2], *Karan Seegobin*[2], *Yujie Zhao*[2], *Yanyan Lou*[2] *and Rami Manochakian*[2]*

[1] Department of Internal Medicine, Mayo Clinic, Jacksonville, FL, United States, [2] Division of Hematology and Oncology, Department of Internal Medicine, Mayo Clinic, Jacksonville, FL, United States

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***Correspondence:**
Rami Manochakian
manochakian.rami@mayo.edu

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Over the last decade, the treatment of advanced non-small cell lung cancer (NSCLC) has undergone rapid changes with innovations in oncogene-directed therapy and immune checkpoint inhibitors. In patients with epidermal growth factor receptor (EGFR) gene mutant (*EGFR*m) NSCLC, newer-generation tyrosine kinase inhibitors (TKIs) are providing unparalleled survival benefit and tolerability. Unfortunately, most patients will experience disease progression and thus an urgent need exists for improved subsequent lines of therapies. The concurrent revolution in immune checkpoint inhibitor (ICI) therapy is providing novel treatment options with improved clinical outcomes in wild-type *EGFR* (*EGFR*wt) NSCLC; however, the application of ICI therapy to advanced *EGFR*m NSCLC patients is controversial. Early studies demonstrated the inferiority of ICI monotherapy to EGFR TKI therapy in the first line setting and inferiority to chemotherapy in the second line setting. Additionally, combination ICI and EGFR TKI therapies have demonstrated increased toxicities, and EGFR TKI therapy given after first-line ICI therapy has been correlated with severe adverse events. Nonetheless, combination therapies including dual-ICI blockade and ICI, chemotherapy, and angiogenesis inhibitor combinations are areas of active study with some intriguing signals in preliminary studies. Here, we review previous and ongoing clinical studies of ICI therapy in advanced *EGFR*m NSCLC. We discuss advances in understanding the differences in the tumor biology and tumor microenvironment (TME) of *EGFRm* NSCLC tumors that may lead to novel approaches to enhance ICI efficacy. It is our goal to equip the reader with a knowledge of current therapies, past and current clinical trials, and active avenues of research that provide the promise of novel approaches and improved outcomes for patients with advanced *EGFR*m NSCLC.

Keywords: Non-small cell lung cancer, lung cancer, EGFR, tyrosine kinase inhibitor, immune-mediated adverse effects, immune checkpoint inhibitor (ICI), cancer immunotherapies

1 INTRODUCTION

Lung cancer remains the leading cause of cancer-related death in the United States with an estimated 235,760 new cases and 131,880 new deaths in 2021 (1). Non-small cell lung cancer (NSCLC) represents approximately 85% of all lung cancer cases in the United States (2), and includes three major histologic subtypes: adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC). More than three quarters of patients with NSCLC have advanced (stage III or IV) disease at time of diagnosis, where clinical outcomes and survival have remained suboptimal (3). Fortunately, systemic treatment options for NSCLC have recently made significant improvements with advancements in oncogene-directed and ICI therapies. Data from the Surveillance, Epidemiology, and End Results (SEER) database demonstrate an approximate two-fold increase in 5-year survival for patients with lung cancer from 1973 to 2010 from 10.7 to 19.8% (4), consistent with improved treatment options, and this trend in improved patient outcomes is expected to continue. Targeted therapies for NSCLC are constantly evolving and there is significant interest in the potential interplay between immunotherapy and targeted therapies.

Multiple targetable genetic alterations have been identified in patients with NSCLC affecting the *EGFR, KRAS*, *BRAF*, *PIK3CA, ALK*, *ROS1*, *NRAS*, and *MET* genes among others (5). Lung ADC harbors more recognized oncogene mutations that SCC or LCC (6, 7), with up to 64% of metastatic lung ADC cases carrying a recognized oncogene driver mutation (8). The frequency of driver mutations is increased in females, never-smokers, and East Asian populations (6). Many clinical trials are underway to expand the number of targeted therapies, therapy combinations, and clinical contexts in which targeted therapies can be offered in NSCLC (9).

EGFR mutations are among the most common NSCLC driver mutations. EGFR is a receptor tyrosine kinase (RTK) that activates Ras/MAPK and PI3K/Akt cell signaling pathways and leads to cell proliferation, metastasis, and resistance to cell death when dysregulated (10). *EGFR* mutations are found in 19-23% of lung ADC in the United States and up to 64-67% of lung ADC in other regions including South East Asia and Peru (11). *EGFR* mutations are less common in lung SCC with a frequency of 2-10% and have only rarely been reported in cases of LCL (12, 13). Almost 90% of *EGFR* mutations are either deletions in exon 19 (ex19del) or leucine to arginine substation in exon 21 (L858R), with less common mutations occurring in exons 18, 20, and elsewhere (14). These mutations structurally activate EGFR signaling *via* different mechanisms and, critically, increase the binding affinity of various EGFR TKIs that inhibit mutant EGFR and spare wild-type EGFR at therapeutic concentrations (15, 16).

First-generation EGFR TKIs including erlotinib and gefitinib were first approved the U.S. Food and Drug Administration (FDA) in 2013 for first line use in metastatic *EGFR* ex19dels or L858R mutant NSCLC in 2013 after multiple studies demonstrated improved clinical outcomes compared to platinum-based chemotherapy (17, 18). The second generation, irreversible EGFR TKI afatanib was approved for first-line use in July 2013 (19). Despite improvement in PFS with first- and second-generation EGFR TKIs, clinical trials failed to uniformly demonstrate improvements in overall survival (OS) due to the development of TKI resistance occurring typically 10-14 months after treatment initiation (20). The most common mechanism of acquired resistance is the EGFR T790M mutation that inhibits the binding of first- and second-generation EGFR TKIs (21), though other mechanisms of resistance have been described (21). Given the predominance of EGFR T790M as the escape strategy for TKI resistance, third-generation EGFR TKIs including osimertinib were developed that are effective against EGFR containing the T790M substitution (16). Osimertinib was initially approved in 2015 for second-line treatment of EGFR T790M-mutant NSCLC after progression on first-line EGFR-TKIs; however, this approval was expanded to first-line use after the landmark FLAURA study demonstrated significantly increased PFS with osimertinib versus standard EGFR-TKIs of 18.9 versus 10.2 months (HR for PFS 0.46, 95% CI 0.37-0.57, $p<0.001$) and improved OS of 38.6 versus 31.8 months (HR for death 0.80, 95% CI 0.64-1.00, $p = 0.046$) (22–24). Given the impressive performance of osimertinib in both first- and second-line contexts, as well as its favorable side effect profile, it is now the standard of care for EGFR targeted therapy in *EGFR*m NSCLC (25). Active research is defining which *EGFR* mutations respond best to different EGFR TKIs and fourth generation inhibitors have been described that are under active investigation (26).

In addition to driver-mutation targeted therapies, the discovery and utilization of immune checkpoint (ICP) inhibitors in NSCLC has provided new and hopeful treatment options for many patients (27).The ICP describes an immunomodulatory process that downregulates T-cell effector responses and is mediated in part by the B7 ligand binding to the cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) receptor and the programmed cell death ligands 1 and 2 (PD-L1 and PD-L2) binding to the programmed cell death protein 1 (PD-1) (28). While the ICP is normally a tissue protective mechanism that prevents autoimmunity, ICP activation is a strategy that many cancer types including NSCLC utilize to impede effective anti-tumor T-cell responses (29). Monoclonal antibodies that bind to CTLA4, PD-1, or PD-L1, known as ICIs, reduce activation of the PD-1/PD-L1 axis to remove inhibitory signals of anti-tumor T-cell activation. ICP blockade enhances anti-tumor T-cell mediated immune responses, especially in immunogenic tumors that rely on ICP activation to escape immune destruction (30, 31). An active challenge is to identify patients who will respond to ICIs before prescribing therapy, as ICIs can cause potent and wide-ranging immune-mediated adverse events (irAEs) including rash, endocrine abnormalities, and interstitial pneumonitis among many others (32).

There are multiple ICIs currently approved by the FDA for the treatment of NSCLC (33). ICIs were first approved in the second-line setting for metastatic NSCLC (mNSCLC) in March 2015 after nivolumab demonstrated superiority to docetaxel for squamous mNSCLC that progressed on platinum therapy (34). Subsequently, both pembrolizumab and atezolizumab were

approved in second-line contexts for mNSCLC by 2016, and since then ICI indications have expanded and are approved in different combinations and settings (35). Pembrolizumab, atezolizumab, and combination of ipilimumab + nivolumab have obtained approval in first-line contexts (36). As such, there now exists a potent repertoire of immunotherapy strategies for patients with advanced NSCLC, though the question of how to integrate ICI therapy with targeted therapies in oncogene-driven NSCLC is an active area of research.

The role of ICI therapy in *EGFR*m advanced NSCLC is the focus of this review. We have discussed the role of ICI therapy in NSCLC with *ALK*, *ROS1*, *BRAF*, *c-MET*, *RET*, and *NTRK* mutations is separate, companion review in this journal. Below, we detail past and current clinical trials evaluating ICI therapy in advanced *EGFR*m NSCLC. We highlight different treatment sequences and combinations as well as subgroups that experienced either improved outcomes or unexpected toxicities. While the role of ICI therapy in *EGFR*m NSCLC is controversial, there is intriguing and hopeful evidence that certain combinations may prove beneficial, and active research is elucidating properties of *EGFR*m tumor biology and TME composition that we anticipate will lead to novel therapies in the near future.

2 CLINICAL TRIALS OF IMMUNE CHECKPOINT THERAPY IN ADVANCED EGFR MUTANT NSCLC

A number of clinical trials have been performed utilizing ICIs in advanced *EGFR*m NSCLC. Initial studies on ICI first-line therapy were overall disappointing and did not reach the efficacy of *EGFR* TKIs. However, second- and later-line ICI therapy has demonstrated promise in certain contexts, with select subgroups demonstrating improved response to ICI strategies. Active clinical studies are addressing important questions including combinations of dual ICIs and ICI + Vascular Endothelial Growth Factor (VEGF) inhibitors + chemotherapy in progressive *EGFR*m NSCLC that are of significant interest to researchers and clinicians in the field. Clinical trials are summarized in **Tables 1**, **2** and are described in detail below.

2.1 First-Line ICI Therapy

Several small studies or subgroups of larger studies have evaluated first-line ICIs either alone or as combination therapy in advanced *EGFR*m NSCLC (**Table 1**). Overall, ICI first-line therapy is unhelpful compared to EGFR TKI monotherapy, especially given the outstanding safety profile of the third-generation EGFR TKI osimertinib for advanced *EGFR*m NSCLC patients with PFS reported at 18.9 months and OS reported at 38.6 months in the FLAURA trial (23). Additionally, combination ICI + EGFR TKI may have enhanced toxicity and ICI treatment before EGFR TKI administration may prime patients for significant later toxicities with second-line EGFR TKI treatment.

2.1.1 ICI Monotherapy

Based on a subgroup of the KEYNOTE-001 trial, in which a small number of *EGFR*m, TKI-naïve patients experienced improved objective response rate (ORR) compared to TKI-pretreated patients, the follow up Phase II NCT02879994 trial evaluated pembrolizumab first-line therapy in TKI-naïve, *EGFR*m advanced NSCLC patients with PD-L1 positivity (TPS ≥ 1%) (38, 58). This trial was halted early due to futility, as only 1/11 initial patients experienced an OR and this patient was found on subsequent analysis to be *EGFR*wt. Importantly, 6/7 patients that switched to second line TKI therapy after PD on pembrolizumab experienced treatment-related adverse events (TRAEs) from the TKI (erlotinib), including one patient who experienced grade 5 pneumonitis. These results suggested potential toxicity of EGFR TKI therapy after pembrolizumab treatment in *EGFR*m NSCLC patients.

A small subgroup of patients in the CheckMate 012 study examining first-line nivolumab monotherapy were *EGFR*m (6/56, 11%). The ORR for *EGFR*m versus *EGFR*wt patients was 14% versus 30%, indicating a comparatively decreased efficacy of ICI therapy in the first-line for *EGFR*m patients (37).

2.1.2 ICI + Chemotherapy

The CheckMate 012 trial compared niviolumab + platinum-doublet chemotherapy in *EGFR*wt versus *EGFR*m advanced NSCLC patients as first-line treatment (39). *EGFR*m patients experienced worse PFS (4.8 *vs* 7.5 months) and median OS (20.5 versus 24.5 months) compared to *EGFR*wt.

2.1.3 ICI + EGFR TKI Therapy

The results of the phase 1 NCT02088112 study, a two-part, dose-escalation study with durvalumab + gefitinib as first-line therapy in advanced *EGFR*m NSCLC patients, were recently published (40). All patients in the study received gefitinib daily. In the dose escalation portion of the study (Part A), 3 patients were randomized to additionally receive durvalumab 3 mg/kg every 3 weeks and 13 were randomized to receive durvalumab 10mg/kg every 3 weeks. Grade 3/4 TRAEs were reported in 68.8% of dose-escalation patients, leading to discontinuation of combined treatment in 94% of patients in this phase. In the dose-expansion phase (Part B), 40 patients were recruited to one of two treatment strategies: 1) gefitinib + durvalumab 10 mg/kg every 2 weeks (Arm 1, 30 patients), or 2) gefitinib for 4 weeks followed by addition of durvalumab 10 mg/kg every 2 weeks (Arm 2, 10 patients). Median PFS was 10.1 months in Arm 1 (95% CI 5.5-15.2 months) and 12.0 months in Arm2 (95% CI: 2.7-15.6 months), which was not considered improved compared to gefitinib monotherapy studies. For example, the phase 4 update of the NCT01203917 first-line gefitinib study demonstrated PFS of 9.7-10.2 months with gefitinib monotherapy (59). 17/40 of the dose-expansion phase patients experienced high-grade hepatic events, suggesting an additive effect of gefitinib and durvalumab for hepatotoxicity. The authors noted a trend towards favorable PFS in patients with

TABLE 1 | First-line ICI Clinical Trials in *EGFR*m NSCLC.

Trial	Phase	Intervention	Outcome	Safety	Reference
ICI Monotherapy					
CheckMate 012	1	Nivolumab	ORR: 14% for *EGFR*m *vs* 30% for *EGFR*wt PFS: 1.8 *vs* 6.6 mo	G3-4[#]: 17%, G5: 0%	(37)
NCT02879994	2	Pembrolizumab	ORR: 0%*	TRAE: 46%, no G4-5 6/7 patients had a TRAE on second-line EGFR TKI, including one G5 pneumonitis	(38)
ICI + Chemotherapy					
CheckMate 012	1	Nivolumab + PT-DC	ORR: 17% for *EGFR*m *vs* 47% for *EGFR*wt PFS: 4.8 *vs* 7.5 mo OS: 20.5 *vs* 24.5 mo	G3-4[#]: 50%, G5: 0%. Pneumonitis most common TRAE (7%)	(39)
ICI + EGFR TKI Therapy					
NCT02088112	1	Gefitinib + durvalumab dose escalation	ORR: 63.3%-70% PFS: 10.1-12.0 mo	TRAE: 100%, 17/40 high-grade hepatic AEs	(40)
KEYNOTE-021	3	Pembrolizumab (P) + erlotinib (E) or gefitinib (G)	ORR: 41.7% P+E, 14.3% P + G PFS: 19.5 mo P+E, 1.4 mo P + G	P+E: TRAE: 100%, G3: 33.3%, no G4-5 P+G: TRAE: 85.7%, G3-4: 71.4% hepatotoxic AEs	(41)
NCT02013219	1b	Atezolizumab + erlotinib	ORR: 75% PFS: 11.3 mo	G3-4[#]: 39%	(42)
Dual ICI Therapy					
CheckMate 012	1	Nivolumab + ipilimumab	ORR: 50%	TRAE[#]: 72-82%, G3-4: 33-37%, no G5	(43)

*Indicated categories of trials with respective trial parameters are given. *1/11 patients initially reported to respond but was found to be EGFRwt. ORR, overall response rate; PFS, progression-free survival; OS, overall survival; TRAE, Treatment related adverse event; G, grade of toxicity; PT-DC, platinum-doublet chemotherapy; [#]TRAEs for entire study population and not selected for EGFRm patients.*

TPS ≥ 20% (HR 0.46, 95% CI 0.19-1.03), but overall concluded that their study did not support combination gefitinib + durvalumab as first line treatment in *EGFR*m advanced NSCLC.

The ongoing open-label, multicohort phase 1/2 KEYNOTE-021 study (NCT02039674) is evaluating pembrolizumab in combination with chemotherapy or immunotherapy in previously untreated stage IIIB/IV *EGFR*m NSCLC patients (41). Two cohorts were reported early and evaluated pembrolizumab + EGFR TKI: Cohort E included 12 patients treated with pembrolizumab + erlotinib and Cohort F included 7 patients treated with pembrolizumab + gefitinib. The pembrolizumab + gefitinib arm was permanently discontinued early due to safety concerns, as 5/7 patients (71.4%) had treatment-related elevations in ALT and AST. However, the pembrolizumab + erlotinib arm was found to be tolerated and this arm experienced an objective response rate (ORR) of 41.7%, which is similar to the ORR seen with erlotinib and pembrolizumab monotherapies. All (4/4) patients with TPS ≥ 50% had an objective response, whereas only 1/4 patients with TPS 1%-49% and 0/2 patients with TPS <1% responded to pembrolizumab + erlotinib (41).

The phase 1b NCT02013219 study evaluated first-line erlotinib + atezolizumab in TKI-naïve patients with *EGFR*m NSCLC (42). The preliminary report included 28 patients and demonstrated a median PFS of 11.3 months, which was similar to erlotinib monotherapy. However, 50% of patients experienced serious TRAEs including 39% of patients who experienced grade 3/4 events. These data suggest that combination atezolizumab + erlotinib enhances toxicity without significant additive benefit. We await the publication of full trial results make a more comprehensive assessment of this combination.

The Phase 1/2 CheckMate 370 study (NCT02574078) is evaluating nivolumab as maintenance or first-line + other standard of care therapies (60). Group D will compare erlotinib versus nivolumab + erlotinib. Results have not yet been announced.

2.1.4 Dual ICI Therapy

As part of the multi-arm phase 1 CheckMate 012 (NCT01454102) trial, patients with chemotherapy-naïve Stage IIIB/IV NSCLC were randomized to receive different dose schedules of nivolumab and ipilimumab as first line treatment. 10-11% of patients in different arms had *EGFR* activating mutations. Of these, 50% (4/8) had objective responses with combined nivolumab + ipilimumab, including 3/3 (100%) of patients with TPS ≥ 50% (43). PFS data are not available. These results, while limited by small sample size, suggest that combination immune checkpoint inhibition therapy may more effectively sensitize *EGFR*-mutant, PD-L1-expressing NSCLC to immune-mediated destruction than ICI monotherapy.

2.2 Second-Line or Later ICI Therapy

Multiple clinical trials have been performed with single or dual agent ICI therapy after patients experienced progressive disease (PD) with EGFR TKIs (**Table 2**). Most of the earlier studies were single agent trials that did not demonstrate benefit versus chemotherapy. However, more recent trials have added intriguing combination ICI strategies that may yield enhanced benefit.

TABLE 2 | Second-line or later ICI Clinical Trials in *EGFR*m NSCLC.

Trial	Phase	Intervention	Outcome	Safety	Reference
ICI Monotherapy					
KEYNOTE-010	3	Pembrolizumab (P) *vs* docetaxel (D)	HR for PFS[$] with P *vs* D: 1.79 in *EGFR*m *vs* 0.83 in *EGFR*wt HR for OS with P *vs* D: 0.88 vs 0.66	P: G3-5[#]: 13-16% D: G3-5: 35%	(44)
CheckMate 057	3	Nivolumab (N) *vs* docetaxel (D)	HR for OS[$] with N *vs* D: 1.18 for *EGFR*m *vs* 0.66 in *EGFR*wt	N: G3-5[#]: 10% D: G3-5: 54%	(45)
POPLAR	2	Atezolizumab (A) *vs* docetaxel (D)	HR for OS[$] with A *vs* D: 0.99 for *EGFR*m *vs* 0.70 in *EGFR*wt	A: G3-4[#]: 40%, G5: 4% D: G3-4: 53%, G5: 4%	(46, 47)
KEYNOTE-001	1b	Pembrolizumab	PFS[$]: 6.0 mo in *EGFR*m *vs* 12.1 mo in *EGFR*wt	N.R.**	(48)
PACIFIC	3	Durvalumab	HR for PD[$] or death: 0.76 in *EGFR*m *vs* 0.47 in *EGFR*wt	TRAE: 96.8%, G3-4: 29.9%	(49)
ATLANTIC	2	Durvalumab	ORR for *EGFR*m/*ALK*m: 3.6% PD-L1 TPS <25%, 12.2% for PD-L1 ≥25% OS for *EGFR*m/*ALK*m: 9.9 mo PD-L1 TPS <25%, 13.3 mo for PD-L1 ≥25%	G3-4: 5%	(50)
ICI + Chemotherapy					
NCT03513666	2	Toripalimab + PT-DC	ORR: 50% PFS: 7.0 mo	G3-5: 55%, including neutropenia (48%), leukopenia (20%), and anemia (13%)	(51)
ICI + EGFR TKI Therapy					
CheckMate 012	3	Nivolumab + erlotinib	ORR: 15%[&] PFS: 5.1 mo OS: 18.7 mo	G3: 24%, no G4-G5	(52)
TATTON	1b	Durvalumab + osimertinib	ORR: 43%	TRAE: 100%, G3-5: 48%. ILD occurred in 22% with G≥3 ILD in 8.7%	(53)
CAURAL	3	Durvalumab + osimertinib	ORR: 64%	TRAE: 100%, G3-5: 8%. One G2 ILD reported	(54)
Dual ICI Therapy					
KEYNOTE-021	1/2	Pembrolizumab + ipilimumab	ORR: 10% for *EGFR*m *vs* 30% for *EGFR*wt	TRAE[#]: 98%, G3-G5: 49%, one G5 pancreatitis	(55)
ICI + VEGF Inhibitor + Chemotherapy					
IMpower150	3	Atezolizumab (A) + bevacizumab (B) + carboplatin-paclitaxel (CP)	ORR[##]: 70.6% for ABCP, 35.6% for ACP, 41.9% for BCP OS: NR for ABCP[##], 17.5 mo for BCP	G3-4: 64% of ABCP, 68% of ACP, and 64% of BCP	(56)
NCT03647956	2	Atezolizumab + bevacizumab + pemetrexed-carboplatin	ORR: 62.5% PFS: 9.43 mo	G3-5: 37.5%, One G5 myocardial infarction, 7.5% blood clot	(57)

*Indicated categories of trials with respective trial parameters are given. ORR, overall response rate; TRAE, Treatment related adverse event; G, grade of toxicity. [#]TRAEs for entire study population and not selected for EGFRm patients. [$]ORR data not given for EGFRm subgroup. **Safety data were not reported in this long-term survival update report. PD, progressive disease; PT-DC, platinum-doublet chemotherapy. [&]Authors note that these patients were all TKI treated for first-line. NR, not reached. [##]These numbers refer to the subgroup of patients with sensitizing EGFR mutations.*

2.2.1 ICI Monotherapy

In regard to single agent ICI as a second-line agent after PD on EGFR TKI, three studies explored different single agent ICIs versus docetaxel. KEYNOTE-010 included a small number of *EGFR*m advanced NSCLC patients (9%) who were randomized to receive 2mg/kg pembrolizumab, 10mg/kg pembrolizumab, or 75 mg/m^2 docetaxel every three weeks after having progressed on at least two cycles of platinum-based chemotherapy and treatment with an EGFR TKI. The *EGFR*m patients experienced worse PFS with pembrolizumab versus docetaxel (HR for PFS 1.79, 95% CI 0.94-3.42, *p* not given), in contrast to the *EGFR*wt patients who had an improved pooled PFS (HR for PFS 0.83, 95% CI 0.71-0.98, *p* not given) (44). CheckMate 057 (NCT01673867) was a large phase 3 study of nonsquamous NSCLC patients who had progressed on or after platinum-doublet therapy that compared nivolumab 3mg/kg every two weeks versus docetaxel 75 mg/m^2 every three weeks. *EGFR*m patients were allowed to have received previous treatment with an EGFR TKI. 14% of study participants were *EGFR*m positive and experienced worse OS with nivolumab compared to docetaxel (HR 1.18, 95% CI 0.69-2.00, *p* not given), whereas the *EGFR*wt patients experienced significant benefit to OS with nivolumab (HR 0.66, 95% CI 0.51-0.86, *p* not given) (45). The POPLAR phase 2 study compared atezolizumab versus docetaxel as second line therapy for NSCLC. Similar to the PD-1 inhibitor studies, atezolizumab improved OS compared to docetaxel among all NSCLC patients (HR 0.73, 95% CI 0.53-0.99, *p*=0.04) (46). However, as reported by Lee et al., the subgroup of *EGFR*m patients did not see an improvement in OS (HR for OS 0.70 in WT versus 0.99 in *EGFR*-mutant, compared to docetaxel) (47). Thus, PD-L1 inhibition, as with PD-1 inhibition, failed to demonstrate improvement in *EGFR*m patients in the second-line context, though ICI therapy was more well tolerated across all three studies with less TRAEs compared to docetaxel.

A meta-analysis published by Lee et al. combined the results of these three studies utilizing ICI single therapy versus docetaxel as second-line therapy. The *EGFR*m NSCLC patients overall did

not benefit from ICI monotherapy compared to docetaxel, whereas *EGFR*wt patients experienced a significant benefit from ICI therapy (HR for OS 1.05 for *EGFR*m *vs* 0.66 for *EGFR*wt) (47), confirming that ICI monotherapy is not advantageous over chemotherapy in the second-line setting for *EGFR*m patients with PD on TKI/chemotherapy.

Hui et al. reported updated results from the KEYNOTE-001 study, which examined pembrolizumab efficacy across several settings for patients with NSCLC and PD-L1 TPS ≥ 1% (48). The subgroup of *EGFR*m patients who had previously received treatment had significantly less benefit from pembrolizumab than *EGFR*wt patients (median OS 12.1 months versus 6.0 months). PD-L1 overexpression (TPS ≥ 50%) did not rescue response to pembrolizumab in the *EGFR*m *vs EGFR*wt patients (median OS 6.5 *versus* 15.7 months) (48). These results suggested that PD-L1 is an imperfect biomarker to predict ICI response in previously treated *EGFR*m patients, as discussed in further detail below.

The PACIFIC trial (NCT02125461) was a phase 3 trial that assessed the addition of durvalumab consolidation therapy after definitive chemoradiotherapy (CRT) for patients with stage III NSCLC (49). *EGFR*m NSCLC patients did not have significant benefit from durvalumab consolidation therapy (HR for PD or death 0.76, 95% CI: 0.35-1.64) whereas the *EGFR*wt patients did experience benefit (HR for PD or death 0.47, 95% CI: 0.36-0.60). In the recently published four-year survival update of the PACIFIC trial, *EGFR*m NSCLC patients again did not demonstrate benefit from durvalumab consolidation (HR for PFS 0.84, 95% CI: 0.40-1.75) whereas the *EGFR*wt patients again demonstrated significant benefit (HR for PFS 0.51, 95% CI: 0.40-0.65) (61).

Similar to the PACIFIC trial, Aredo et al. recently published the results of a multi-center retrospective study of patients (n=13) with unresectable *EGFR*m NSCLC who received consolidation durvalumab after CRT (62). They compared these patients to a cohort of *EGFR*m NSCLC patients who instead received consolidation EGFR TKI after CRT (n=24). Median PFS was 10.3 months for the CRT + durvalumab cohort versus 26.1 months for the CRT + EKGFR TKI group ($p = 0.023$). Notably, six patients opted to switch to EGFR TKI after experiencing PD on CRT + durvalumab and one of these patients developed Grade 4 pneumonitis 17 days after initiating osimertinib, again highlighting the safety signal of initiating EGFR TKIs after ICI therapy.

The ATLANTIC trial was a phase 2 open-label trial of durvalumab monotherapy as third-line or later treatment in patients with advanced NSCLC (63). Enrolled patients had to have received at least two previous lines, with one platinum-containing regimen and a TKI if indicated. Cohort 1 included both *EGFR*m and *ALK*m NSCLC patients and was stratified by PD-L1 TPS: median OS was 13.3 months in the TPS ≥ 25% subcohort versus 9.9 months in the TPS < 25% subcohort. Notably, this was higher than in the *EGFR*wt and *ALK*wt Cohort 2 that demonstrated median OS of 10.9 versus 9.3 months for TPS ≥ 25% and TPS <25% subcohorts, respectively. The safety profile of *EGFR*m/*ALK*m cohort was similar to the *EGFR*wt/*ALK*wt profile, with 6-8% of patients experiencing Grade 3-4 TRAEs, supporting the safety of ICI administration after EGFR TKI treatment. Of note, the ATLANTIC trial has multiple limitations, including lack of descriptive statistics, single-arm design, and variations in testing platform for PD-L1 that made direct comparisons with other trials not possible (64). Despite this, the ATLANTIC trial suggested that ICI therapy may have efficacy in heavily pre-treated *EGFR*m NSCLC patients and further supported the safety of ICI therapy after patients progressed on TKI therapy.

2.2.2 ICI + Chemotherapy

The NCT03513666 trial was a phase 2 study that evaluated toripalimab (anti-PD-1) + platinum doublet chemotherapy in patients with *EGFR*m advanced NSCLC who developed PD on first- and second-generation EGFR TKIs without T790M mutation (51). Median PFS was 7.0 months, and interestingly the authors identified that *TP53* co-mutation patients experienced significantly improved ORR compared to *TP53*wt patients (62% *vs* 14%, $p = 0.04$). This combination was found to have manageable safety profile and efficacy, and a follow up randomized Phase III trial (NCT03924050) that will compare this combination to standard chemotherapy with planned enrollment for 350 patients (65).

The CheckMate 722 trial (NCT02864251) is a currently active phase 3 study of patients with *EGFR*m, T790M-negative recurrent or stage IV NSCLC who have previously been treated with EGFR TKI therapy (66). Arm A comprises nivolumab + platinum-doublet therapy and Arm C involves platinum-doublet alone. Additionally, the KEYNOTE-789 trial (NCT03515837) is another currently active Phase III trial evaluating pemetrexed-platinum combined with pembrolizumab versus placebo in *EGFR*m advanced NSCLC that has progressed on EGFR TKI (67). The results of these studies will provide highly valuable information on the efficacy and safety of second line ICI + chemotherapy. The CheckMate 722 trial has the additional benefit of comparing this strategy to dual ICI therapy (Arms A versus B).

2.2.3 ICI + EGFR TKI Therapy

A number of trials have evaluated combination ICI therapy and EGFR-targeted therapy in the second-line and beyond. These studies were predicated on pre-clinical studies that suggested added benefit to the combined approach in animal models (68–71). However, results have mostly been disappointing and in many cases demonstrated increased and severe toxicities. Nonetheless, several ongoing trials are assessing different combination therapies that will be of interest when results are available.

2.2.3.1 Nivolumab + EGFR TKI

Arm E of the CheckMate012 study evaluated nivolumab + erlotinib in 21 *EGFR*m NSCLC patients (52). 20/21 patients had discontinued prior erlotinib treatment due to PD, and 1/21 patients was TKI-naïve. 3/21 patients had an OR to nivolumab + erlotinib, including the treatment naïve patient who also had atypical *EGFR* mutation status (double L858R, S768I). 24-week

PFS was rate was 48%. The PFS for the previously TKI-tread patients (n=20) was 5.1 months. Overall, most patients had PD and were switched to other treatment regimens (52). Of note, the patients who responded were either PD-L1 positive (10% or 65%) or had unknown PD-L1 status. While not significantly efficacious in this small study, the dual nivolumab + erlotinib therapy was overall tolerated well, with no grade 4 or 5 TRAEs reported and 2/21 patients discontinuing study drugs due to toxicity.

2.2.3.2 Durvalumab + EGFR TKI

The TATTON phase 1b study (NCT02143466) is highly significant in regard to the safety of combination EGFR-targeted + ICI combination therapy. In TATTON, osimertinib was combined with durvalumab in one of the three study arms to treat 23 patients with advanced *EGFR*m NSCLC that had progressed on previous EGFR TKI therapy (53). The ORR was 43%; however, significant safety concerns arose as 48% of patients had at least one grade 3 TRAE and 5/23 patients developed interstitial lung disease (ILD), leading all patients to discontinue the study. This study is very relevant now that osimertinib is the standard of care for first-line *EGFR*m NSCLC and as second-line for *EGFR*m NSCLC that progressed on a previous EGFR TKI.

In the CAURAL phase III study (NCT02454933), patients with *EGFR*m T790M-positive advanced NSCLC with PD after initial EGFR TKI therapy were randomized to receive either osimertinib or osimertinib + durvalumab (54). CAURAL was terminated early after one patient developed ILD given the contemporaneously reported results of the TATTON trial, though partial results were reported. In all, 15 patients received osimertinib and 14 received osimertinib + durvalumab. The ORR in the osimertinib arm was 80% versus 64% in the combination arm, and the median 12-month PFS rates were 82% and 76% for the osimertinib and combination arms respectively, indicating no evidence of increased efficacy of the combined approach. Aside from the one patient who developed grade 2 ILD in the combination arm (after receiving only a single dose of durvalumab and remaining on osimertinib), the safety profile was otherwise relatively unremarkable with no other ILD events reported.

2.2.3.3 Atezolizumab + EGFR TKI

A phase 1b/2 study (NCT02630186) evaluating rociletinib, a third generation EGFR TKI, + atezolizumab in *EGFR*m patients who progressed after prior EGFR TKI was terminated after only three patients were recruited (72). No efficacy data were reported, and it was noted that 1/3 patients experienced a serious AE (pancreatitis), and all patients experienced AEs that included diarrhea (3/3), nausea (2/3), and bilateral hearing loss (1/3) among others.

2.2.3.4 Tremelimumab + EGFR TKI

The phase 1 GEFTREM trial (NCT02040064) evaluated the safety of dose-escalation of the CTLA-4 inhibitor tremelimumab (3 mg/kg, 6 mg/kg, and 10 mg/kg) in combination with gefitinib in previously treated *EGFR*m NSCLC patients (73). The preliminary report indicated that dose-limiting toxicities occurred in 5/26 patients, and multiple grade 3/4 TREAs were reported that resolved upon discontinuation of tremelimumab. However, the overall safety profile of the 3 mg/kg tremelimumab + gefitinib combination was considered acceptable and an expansion cohort is planned.

2.2.3.5 Ipilimumab + EGFR TKI

The phase 1 NCT01998126 study evaluated the addition of ipilimumab to erlotinib in *EGFR*m mNSCLC patients already on erlotinib for at least 28 days (74). Dose limiting toxicity (DLT) was reached in 3/8 patients, and excessive toxicity led to the study being closed after 14 patients. 4/11 *EGFR*m patients developed grade 3 colitis. However, PFS from start of ipilimumab was 17.9 months in 11 *EGFR*m patients, well above the typical observed for monotherapy, leading the authors to conclude that while ipilimumab + erlotinib caused excessive toxicity, targeted therapies with immunotherapy merited further study.

2.2.4 Dual ICI Therapy

Cohort H of the KEYNOTE-021 phase 1/2 study assessed pembrolizumab 2 mg/kg plus ipilimumab 1 mg/kg as second-line or later therapy (55). Of the 10 *EGFR*m patients, only 1 (10%) patient responded to therapy compared to an ORR of 30% for the entire study population. 98% of patients experienced a TRAE, including 49% with grade 3-5 AEs.

Multiple active studies are investigating dual ICI therapy in second line or later *EGFR*m NSCLC. The ILLUMINATE Phase 2 study (NCT03994393) is evaluating the safety and tolerability of combined durvalumab and tremelimumab plus platinum-pemetrexed in *EGFR*m NSCLC following progression on EGFR TKIs (75). 100 patients will receive induction durvalumab + tremelimumab with platinum-pemetrexed every three weeks, followed by maintenance durvalumab + pemetrexed every four weeks until disease progression. *EGFR*m T790M negative and positive patients will be included. Additionally, two arms of the CheckMate 722 phase 3 trial, described above, will compare nivolumab + ipilimumab (Arm B) to platinum-doublet chemotherapy (Arm C). We eagerly await the results from these study that will leverage the potentially enhanced immune response of dual ICI therapy.

2.2.5 ICI + VEGF inhibitor + Chemotherapy

The IMpower150 Phase 3 study (NCT02366143) assessed the addition of PD-L1 inhibition with atezolizumab and VEGF inhibition with bevacizumab to carboplatin + paclitaxel (CP) in patients with mNSCLC. Regimens included bevacizumab + CP (BCP), atezolizumab + CP (ACP), and atezolizumab + BCP (ABCP) (76). A subgroup analysis was performed of *EGFR*m patients; notably, 85-88% of the patients had previously received at least one EGFR TKI therapy (56). In the initial subgroup analysis, median OS was not reached with the ABCP group in *EGFR*m patients. Fortunately, the updated results were recently published and demonstrated that among *EGFR*m patients who had received prior TKI therapy a significant increase in median OS was observed with the ABCP regimen (27.8 months versus 14.9 months with ACP and 18.1 months with BCP) (77). The HR

for ABCP versus BCP was 0.74. The toxicity profile was similar between different regimens in *EGFR*m patients: 64-68% of patients across all three regimens experienced at least one Grade 3-4 TRAE, and 1-3% experienced a Grade 5 TRAE. These results suggested the hopeful possibility of an effective ICI-combination therapy for patients who experienced PD on an EGFR TKI.

Lam et al. recently reported the results of the Phase II NCT03647956 trial that enrolled 40 patients with metastatic *EGFR*m NSCLC that had progressed on EGFR TKI (57.5% osimertinib) (57). Patients were treated with atezolizumab + bevacizumab + pemetrexed-carboplatin until progression. Median PFS was 9.43 months and median OS was not mature yet at time of publication (1-year OS was 72.5%). 37.5% of patients experienced a grade 3 or above TRAE but only 1/40 patients discontinued treatment due to toxicity. These encouraging results, coupled with the IMpower150 *EGFR*m subgroup results, support the potential efficacy of adding VEGF inhibition to ICI and chemotherapy as second-line in *EGFR*m NSCLC that has progressed on EGFR TKI.

The phase 2 NCT04517526 trial is planning to enroll 60 patients with stage IV *EGFR*m NSCLC with PD after first-line osimertinib. Patients will receive platinum-based chemotherapy + bevacizumab + durvalumab + stereotactic radiotherapy to oligometastatic or oligoprogressive sites (78). The results of this study will be of great interest as it will combine advanced combination immunotherapy and radiation therapy approaches.

3 DISCUSSION

The treatment of *EGFR*m NSCLC has made significant progress with the advent of osimertinib, a third-generation TKI, that is now the standard of care for first-line treatment. Unfortunately, patients will almost uniformly experience PD. Meanwhile, the role of ICI therapy in *EGFR*m NSCLC is complex, with many studies describing additive toxicities without clinical benefit in combination ICI + EGFR TKI treatment models as described above. Given this, there is controversy around the standard of care for *EGFR*m NSCLC patients who have progressed on EGFR TKIs, with some advocating for chemotherapy alone and some advocating for chemotherapy combined with ICI therapy (79, 80). The National Comprehensive Cancer Network (NCCN) guidelines recommend tailoring response by symptomatology and location and number of metastatic sites, with osimertinib continuation recommended for asymptomatic *EGFR*m patients with PD on EGFR TKI and consideration of definitive local therapy for oligometastatic disease (81). For patients with symptomatic and widely metastatic PD on osimertinib, the NCCN guidelines recommend standard therapeutic strategies and clinical trial enrollment. The European Society of Molecular Oncology (ESMO) 2020 clinical practice guidelines recommend osimertinib as second-line if another EGFR TKI was utilized first-line and resistance is found to be due to the EGFR T790M mutation, followed by platinum doublet chemotherapy after progression on osimertinib (82). The ESMO guidelines briefly mention ICI therapy as a non-EMA approved option that can be considered after targeted therapies have been exhausted. Fortunately, active research is delineating important and unique characteristics in the tumor biology and TME of *EGFR*m NSCLC as well as identifying subgroups of *EGFR*m NSCLC patients who may have an improved response to ICI therapy, as discussed below.

3.1 Unique Biology of *EGFR*m NSCLC and Future Research Directions

There is a growing appreciation that the TME is the master regulator of response to ICI therapy (83), and *EGFR*m NSCLC tumors are no exception with their unique and complex tumor biology. On average, *EGFR*m tumors generate an immunosuppressive TME with less PD-L1 expression, reduced TMB and neoantigen presentation, decreased TIL infiltration, and activation of the immunosuppressive CD73/adenosine axis, all of which decrease ICI efficacy (**Figure 1**). Furthermore, the standard biomarker for ICI therapy, PD-L1 TPS, has less straightforward utility in *EGFR*m tumors and biomarkers to predict ICI response are not yet standardized in *EGFR*m NSCLC patients. The unique aspects of *EGFR*m NSCLC tumor biology are active areas of research, with multiple areas of interest for current and future clinical trials (**Table 3**).

3.1.1 PD-L1 Expression

PD-L1 TPS is a standard biomarker for stratifying patients in clinical trials of ICI therapy in *EGFR*wt cells, with evidence from multiple trials that higher PD-L1 TPS tumors (e.g. with TPS ≥ 50%) have an enhanced response to ICI therapy (84, 85). Of note, PD-L1 expression is not uniformly prognostic of response to ICI therapy (86), and it has been demonstrated that NSCLC patients without PD-L1 immunohistochemical staining can still derive benefit from ICI therapy (87), supporting the now widely accepted notion that PD-L1 status by itself is insufficient to predict ICI response.

In *EGFR*m NSCLC, the value of PD-L1 expression is even less clear. Mechanistic studies have demonstrated that upregulation of *EGFR* signaling *in vitro* leads to increased PD-L1 expression by pathways including the IL-6/JAK/STAT3 pathway (88). However, immunohistochemical and mRNA expression profiling analysis of *EGFR*m NSCLC patient tumor samples demonstrated decreased PD-L1 expression across multiple datasets (89, 90), leading to an unresolved discrepancy between preclinical and clinical studies. Of note, a weakness of these studies is the inability to assess the half-life of PD-L1 between subgroups.

In regard to treatment response, multiple studies have demonstrated that increased PD-L1 expression on *EGFR*m NSCLC cells predicts worse outcomes with TKIs but improved outcome with ICI therapy (91–93). Liu et al. recently published a correlation analysis of 57 *EGFR*m NSCLC patients who received ICI treatment after developing PD on EGFR TKIs (94).They identified by using a TKI-PFS cutoff of 10 months that *EGFR*m patients with <10 month TKI-PFS had significantly improved ICI-PFS of 15.1 versus 3.8 months, respectively (HR 0.26, 95% CI: 0.12-0.5, $p = 0.0002$), strongly suggesting that *EGFR*m tumors

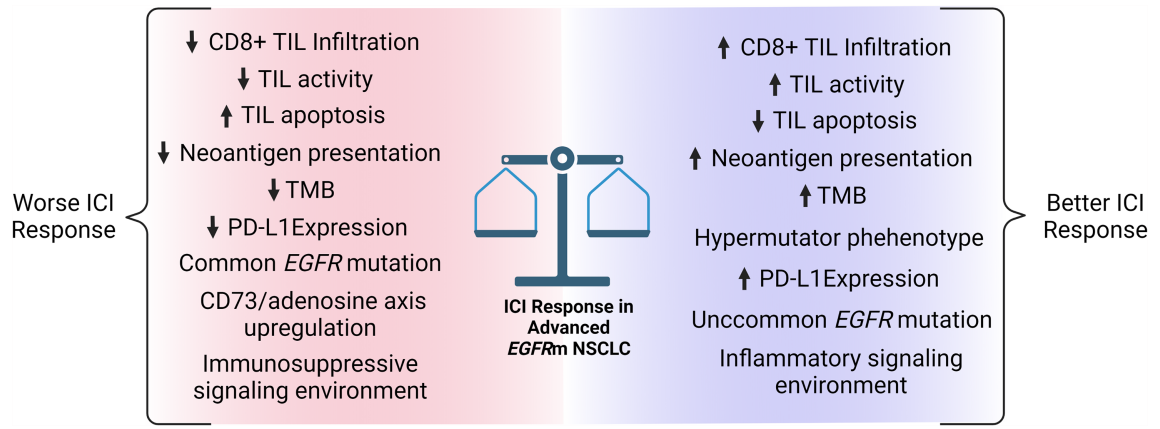

FIGURE 1 | Factors that may influence immune checkpoint inhibitor response in advanced *EGFR*m NSCLC. ICI, immune checkpoint inhibitor; TIL, tumor infiltrating lymphocyte; TMB, tumor mutational burden; PD-L1, programmed cell death 1 ligand. Figure created with BioRender.com.

TABLE 3 | Active or planned clinical trials addressing important questions of ICI use in advanced *EGFR*m NSCLC.

Trial	Phase	Population	n	Intervention	Primary End Point(s)	Status
Question: Activity of second-line dual ICI therapy						
ILLUMINATE/NCT03994393	2	*EGFR*m NSCLC that failed third generation TKI	100	Durvalumab + Tremelimumab + Platinum-Pemetrexed	OTRR*	Recruiting
CheckMate722/ NCT02864251	3	*EGFR*m NSCLC that failed first- or second-line EGFR TKI therapy	365	(Arm B) Nivolumab + Ipilimumab *vs* (Arm C) Platinum-doublet	PFS	Active
Question: Activity of second-line combination ICI + chemotherapy						
NCT03924050	3	Advanced *EGFR*m NSCLC that has progressed on EGFR TKI	350	Toripalimab + standard chemotherapy	PFS	Recruiting
CheckMate722/ NCT02864251	3	*EGFR*m NSCLC that failed first- or second-line EGFR TKI therapy	365	(Arm A) Nivolumab + Platinum-doublet *vs* (Arm C) Platinum-doublet	PFS	Active
KEYNOTE-789/ NCT03515837	3	EGFR*m* NSCLC resistant to EGFR TKI	492	Pembrolizumab + Pemetrexed + Chemotherapy *vs* Placebo + Pemetrexed + Chemotherapy	PFS, OS	Active
Question: Activity of second-line ICI + chemotherapy + antiangiogenic therapy						
NCT04517526	2	Stage IV *EGFR*m NSCLC that has progressed on EGFR-TKI	60	Pemetrexed + Cisplatin/Carboplatin + Bevacizumab + Durvalumab + SBRT	PFS, OS	Not yet recruiting
Question: Activity of CD73/adenosine axis inhibition + ICI therapy in EGFRm NSCLC						
*No active studies**						
Question: Activity of TNF-α agents + ICI therapy in EGFRm NSCLC						
No active studies						

*Trial information obtained from ClinicalTrials.gov *A phase 1b/2 trial of oleclumab (CD73-ab) + osimertinib versus AZD4635 is currently recruiting (NCT03381274). This study does not have an ICI arm, but will provide helpful information on the utility and tolerability of oleclumab in EGFR NSCLC patients. OTRR, overall treatment response rate; PFS, progression-free survival; OS, overall survival; SBRT, stereotactic body radiation therapy.*

are either TKI or ICI responsive. Intriguingly, this relationship was independent of PD-L1 status, again reiterating the importance of other elements of the TME in treatment response. To further probe differences in the TME between these groups, they performed single-cell RNA sequencing of patients with TKI-PFS <10 months (group A) and >10 months (group B). Group A demonstrated significantly higher proportion of T-cell TILs along with increased CD8+ effector proportion of T-cells. These results suggest a critical role for CD8 + effector TILs in determining response to ICI therapy in *EGFR*m NSCLC, as discussed in more detail below.

While PD-L1 expression is not uniformly predictive of ICI response, there is some evidence that *EGFR*m NSCLC tumors with increased PD-L1 TPS have improved response to third or later line ICI therapy (63). Thus, while ICI monotherapy is inappropriate for first line treatment as described above, PD-L1 analysis may be a valuable component of a holistic evaluation of the TME in *EGFR*m, along with other elements including TMB, TILs, and other discussed below to assist oncologists in deciding on later-line ICI treatment strategies for *EGFR*m patients who fail EGFR TKI therapy. As such, future clinical trials should continue to gather and report PD-L1 expression data so that these relationships can be better elucidated.

3.1.2 Tumor Infiltrating Lymphocytes

The efficacy of ICI therapy depends on the intratumoral migration and activation of $CD8^+$ effector T-cells where they perform cytotoxic functions after interaction of the T-cell receptor with tumor-specific peptides displayed on MHC-I complexes on tumor cells (29). Multiple studies have demonstrated that *EGFR*m NSCLC tumors have reduced $CD8^+$ TIL presence compared to *EGFR*wt tumors (89, 95, 96). Interestingly, Zhao et al. recently published an analysis of 190 surgical lung ADC samples that demonstrated increased apoptosis in the *EGFR*m patient tumor samples (96). They further went on to demonstrate that exosomes secreted from *EGFR*m cells were more capable of inducing $CD8^+$ T-cell apoptosis *in vitro* than exosomes from *EGFR*wt cells. These results suggest that, in addition to reduced TIL density in *EGFR*m tumors, there may also be increased TIL apoptosis that impairs immune-mediated tumor destruction. Further study of this mechanism may provide valuable new information on the TME in *EGFR*m NSCLC and possibly provide a novel therapeutic avenue to enhance antitumor immunotherapy (97).

Strategies to increase TIL trafficking and activity in tumors including NSCLC are an active area of research (98). Possible approaches include targeted tumor necrosis factor α (TNF-α) delivery and anti-angiogenic drugs including inhibitors of VEGF. TNF-α causes endothelial cell activation and increased vessel permeability that can enhance the ability of both chemotherapy and immune cells to penetrate solid tumors (99); however, systemic TNF-α administration is quite toxic (100). A compound containing the tumor vasculature-homing peptide Cys-Asn-Gly-Arg-Cys (NGR) has been fused to TNFα to create a tumor vasculature-homing version of TNF-α that avoids the toxicity of systemic TNFα administration (NGR-hTNF) (101). A phase II trial of NGR-hTNF combined with chemotherapy in patients with chemotherapy-naïve NSCLC was previously reported. Patients with nonsquamous NSCLC in the chemotherapy + NGR-hTNF arm experienced improved PFS at 8 months (38% versus 18% with chemotherapy alone) and a tolerable safety profile (102). While preclinical models support the ability of TNF-α to also enhance ICI therapy (103), there are no active clinical trials involving NGR-hTNF and ICI therapy in NSCLC due to the manufacturer of NGR-hTNF discontinuing the product after a Phase III mesothelioma trial did not meet its primary endpoint (104). Importantly, an NGR-TNF derivative with an additional serine at the N-terminus that demonstrates increased stability, S-NGR-TNF, has been recently developed (105). It will be intriguing to see if TNF-α strategies such as S-NGR-TNF can restore TIL trafficking, enhance ICI therapy, and augment chemotherapy delivery to *EGFR*m NSCLC tumors.

VEGF is also known to suppress TILs *via* multiple mechanisms, including suppressing endothelial cell activation, inhibiting TNFα-mediated gene regulation, and blocking dendritic cell maturation thereby reducing T-cell activation (106, 107). In preclinical models, VEGF inhibition synergized with PD-1 blockade and reduced T-cell exhaustion, and in clinical studies combinations of therapies including ICIs and TKIs have demonstrated improved TIL recruitment and improved PFS (108). The IMpower150 study, for example, demonstrated improved PFS of *EGFR*m NSCLC patients who had disease progression on or did not tolerate an EGFR TKI when they were treated with a combination of chemotherapy, ICI therapy, and VEGF-inhibition as described above (56, 76). The success of VEGF-inhibition in second-line combination chemotherapy + ICI therapy for *EGFR*m NSCLC patients with PD on EGFR TKI is one of the few bright signals currently in the field, and we eagerly await the results of current trials that are ongoing further exploring this question (**Table 3**).

3.1.3 Tumor Mutation Burden

On average, *EGFR*m NSCLC patients have a decreased TMB compared to *EGFR*wt patients (89, 109). This is thought to be at least partly due to the fact that *EGFR*m patients tend to have a lighter smoking history. Increased TMB classically correlates to decreased response to chemotherapy and an increased response to ICI therapy in NSCLC (110, 111), and in *EGFR*m NSCLC patients increased TMB correlates negatively with response to EGFR TKIs (112). Increased TMB is thought to potentiate ICI therapy by creating an environment where more tumor-specific neoantigens are generated, thus creating more targets for TILs to recognize and enhancing the adaptive anti-tumoral response. Indeed, significantly fewer candidate MHC class-I neoantigens were identified in *EGFR*m versus *EGFR*wt NSCLC tumors in a whole-genome DNA sequencing study (113). While the decreased TMB in *EGFR*m NSCLC patients overall may contribute to decreased efficacy of ICI therapy, there is some evidence that TMB may still be of significance in this population. For example, Hastings et al. retrospectively analyzed 171 cases of *EGFR*m NSCLC and demonstrated that $EGFR^{\Delta 19}$ tumors had a lower TMB and a worse response to ICI therapy compared to $EGFR^{L858R}$ tumors (114). Additionally, certain hypermutator

phenotypes such as DNA mismatch repair (MMR) deficient tumors and DNA polymerase delta and epsilon proofreading mutants, while uncommon in NSCLC, may respond well to ICIs (115, 116). As such, subgroups of *EGFR*m patients with increased TMB, while less common than in *EGFR*wt context, are predicted to still receive increased benefit from ICI therapy compared to their low TMB counterparts. TMB analysis is an intriguing and significant element that should be strongly considered for clinical studies of *EGFR*m NSCLC patients.

3.1.4 CD73/Adenosine Axis

CD73 is an ecto-nucleotidase that catabolizes the breakdown of extracellular ATP to adenosine (117). There is a growing appreciation that the CD73/adenosine axis plays a significant and complex role in the TME. Increased intratumoral adenosine contributes to localized immunosuppression and impairment of T-cell effector function (118, 119), and the CD73/adenosine axis is becoming considered an immune checkpoint in its own right (120). Pre-clinical data demonstrated that anti-CD73 monoclonal antibodies (mAbs) significantly enhanced the activity of anti-CTLA-4 and anti-PD-1 mAbs in animal studies of colon, prostate, and breast cancer (118). An intriguing, recently published study by Le et al. analyzed upregulated genes in *EGFR*m NSCLC tumors and found that two of the top upregulated genes (*NT5E* and *ADORA1*) belonged to the CD73/adenosine pathway (89), suggesting that *EGFR*m NSCLC may leverage the CD73/adenosine axis to generate an immunosuppressive TME. They assessed the efficacy of an anti-CD73 mAb in a mouse model of *EGFR*m murine lung cancer and found that anti-CD73 treatment significantly reduced tumor size. As such, an active question is whether suppression of the CD73/adenosine axis can enhance the treatment of *EGFR*m NSCLC. Along these lines, a human mAb targeting CD73, Oleclumab, is being assessed in a phase 1b/2 study (NCT03381274) in combination with either osimertinib or AZD4635, which is an adenose 2a receptor (A2aR) inhibitor (121). Given the encouraging preclinical data, we eagerly look forward to further clinical trials utilizing anti-CD73 mABs or A2aR inhibitors in conjunction with ICI therapy.

3.1.5 Role of Specific EGFR Mutations

There is intriguing evidence that the specific *EGFR* mutation impacts the immunogenicity of the TME and response to ICI therapy. Chen et al. performed a large single-study of 600 NSCLC patients in China with *EGFR*m NSCLC and identified 49 with uncommon mutations (Ex20ins, S767I, L861Q, G719X, and double mutations) (91). They found a much higher proportion of PD-L1 expressing tumors with uncommon mutations compared to classic mutations (49% versus 12.2%), and $CD8^+$ TIL infiltration was more abundant in this group (91). They reported worse OS for patients with PD-L1 positive *EGFR*m NSCLC versus PD-L1 negative (median OS 15.2 versus 29.3 months, $p = 0.006$), though most of these patients received EGFR TKI monotherapy across all lines of treatment. Negrao et al. reported that metastatic *EGFR*m exon 20 mutation NSCLC patients had increased benefit from ICIs compared to classic mutation patients (exon 19 del, exon 21 L858R) with an ORR of 25% versus 0% and disease control rate (DCR) of 50% versus 15% (122). Mazieres et al. analyzed the IMMUNOTARGET registry and compared the molecular characteristics of *EGFR*m patients to response to ICIs (123). *EGFR* exon21 mutation patients derived significantly longer PFS from single-agent ICI therapy (2.5 months) in this database than patients with T790M and exon 19 mutations (1.4 and 1.8 months, respectively, $p < 0.001$). As such, these studies suggest that uncommon *EGFR* mutations including exon 21 mutations may have increased immunogenicity and response to ICIs. Future clinical trials should ensure that the specific *EGFR* genetic alterations are reported and provide mutation subgroup data so that further evidence on this subject can be obtained.

3.1.6 ICI Response Prediction

One of the greatest needs in the field currently is the development of a scoring/stratification system that will predict which *EGFR*m patients will benefit from ICI therapy. As more studies publish the results of detailed molecular and immunohistochemical analysis, this will empower a more comprehensive understanding of the cellular composition of *EGFR*m TMEs and tumor biologies (**Figure 1**). Complex multivariate analyses should be employed to delineate subgroups of *EGFR*m patients that will benefit from ICI therapy. The use of artificial intelligence (AI) including artificial neural networking is being studied for the analysis of TMEs (124, 125), and may prove invaluable to identify signatures of *EGFR*m tumors that predict ICI response. The value of machine learning in *EGFR*m tumor biology was recently demonstrated by Song et al. who utilized a machine learning model to analyze pre-treatment computed tomography (CT) images of stage IV *EGFR*m NSCLC patients (126). Their machine learning approach successfully identified an imaging signature able to stratify *EGFR*m patients most likely to rapidly progress despite TKI therapy. It is a logical next step to apply machine learning to stratify patients likely to respond to ICI therapy based on tumor biological characteristics.

3.1.7 Effect of EGFR TKIs on the TME

Multiple lines of pre-clinical evidence suggested synergy between EGFR TKI inhibition and ICI therapy. In pre-clinical studies, EGFR inhibition enhanced antigen presentation to T-cells, stimulated immunogenic apoptosis of tumor cells, boosted T-cell chemoattractants, and stimulated MHC-1 upregulation, all of which are predicted to enhance the anti-tumor immune response (68–71). Despite this, early clinical studies demonstrated that preclinical studies would not translate in a straightforward manner. IHC analysis of tumors from early *EGFR*m patients treated with ICIs, against expectation, demonstrated decreased PD-L1 expression and decreased CD8+ TILs (95), data that has since been recapitulated in multiple studies described above. This has led to the active research question of the effect of EGFR TKIs on the TME of *EGFR*m NSCLC *in vivo* during and after therapy.

Multiple groups have addressed this question with TME analysis at various time points of treatment. Isomoto et al. performed serial immunohistochemical analysis of 138 patients

who underwent rebiopsy after progression on EGFR TKI treatment (127). They found multiple significant changes in the TME after PD, including an expanded proportion of high (≥50%) PD-L1 expressing tumors and decreased CD8+ TILs in PD-L1 <50% tumors. Notably, they identified subgroups with opposing clinical courses: tumors with high PD-L1 expression after progressing on EGFR TKI had significantly longer PFS with ICI therapy (7.1 versus 1.7 months, $p = 0.0033$) and increased $CD8^+$ TIL presence. In contrast, PD-L1 <50% tumors had significantly decreased $CD8^+$ TIL density. Also of interest, the PD-L1 high tumors had increased FOXP3+ and CD73 TIL density, suggesting that regulatory T-cell (Treg) and CD73 axis activation may contribute to ICI treatment failure.

Sugiyama et al. analyzed surgically resected *EGFR*m tumors and found decreased CD8+ TILs and increased FOXP3+CD4+ Tregs, further supporting a role for Treg suppression of the immune response in *EGFR*m tumors (128). Gurule et al. performed RNA sequencing of patient tumors before and 2 weeks after TKI treatment and demonstrated induction of an interferon response program (71). Interestingly, higher enrichment of interferon gamma (IFNγ) was correlated with longer time to progression. Taken together, these results suggest that *EGFR*m NSCLC tumors undergo diverse responses to EGFR TKIs with some tumors becoming more immunogenic and some becoming more immunosuppressive with resulting divergent responses to ICI therapy (129). While the mechanism behind the divergent TME responses to TKI therapy in *EGFR*m NSCLC is unclear, these studies suggest that rebiopsy may have clinical benefit in identifying subpopulations of patients who are more likely to respond to ICI as subsequent therapy.

3.2 ICI + EGFR TKI Toxicity

Despite the pre-clinical evidence of synergy between EGFR TKIs and ICI therapy, the clinical trials of combined or sequential ICI and EGFR TKI therapies as described above failed to demonstrate additive clinical benefit and generated safety concerns in two major regards. First, multiple combination of ICI + EGFR TKI therapies were found to generate severe toxicities. As described above, durvalumab + gefitinib and pembrolizumab + gefitinib were correlated with high grade hepatotoxicity (40, 41), durvalumab + osimertinib was correlated with an increased incidence of ILD (53), and both azetolizumab + erlotinib and ipilimumab + erlotinib were poorly tolerated with an increased risk of various grade 3/4 TRAEs (42, 74). In line with these studies, Oshima et al. performed an analysis of adverse events reported through the FDA adverse event reporting system and compared the incidence of interstitial pneumonitis (IP) between patients treated with and EGFR TKI, nivolumab, or combination nivolumab + EGFR TKI (130). Their analysis identified a significant elevation in IP in the combination group (25.7%) versus 6.4% for nivolumab alone and 4.6% for EGR TKI alone, suggesting an additive interaction between EGFR TKIs and nivolumab in favor of developing IP. We note that certain ICI + EGFR combinations were well tolerated, including pembrolizumab + erlotinib and nivolumab + erlotinib (41, 52). Given the small number of patients in most of these studies, caution must be taken in interpreting these results, though a clear theme of concerning safety signals without added benefit for most tested ICI + EGFR TKI strategies emerges.

Second, the sequence of ICI and TKI therapies appears critical in determining toxicity. Schoenfeld et al. analyzed 126 patients treated with ICI and EGFR TKI at a single institution in various sequences and found that 15% of patients treated with ICI followed by osimertinib developed severe irAEs whereas 0% of patients treated with osimertinib followed by ICI therapy developed severe irAEs (131). In other words, osimertinib after ICI was dangerous, whereas ICI after osimertinib was tolerated. This study is congruent with findings reported above, including the increased incidence of TRAEs in patients who experienced PD on first-line ICI monotherapy and then switched to second-line EGFR TKI in the KEYNOTE-001 study (38, 58), as well as the lack of any increased toxicity noted in patients who switched to either pembrolizumab, nivolumab, or atezolizumab after PD on first-line EGFR TKI therapy (44–46). Given these combined results, we urge oncologists not to empirically start advanced NSCLC patients on ICI therapy until the oncogene status of their cancer is known, as inadvertent ICI treatment of *EGFR*m NSCLC will increase the risk of severe TRAEs on subsequent EGFR TKI therapy.

While the mechanism for checkpoint inhibitor toxicity is currently unknown, Zhai et al. recently reviewed possible causes that may include increased immune activity against cross-antigens in tumor and normal tissues, increased levels of pre-existing autoantibodies, and increased inflammatory cytokines in patients who experience irAEs (132). Given that EGFR inhibitors have been demonstrated to increase the expression of MHC class I and class II molecules (133), this suggests that increased autoreactivity stimulated by increased expression of cross-antigens *via* MHC class I and II molecules may at least a partially explain severe TRAEs such as IP. One possible approach to combine ICI and EGFR TKI therapy more safely would be to target treatments specifically to tumor cells, for example by tumor-homing nanoparticles (134), thereby bypassing adverse effects due to systemic impact of the drugs. Alternatively, if biomarkers predicting which patients are at risk of developing severe TRAEs from combination ICI + EGFR TKI can be identified, then patients could be stratified by likelihood to develop severe combination TRAEs so that combination therapy could be applied more safely in a first line setting. In this way, future clinical trials could attempt to realize the potential of combination ICI + EGFR TKI therapy seen in preclinical studies.

4 CONCLUDING REMARKS

NSCLC remains the deadliest malignancy on the planet. 15-67% of NSCLC tumors harbor *EGFR* mutations based on geographic region, lending urgency to the development of better therapeutic strategies for *EGFR*m NSCLC patients. First-line ICI therapy is clearly inferior to *EGFR*-targeted therapy, and first-line combination EGFR TKI + ICI therapy has so far demonstrated synergy only in regard to toxicity without any consistent clinical benefit. Furthermore, pre-treatment of *EGFR*m NSCLC patients with ICIs can prime patients for serious TRAEs on subsequent

EGFR TKI therapy due to an unknown mechanism. As such, oncologists must take great caution to avoid treating NSCLC patients with ICI therapy until molecular analysis has been performed and *EGFR* mutation status is ascertained.

While first-line ICI monotherapy and ICI + EGFR TKI combination therapy in *EGFR*m NSCLC patients has thus far been disappointing, intriguing results have been obtained from trials of second-line ICI therapy combinations and multiple open research questions are under clinical investigation (**Table 3**). Recent trials have demonstrated encouraging signals with dual ICI blockade and combination of ICI, chemotherapy, and VEGF inhibitors. Furthermore, accumulating evidence suggests that multiple components of *EGFR*m tumor biology may predict response to ICI therapy, including specific *EGFR* mutation, TMB, PD-L1 expression, and TIL density among others (**Figure 1**). Multiple active areas of research are identifying other significant *EGFR*m TME differences including CD73/adenosine axis activation that may prove fruitful for the development of novel therapeutic interventions to enhance the immunogenicity of *EGFR*m tumors (135). As such, there exists a great deal of hope for improved therapies for *EGFR*m NSCLC patients in the near future. We believe that as researchers and clinicians continue to advance our understanding of *EGFR*m NSCLC tumor and TME biology that outcomes for patients will only continue to improve.

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NW wrote the manuscript. RM provided major editorial and intellectual contributions. UM, KS, YL, and YZ provided significant intellectual contributions. All authors contributed to the article and approved the submitted version.

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REVIEW
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Immunotherapy in Non-Small Cell Lung Cancer With Actionable Mutations Other Than *EGFR*

Karan Seegobin[1], *Umair Majeed*[1], *Nathaniel Wiest*[2], *Rami Manochakian*[1], *Yanyan Lou*[1] *and Yujie Zhao*[1]*

[1] Division of Hematology and Oncology, Mayo Clinic, Jacksonville, FL, United States, [2] Department of Medicine, Mayo Clinic, Jacksonville, FL, United States

While first line targeted therapies are the current standard of care treatment for non-small cell lung cancer (NSCLC) with actionable mutations, the cancer cells inevitably acquire resistance to these agents over time. Immune check-point inhibitors (ICIs) have improved the outcomes of metastatic NSCLC, however, its efficacy in those with targetable drivers is largely unknown. In this manuscript, we reviewed the published data on ICI therapies in NSCLC with *ALK*, *ROS1*, *BRAF*, *c-MET*, *RET*, *NTRK*, *KRAS*, and *HER2 (ERBB2)* alterations. We found that the objective response rates (ORRs) associated with ICI treatments in lung cancers harboring the *BRAF* (0–54%), *c-MET* (12–49%), and *KRAS* (18.7-66.7%) alterations were comparable to non-mutant NSCLC, whereas the ORRs in *RET* fusion NSCLC (less than10% in all studies but one) and *ALK* fusion NSCLC (0%) were relatively low. The ORRs reported in small numbers of patients and studies of *ROS1* fusion, *NTRK* fusion, and *HER 2* mutant NSCLC were 0–17%, 50% and 7–23%, respectively, making the efficacy of ICIs in these groups of patients less clear. In most studies, no significant correlation between treatment outcome and PD-L1 expression or tumor mutation burden (TMB) was identified, and how to select patients with NSCLC harboring actionable mutations who will likely benefit from ICI treatment remains unknown.

Keywords: targeted mutations, immunotherapy, *c-MET*, *RET*, *BRAF*, *ROS-1*, *ALK*, *NTRK*

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Laura Bonanno,
Veneto Institute of Oncology (IRCCS), Italy

Reviewed by:
Min Li,
Sun Yat-sen University Cancer Center (SYSUCC), China
Alberto Pavan,
Azienda ULSS 3 Serenissima, Italy

***Correspondence:**
Yujie Zhao
Zhao.Yujie@mayo.edu

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INTRODUCTION

NSCLC accounts for 85% of all lung cancers, with lung adenocarcinoma being the major subtype (1). Platinum-based combination chemotherapy is the historical first-line standard of care for patients with advanced NSCLC who have no actionable mutations (2). The introduction of ICIs, such as anti-programmed cell death protein ligand 1 (anti-PD-L1) and anti-programmed cell death protein 1 (anti-PD-1) antibodies, as well as the anti-cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA-4) antibody, have revolutionized the treatment of NSCLC, and is typically offered with or without chemotherapy in the front-line setting for incurable NSCLC that does not have any actionable mutations (2). A number of actionable genetic alterations have been identified in NCSLC, including *ALK*, *ROS1*, *c-MET*, *RET*, *NTRK*, *BRAF* V600E, *KRAS*, and *ERBB2 (HER2)* (3–11). MET, RET, HER2, ALK, NTRK, and ROS-1 are receptor tyrosine kinases; BRAF is a serine/threonine kinase mediating cellular signal from RAS to MEK1/2; KRAS is a RAS protein which functions as a

GDP–GTP-regulated binary on-off switch. While *c-MET*, *BRAF* and *KRAS* altered NSCLC may develop in both smokers and non-smokers, *ALK*, *ROS1*, *RET*, *NTRK*, and *HER2* alerted NSCLC tend to occur in non-smokers. In patients with actionable driver mutations, namely, *EGFR*, *ALK*, *BRAF*V600E, *RET*, *c-MET*, *NTRK* or *ROS1* alterations, the standard of care is to treat with a Food and Drug Administration (FDA) approved targeted agent, which typically can achieve ORRs of 60–80% in treatment naive patients (2, 12). After targeted therapies are exhausted in these patients, systemic therapy with chemotherapy is typically available for them. While incorporating immunotherapy in the regimen is a standard of care option for them, the efficacy of immunotherapy in those with actionable mutations remains poorly defined due to the limited numbers of these patients included in the randomized prospective trials. In addition to the genetic alterations for which targeted therapies have been approved by FDA in the first line setting in NSCLC, *KRAS G12C* has a targeted agent that was approved recently in the beyond first-line setting. Moreover, *HER2* mutations have emerged as new therapeutic targets with promising therapeutic agents in development. The efficacy of ICI in the *KRAS* G12C or *HER2* mutant NSCLC is also of great clinical interest.

In this modern era with a booming number of treatment options for NSCLC and continued improvement in survival, further guidance is needed on what to expect from the use of immunotherapy in those with these genetic abnormalities. The goal of this review is to add valuable information on the use of immunotherapy in NSCLC with actionable alterations in genes including *ALK*, *ROS1*, *BRAF*, *c-MET*, *RET*, *NTRK*, *KRAS*, and *HER2*. Epidermal growth factor receptor (*EGFR*) mutations are not included in this review as they are included in another manuscript by our group which was submitted separately. In this review, we showed that the sensitivity to ICIs can be heterogenous and differs according to the driver alteration considered. *ALK* and *RET* fusions were found to be associated with low responses to ICI while *BRAF*, *KRAS*, and *c-MET* alterations were associated with responses that were comparable to non-mutant NSCLC, and PD-L1 positive *KRAS* mutant NSCLC may be associated with better outcome when treated with ICI monotherapy as suggested by two retrospective studies. The responses to ICIs are less clear in *HER2*, *ROS1* or *NTRK* altered NSCLCs due to low patient numbers. While an association between PD-L1 expression level or TMB and the responses to ICI has not been consistently observed across all driver alterations, the overall lack of response to ICI treatment appeared to be more common among NSCLC with driver alterations that are typically associated with non-smokers, raising the question whether the absence of tobacco exposure may predict the lack of benefit from ICI treatment. Moreover, the emerging data in the role of co-mutations in response to ICI had also shed a light in the potential underlining mechanism of resistance to ICI, and particularly in the presence of *KRAS* mutation, co-mutations in *TP53*, *STK11*, and *KEAP1* have been found to modulate the response to ICIs in several studies (13–15).

ALK

Anaplastic lymphoma kinase (*ALK*), a member of the insulin receptor tyrosine kinase family, has been identified as a fusion partner of nearly 30 different proteins in oncogenic signaling in many different cancer types (3). While there are now over 20 *ALK* fusion partners identified in NSCLC, *EML4* represents the most common fusion partner with 29–33% of gene fusions identified to date (16, 17). The fusion of the 5′ end partner *EML4* to the coding region of the intracellular tyrosine kinase domain of *ALK* leads to aberrant expression of the ALK fusions in the cytoplasm. The domains in the partner proteins also promote dimerization and oligomerization of the fusion proteins, leading to constitutive activation of *ALK* kinase and its downstream signaling pathways including RAS–mitogen-activated protein kinase, phosphoinositide 3-kinase-AKT, and JAK-STAT pathways. This subsequently results in uncontrolled cellular proliferation and promotes survival (3, 18). *ALK* fusions are seen in 3–5% of NSCLC patients and are more common among the following groups: no prior smoking history, adenocarcinoma histology, younger age, female gender, and tumors with wild type *EGFR* and *KRAS* (16, 19–21). Several *ALK* inhibitors have been approved by the FDA for metastatic NSCLC, including crizotinib, brigatinib, alectinib, lorlatinib and ceritinib (22–29). The data on the efficacy of ICIs in the *ALK* fusion positive NSCLC has been scarce. It has been postulated that EML4-ALK oncoprotein can upregulate the PD-L1 expression in lung cancer cells. In one report of 100 patients, fifty patients (50.0%) were PD-L1 negative, 34 patients (34.0%) were PD-L1 low expression (tumor proportion score [TPS] 1–50%), and 16 patients (16.0%) had a strong PD-L1 expression (TPS ≥ 50%) (30). Despite the expression of PDL1 in these tumors, the overall response to ICIs in the *ALK* fusion positive population has been disappointing except in one study (**Table 1**).

Although small numbers of patients with *ALK* fusion NSCLC were included in the randomized phase 3 CheckMate 057 and KEYNOTE-010 studies comparing ICI versus docetaxel in

TABLE 1 | Efficacy of ICIs in NSCLS with *ALK* mutations.

Reference	Characteristics	ORR, %	mPFS, months	mOS, months since start of ICI
Mazieres J., et al. (31)	*ALK* (n=23)	0	2.5	17
Gainor JF.,et al. (32)	*ALK* (n=6)	0		
Jahanzeb M., et al. (33)	*ALK* (n=83)		2.34	
Gadgeel SM. et al. (34)	*ALK (n=7)*	28.6%	2.9	2.9

ICIs, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; ORR, overall response rate; mPFS-median progression-free survival; mOS, median overall survival.

previously treated NSCLC patient population, the outcomes in this specific population were not reported (35, 36).

In a retrospective study using the IMMUNOTARGET registry which included 551 patients receiving ICI monotherapy for advanced NSCLC with at least one oncogenic driver alteration, 23 patients with *ALK* fusion NSCLC were identified (31). The objective response rate to ICI treatment was 0%. The Median PFS was 2.5 (1.5; 3.7) months. The median OS from start of ICI therapy was 17.0 (3.6; NR) months. Among the 10 patients with available PD-L1 status, the median percentage of cells expressing PD-L1 was 7.5% (**Table 1**).

In a retrospective study conducted at the Massachusetts General Hospital, the ORR to ICI treatment among patients with *EGFR* mutations or *ALK* rearrangements was only 1/28 (3.6%) while the ORR among *EGFR WT/ALK*-negative patients was 7/30 (23.3%) (P = 0.053) (32). Since the lone partial response was seen in an *EGFR*-mutant patient, it appeared that none of the six *ALK* fusion NSCLC patients had a response (**Table 1**).

In the randomized Impower130 study, atezolizumab plus chemotherapy (Nab-Paclitaxel and Carboplatin) did not show improved overall survival versus chemotherapy alone in the subset of 44 patients with *EGFR* or *ALK* genomic alterations in the first line setting (37). However, in the Impower150 study, the addition of Atezolizumab to Bevacizumab, Carboplatin, and Paclitaxel improved the median PFS for patients with *EGFR* or *ALK* genomic alteration whose diseases had progressed on TKI or who were unable to tolerate TKI (median, 8.3 months vs. 6.8 months; stratified hazard ratio, 0.61; 95% CI, 0.52 to 0.72). Of note, 34 patients with *ALK* fusion and 80 patients with *EGFR* mutant nonsquamous metastatic lung cancer were included in this study, and information on the benefit of atezolizumab in *ALK* fusion NSCLC was not reported separately (38). In another report of 83 patients with *ALK* mutation treated with ICI, a mPFS of 2.34 months was reported (33).

A recent prospective multicenter trial presented at the World Conference on Lung Cancer evaluated pembrolizumab and chemotherapy in the setting of recurrent *EGFR*/*ALK*-positive NSCLC. The study enrolled a total of 33 patients, including 26 *EGFR* mutant NSCLC and seven *ALK* fusion positive NSCLC patients. Most of the patients had one prior targeted therapy. No more than one prior line of platinum-based chemotherapy for advanced NSCLC was allowed. In those with ALK-positive tumors, the ORR was seen in 2/7 (28.6%), and the mPFS and mOS were both 2.9 months, suggesting lack of benefit of ICI in this group of patients (34).

BRAF

BRAF is a serine/threonine kinase mediating cellular signal from RAS to MEK1/2, and BRAF activation can result in phosphorylation and activation of extracellular signal-regulated kinase (ERK)1/2, leading to cell survival and proliferation (4). *BRAF* mutations are found in 1.5–3.5% of NSCLC with V600E accounting for approximately half of those mutations (39). Besides adenocarcinoma, *BRAF* mutations have been reported in sarcomatoid carcinomas, large-cell neuroendocrine carcinomas, and squamous cell lung cancer (40, 41). *BRAF* mutations can occur in both smokers and non-smokers (42). Selective kinase inhibitors have been recommended for the first-line and second-line treatments of *BRAF* V600E mutant advanced NSCLC with a reported ORR as high as 64% in this group of patients (39). The outcomes associated with ICIs in this population have been studies in multiple retrospective analyses (**Table 2**). Although the data vary significantly among different studies, responses to ICI have been seen in most of the studies.

In a retrospective study including seven participating Israeli cancer centers reported by Dudnik et al., PD-L1 expression level,

TABLE 2 | Efficacy of ICIs in NSCLS with *BRAF* mutations.

Reference	Characteristics		ORR, %	mPFS, months	mOS, months since start of ICI
Dudnik E., et al. (43)	Total (n=22)		28		
	mutation type	V600E (n=12)	25	3.7	Not reached (median follow-up of 5.5 months)
		nonV600E (n=10)	33	4.1	Not reached (median follow-up of 5.5 months)
	PD-L1 expression	PD-1L ≥50%	36	5.3	
		PDL-1 0-49%	14	2.2	
Rihawi K., et al. (44)	*BRAF*, 2nd line immunotherapy (n=11)		9		10.3
Tan I., et al. (45)	*BRAF*, 1st line immunotherapy (n=3)			0.17, 1.4, and 4.4 for each patient respectively	0.17, 6.8, and 7.5 for each patient respectively
	BRAF, 2nd line immunotherapy (n=8)			2.5	
	BRAF, 1st line chemoimmunotherapy (n=2)			1.5 and 2.1 for each patient respectively	6.6 and 5.6 for each patient respectively
Mazieres J., et al. (31)	*BRAF* (n=43)		24.3	3.1 (1st-3rd line ICI) 2.7 (>3rd line)	20.3
Dudnik E., et al. (46)	*BRAF* V600E (n=5)		25	1.5	NR (not reached)
	BRAF non-V600*E* (n=5)		20	2.6	NR (not reached)
Guisier F., et al. (47)	*BRAF* V600E (n=26)		26.1%	5.3	22.5
	BRAF non-V600E (n=18)		35.3%	4.9	12
Mu Y., et al. (48)	*BRAF*(n=9)		25%	3.0	

ICIs, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; ORR, overall response rate; mPFS-median progression-free survival; mOS, median overall survival.

tumor mutational burden (TMB), and microsatellite instability status were assessed in both *BRAF* V600E and non-V600E *BRAF* mutation positive NSCLC, and the outcome with ICI treatment was reported (43). High (≥50%) PD-L1 expression was found to be more common in the *non-BRAF* V600E mutant group than the V600E *BRAF* mutant group (50% vs 42%, $p = 0.05$). No MSI-H was found in both groups, and the median TMB was 5 (1–42) muts/Mb and 11 (7–14) muts/Mb in the *BRAF* V600E and the non-V600E *BRAF* mutant groups, respectively. ICI therapy was associated with ORRs of 25 and 33% in the *BRAF* V600E and the non-V600E *BRAF* mutant positive groups, respectively (p = 1.0) (**Table 2**). Among the six patients with high PD-L1 and *BRAF* V600E mutant NSCLC, two patients had major tumor shrinkage while two other patients had hyperprogression (43).

Among the 1,588 advanced non-squamous NSCLC patients enrolled in the Italian Expanded Access Program of second line nivolumab, 210 patients were assessed for *BRAF* mutations, and 11 patients (5%) were found to be positive. Median OS was comparable among different groups, and was found to be 11.0 months (range: 9.8 to 12.2 months), 11.2 months (range: 9.2 to 13.2 months) and 10.3 months (range: 2.1 to 18.5 months) in the population with unknown *BRAF* status, *BRAF* wild-type subgroup, and *BRAF* mutated subgroup, respectively (44) (**Table 2**).

A retrospective study was conducted to evaluate the clinical response to immunotherapy and chemotherapy among 31 patients with *BRAF* mutant metastatic NSCLC treated at the Duke University Hospital (45). PD-L1 expression information was only available for 11 patients. PD-L1 expression levels ranged from 0 to 90%, with six patients with PD-L1 expression levels greater than 50%. TMB was only available on five patients, ranging from 3 to 18 mutations/Mb. The median PFS in patients who received first-line chemotherapy was 6.4 months (95% CI, 2.3 to 13.0) while the PFS of each of the three patients who received first-line immunotherapy was 0.17, 1.4, and 4.4 months. The median OS in patients who received first-line chemotherapy was 18.4 months (95% CI, 7.4 to 28.6), and the OS of each of the three patients who received first-line immunotherapy was 0.17, 6.8, and 7.5 months (**Table 2**).

In the retrospective study using the IMMUNOTARGET registry, among the 43 patients with *BRAF* mutations, PFS was significantly higher in smokers than never smokers (4.1 versus 1.9 months, $P = 0.03$), however shorter in the V600E subgroup (1.8 months) compared with other *BRAF* mutations (4.1 months, $P = 0.20$) (31) (**Table 2**). The ORR was 24.3%. Among the nine patients with available PD-L1 status, the median percentage of cells expressing PD-L1 was 50%.

In the IMAD2 (GFPC 01-2018), a retrospective study that included 21 centers in France reported by Guisier et al., 44 ICI-treated *BRAF* mutant (*BRAF* V600E, n = 26; *BRAF* non-V600E, n = 18) NSCLC patients were identified (47). Most of the patients received ICI in the beyond-first line setting. Response rates for *BRAF*-V600E- and *BRAF*-non-V600E- mutant NSCLC were 26 and 35%, respectively. The median DORs to ICI were NR (95% CI 12.6–NR) and 13.1 months (95% CI 7.6–NR) in the *BRAF*-V600E- and *BRAF*-non-V600E groups. The PFS in the *BRAF*-V600E- and *BRAF*-non-V600E groups were 5.3 months (95% CI 2.1–NR) and 4.9 months (95% CI 2.3–NR), and the OS in the *BRAF*-V600E- and *BRAF*-non-V600E groups were 22.5 months (95% CI 8.3–NR) and 12 months (95% CI 6.8–NR). The 12-month OS in the *BRAF*-V600E- and *BRAF*-non-V600E- groups were 53.4 and 44%, respectively (**Table 2**).

In a cohort of 10 patients with tumors harboring *BRAF* mutations (*BRAF* V600E, n–5; *BRAF* non-V600E, n–5) who received ICI treatment, ORR of 25% (1/4) and 20% (1/5) were seen in patients with *BRAF* V600E mutation and *BRAF* non-V600E mutation, respectively (46). Median PFS comprised 1.5 months (95% CI, 1.2–8.3) in patients with *BRAF* V600E mutation and 2.6 months (95% CI, 2.0–4.2) in patients with *BRAF* non-V600E mutation. Median OS was not reached in patients with *BRAF V600E* mutation (95% CI, 1.2–NR) or *BRAF* non-V600E mutation (95% CI, 2.3–NR) (46) (**Table 2**). Among patients with known PD-L1 TPS, TPS high (≥50%) was seen in 25 and 60% of the *BRAF* V600E- and non-*BRAF* V600E-mutant NSCLC cases, respectively. TMB high (≥10 mut/Mb) was seen in 3 and 1% of the *BRAF* V600E- and non-*BRAF* V600E-mutant NSCLC cases, respectively. No MSI-H/I was seen.

In another report of nine patients with *BRAF* (*BRAF* V600E, n–6; *BRAF* non-V600E, n–3) who received ICI with chemotherapy or antiangiogenic treatment, the ORR was 25% and mPFS was three months (95%CI 2.9, 3.1) (48).

MET

MET is a proto-oncogene receptor tyrosine kinase that mediates cell proliferation, survival, and metastasis (5). Recurrent somatic splice site alterations at *MET exon 14 (METex14)* can result in exon skipping, decreased MET degradation, and MET activation. *METex14* is involved in cancer through promoting angiogenesis, cell migration, and invasion (49, 50). *METex14* occurs in 3–4% of lung cancers and 8–30% of sarcomatoid lung cancers (51, 52). The occurrence of *METex14* appears to be independent of smoking status (53). FDA has granted accelerated approval to capmatinib and tepotinib for adult patients with metastatic NSCLC whose tumors have a mutation that leads to *METex14* alterations (54, 55).

In a retrospective study that included 147 patients with *METex14* lung cancers, PD-L1 expression of ≥50% was identified in 41% of 111 evaluable tumor samples. The median TMB of *METex14* lung cancers was lower than that of unselected non-small-cell lung cancers (NSCLCs). In 24 response-evaluable patients, the ORR was 17% (95% CI 6 to 36%) and the median PFS was 1.9 months (95% CI 1.7–2.7). Responses were not associated with PD-L1 expression ≥50% or high TMB (12) (**Table 3**).

Among the 551 patients in the IMMUNOTARGET registry, 13 patients with *MET* amplification and 23 patients with *METex14* were identified (31). Median OS from ICI initiation of this 36-paitent cohort was 18.4 months (7.0; NR) (31). Progressive disease (PD) was found to be the best response to ICI among 50% of patients, and median PFS was found to be 3.4

TABLE 3 | Efficacy of ICIs in NSCLS with *c-MET* mutations.

Reference	Characteristics	ORR, %	mPFS, months	mOS, months since start of ICI
Sabari JK. et al. (12)	*cMET exon 14* skipping mutation (n=147)	17	1.9	18.2
Mazieres J., et al. (31)	*cMET exon 14* skipping mutation and *cMET* amplification (n=36)	49	3.4	18.4
Guisier F., et al. (47)	*cMET* mutant (n=30)	36	4.9	13.4
Dudnik- E., et al. (46)	*cMET exon 14* skipping mutation (n=148)	12	4	NR (not reached)
	cMET amplification (n=54)	25	4.9	NR (not reached)
Mayenga M., et al. (56)	*cMET exon 14* skipping mutations, 2nd line immunotherapy (n=13)	46.2		

ICIs, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; ORR, overall response rate; mPFS, median progression-free survival; mOS, median overall survival.

months (1.7; 6.2). Long-term responders were seen in 23.4% of patients (**Table 3**). Among the 15 patients with available PD-L1 status, the median percentage of cells expressing PD-L1 was 30%.

In the French retrospective study IMAD2 (GFPC 01-2018), 30 cases of ICI-treated *MET* mutant NSCLC were identified (47). Most patient received ICI in the beyond-first line setting. The response rate for *MET*-altered NSCLC was 36%. The median duration of response (mDOR) was 10.4 months (95% CI 4.6–NR). The mPFS was 4.9 months ((95% CI 2.0–11.4), and the mOS was 13.4 months (95% CI 9.4–NR) (**Table 3**).

In a retrospective study that included eight cases of NSCLC with *METex14* and four cases of NSCLC with *MET* amplification treated with ICI, median PFS with ICI was 4.0 months (95% CI, 2.4–NR) in patients with *METex14* and 4.9 months (95% CI, 2.4–NR) in patients with *MET* amplification (46). ORR comprised 12% (1/8) and 25% (1/4) in patients with *METex14* and *MET* amplification respectively. Median OS with ICI was not reached in patients with *METex14* (95% CI, 4.1–NR) or in patients with *MET* amplification (95% CI, 3.5–NR) (**Table 3**). Among patients with known PD-L1 TPS, TPS high (≥50%) was seen in 67% of the cases. TMB high (≥10 mut/Mb) or MSI-H/I was not seen.

In a case series, among 13 patients with *METex14* NSCLCs treated with ICI, 46.2% (6/13) patients responded to immunotherapy. Six patients had prolonged duration of responses ranging from 18 months (still ongoing) to 49 months (56).

RET

RET is a proto-oncogene receptor tyrosine kinase that binds with the ligand–co-receptor complex of glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) and subsequently activates signaling pathways such as RAS/mitogen activated protein kinase (MAPK), RAS/ERK, phosphatidylinositol 3-kinase (PI3K)/AKT, and c-Jun N-terminal kinase (JNK). Aberrant activation of the RET receptor have been associated with multiple endocrine neoplasia 2 (*MEN2*), sporadic medullary thyroid cancer, papillary thyroid carcinoma (PTC), and non-small cell lung cancer (NSCLC) (6, 7). *RET* rearrangements have been identified in 1–3% of NSCLC and were found to have significantly higher frequencies in younger (<60 years of age), female, non-smokers, and adenocarcinoma histology (57–60). *RET* fusion positive NSCLC is usually associated with low PD-L 1 expression (61). Two potent selective RET inhibitors, selpercatinib and pralsetinib, have been approved by the FDA for *RET* fusion-positive NSCLC (62, 63). The activity of ICI in *RET* altered NSCLC has been evaluated in multiple studies, and the benefit of ICI was found to be low in most of the studies (**Table 4**).

In the French retrospective study IMAD2 (GFPC 01-2018), nine patients with *RET* translocation NSCLC received ICI, all in the beyond-first line setting. The response rate for *RET*-altered NSCLC was 38%. The mDOR response to ICI was 12.1 months (95% CI 8.4–NR). The median PFS was 7.6 months (2.3–NR), and the median OS was not reached (95% CI 26.8–NR) (47) (**Table 4**).

In a single center retrospective study conducted in Korea, the median progression-free survival for ICI among 13 patients with *RET* fusion-positive NSCLC treated with ICI was 2.1 (95% CI: 1.6–2.6) months, and the ORR was 7.7% (64). The median PFS and OS were 2.1 (95% CI: 1.6–2.6) and 12.4 (95% CI: 2.9–21.8) months, respectively. Among patients with PD-L1 expression 25% and above, 2/5 patients demonstrated stable disease, while the best response in the other three patients was disease progression (**Table 4**). In contrast, the ORR and DCR among 46 patients treated with pemetrexed-based regimens in this study was 63.0 and 91.3%, respectively, and the median PFS was 9.0 (95% CI: 6.9–11.2) months.

Among the 16 patients with *RET* fusion-positive NSCLC in the IMMUNOTARGET registry, the median OS from the start of

TABLE 4 | Efficacy of ICIs in NSCLS with *RET* mutations.

Reference	Characteristics	ORR, %	mPFS, months	mOS, months since start of ICI
Guisier F., et al. (47)	*RET* fusion (n=9)	37.5	7.6	NR (not reached)
Lee J., et al. (64)	*RET* fusion (n=13)	7.7	2.1	12.4
Mazieres J., et al. (31)	*RET* fusion (n=16)	6	2.1	21.3
Offin M., et al. (61)	*RET* fusion (n=16)	0	3.4	
Dudnik E., et al. (46)	*RET* fusion (n=4)	0	3	14.9
	RET mutation (n=1)	0	6.9	15.3

ICIs, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; ORR, overall response rate; mPFS, median progression-free survival; mOS, median overall survival.

ICI therapy was 21.3 (3.8; 28.0), and the median PFS was only 2.1 (1.3; 4.7) (31). The rate of any partial or complete response was very low and was 6.3% (1/16) (**Table 4**). Among the six patients with available PD-L1 status, the median percentage of cells expressing PD-L1 was 26%.

In a retrospective study conducted at the Memorial Sloan Kettering Cancer Center, 13 patients with *RET*-rearranged NSCLC treated with ICI were assessed for clinical and/or radiologic response (30). No response to immunotherapy was observed. The median PFS was 3.4 months (95% CI, 2.1 to 5.6 months). No difference in OS between patients with advanced *RET*-rearranged lung cancers who received immunotherapy (n = 16) and those who did not receive immunotherapy (n = 46), (hazard ratio, 1.4 [95% CI, 0.7 to 2.9]; log-rank P = .35) (**Table 4**). Only one patient was found to have PD-L1 expression ≥50%, and the disease of this patient did not respond to ICI. No patient had TMB >10 mut/Mb.

In the single institution retrospective study published by Dudnik et al., four patients with *RET* fusion NSCLC and one patient with *RET* mutant NSCLC were treated with ICI (46). No objective response was observed. Median PFS was 3.0 months (95% CI, 1.9–3.1) in patients with *RET* fusion and 6.9 months in patient with *RET* mutation. Median OS since start of ICP were 14.9 months (95% CI, 7.2–19.7) in patients with *RET* fusion and 15.3 months in patient with *RET* mutation (**Table 4**). Among patients with known PD-L1 TPS, TPS high (≥50%) was seen in 13 and 0% of the *RET* fusion and the *RET* mutant NSCLC cases, respectively. TMB high or MSI-H/I was not seen.

ROS1

ROS proto-oncogene 1 (*ROS1*) belongs to the subfamily of tyrosine kinase insulin receptors (65). *ROS1* fusion can lead to constitutive activation of kinase activity, resulting in increased cell proliferation, survival, and migration due to the upregulation of JAK/STAT, PI3K/AKT, and MAPK/ERK signaling pathways (8). *ROS1* rearrangements account for 1–2% of NSCLC patients (66, 67). This alteration more frequently occurs in adenocarcinoma and in younger patients with no or light smoking history (68, 69).

Seven patients with *ROS1* fusion NSCLC treated with ICI were identified in the IMMUNOTARGET registry (31). The objective response rate to ICI treatment was 17% (**Table 5**).

In the single institution retrospective study published by Dudnik et al., only one patient with *ROS1* fusion NSCLC treated with ICI was identified, and the reported PFS and OS were both 0.1 month (46) (**Table 5**). Among the five patients with available PD-L1 status, the median percentage of cells expressing PD-L1 was 90%.

In the Japanese retrospective study, 15 *ROS1* altered NSCLC cases were identified. High expression of PD-L1 (>50% of tumor cells by 22C3) were observed in 53% cases, however, no response to immunotherapy was observed (70).

NTRK

The *NTRK* genes (*NTRK1*, *NTRK2* and *NTRK3*) encode tropomyosin receptor kinases (*TRKA*, *TRKB* and *TRKC*) (9). The *TRK* fusion protein leads to constitutive activation of various downstream signal transduction pathways including the PI3k/Akt and RAS/RAF/MAPK pathways, and subsequently causes proliferation of cancer cells (9). Rearrangements including *NTRK1*, *NTRK2*, and *NTRK3* occur in approximately 2–3% of NSCLC patients (10). Selective *TRK* inhibitors, Entrectinib and Larotrectinib, have been approved for patients with *NTRK* fusion-positive solid tumors, including NSCLC (71, 72).

In the single institution retrospective study published by Dudnik et al., two patients with *NTRK* fusion NSCLC were treated with ICI. The objective response rate was 50% (1/2). Median PFS was as not reached (95% CI, 3.2–NR). Median OS since start of ICP not reached (95% CI, NR–NR) (46) (**Table 6**). One patient had PD-L1 TPS ≥50%. No patient had TMB ≥10 muts/Mb.

KRAS G12C

KRAS is one of the RAS proteins (KRAS4A, KRAS4B, NRAS, and HRAS) which function as GDP–GTP-regulated binary on-off switches and regulate cell survival, cell cycle progression, cell polarity, movement, and nuclear transport by transducing signals from transmembrane receptors to cytoplasmic signaling pathways such as the MAPK pathway (10, 11). It is the most common proto-oncogene identified in NSCLC. *KRAS* mutations occur in 15–25% of lung adenocarcinomas and are more prevalent in smokers than nonsmokers (73, 74). Majority of the *KRAS* mutations in NSCLC occur on exon 2 or 3 (G12, G13, and Q61), with the most frequent being the G12C followed by G12V and G12D (75, 76). Sotorasib has been approved by the FDA for patients with *KRAS* G12C mutant locally advanced or metastatic NSCLC in the beyond the first line setting (72). It is associated with an objective response rate of 37.1% in this group of patients (77). The efficacy of ICIs in

TABLE 5 | Efficacy of ICIs in NSCLS with *ROS-1* mutations.

Reference	Characteristics	ORR, %	mPFS, months	mOS, months since start of ICI
Mazieres J., et al. (31)	*ROS1* (n=7)	17		
Dudnik E., et al. (46)	*ROS 1* (n=1)		0.1	0.1

ICIs, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; ORR, overall response rate; mPFS, median progression-free survival; mOS, median overall survival.

TABLE 6 | Efficacy of ICIs in NSCLS with *NTRK* mutations.

Reference	Characteristics	ORR, %	mPFS, months	mOS, months since start of ICI
Dudnik E., et al. (46)	*NTRK* (n=2)	50%	Not reached	Not reached

ICIs, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; ORR, overall response rate; mPFS, median progression-free survival; mOS, median overall survival.

KRAS mutant NSCLC has been studied in several retrospective studies, and most of the data support the benefit on ICIs in *KRAS* mutant NSCLC (**Table 7**).

In a retrospective analysis in patients enrolled in the KEYNOTE-042 evaluating pembrolizumab monotherapy vs platinum-based chemotherapy as the first-line therapy among patients with PD-L1-positive (TPS ≥1%) advanced non-squamous histology NSCLC, 301 patients were evaluable by whole-exome sequencing (WES). *KRAS* mutations were found in 69 (23%) patients, among which, 29 (10%) patients were found to have *KRAS* G12C (78). PD-L1 TPS and TMB were found to be higher in patients with *KRAS* mutations than without *KRAS* mutations, although the differences were not significant. The OS associated with pembrolizumab was better than chemotherapy in both the *KRAS* mutant group and *KRAS* G12C subgroup, with the HRs being 0.42 (0.22–0.81) and 0.28 (0.09–0.86), respectively. Conversely, there was no significant OS difference seen between pembrolizumab and chemotherapy in the *KRAS* wild-type patients, and HR was 0.86 (0.63–1.18). A superior PFS was also observed when pembrolizumab was compared with chemotherapy in the *KRAS* mutant patients. The data supported the benefit of single agent pembrolizumab in the PD-1 TPS >1% *KRAS* mutant (including *KRAS* G12C) NSCLC patients, underlining the important role of ICI in the treatment of this group of patients.

The efficacy of ICIs in the first line setting in PD-L1 TPS ≥50% advanced NSCLC was also investigated in a retrospective analysis using the Flatiron Health database (79). Among the1,127 patients with PD-L1 expression of 50% or greater who were treated with either ICI monotherapy or chemoimmunotherapy, 573 (50.8%) had *KRAS* alterations and 554 (49.2%) had wild type *KRAS*. Among the patients treated with ICI monotherapy, a better mOS was seen in the *KRAS* mutant group when compared with the wild-type group (mOS, 21.1 vs 13.6 months; P = .03). Interestingly, this OS advantage was not observed among patients treated with chemoimmunotherapy, and the mOS was 20.0 vs 19.3 months; P = .93 in the *KRAS* mutant and wild type patients. Furthermore, no mOS difference was seen between ICI monotherapy and chemoimmunotherapy in the *KRAS* mutant NSCLC patients (mOS, 21.1 vs 20.0 months; P = .78), suggesting that the use of ICI monotherapy in the PD-L1 TPS ≥50% is an acceptable option in the *KRAS* mutant advanced NSCLC.

The efficacy of chemoimmunotherapy in *KRAS* mutant NSCLC was also analyzed retrospectively in the participants of another randomized trial, the KEYNOTE-189 study of pembrolizumab plus pemetrexed and platinum chemotherapy vs placebo plus chemotherapy as first-line therapy for metastatic non-squamous NSCLC (80). Among the 289 patients who had evaluable WES data, 89 (31%) patients were found to have *KRAS* mutations including *KRAS* G12C, which was found in 37 (13%) patients. As observed in the KEYNOTE-042 study, the higher PD-L1 TPS and TMB tended to be seen with KRAS mutant patients. Although unlike the observation in the KEYNOTE-042, the OS benefit associated with the addition of ICI was only detected in the *KRAS* wild-type patients. PFS improvement associated with the additional of ICI was seen in both the *KRAS* mutant and wild type group but not in the *KRAS* G12C subgroup, which could be related to the small sample number.

In addition to ICI monotherapy and chemoimmunotherapy, the combination of VEGF receptor targeted agent and chemoimmunotherapy represents another first-line treatment option for advanced NSCLC based on the IMpower150 study (84). A *post hoc* analysis evaluated the efficacy outcomes in

TABLE 7 | Efficacy of ICIs in NSCLS with *KRAS* mutations.

Reference	Characteristics	ORR, %	mPFS, months	mOS, months since start of ICI
Mazieres J., et al. (31)	*KRAS* (n=271)	26%	3.2 (1st-3rd line ICI) 3.1 (>3rd line) G12C: 5.5 G12A: 4.4 G12D: 3.2 G12V: 1.9 G12S: 2.1	13.5
Herbst RS., et al. (78)	Any *KRAS* (n=69), first line immunotherapy	56.7%	12	28
	KRAS G12C	66.7%	15	NR
Sun L., et al. (79)	Any *KRAS* (n=573), first line monotherapy or chemoimmunotherapy			21.1 (ICI monotherapy) 20 (chemoimmunotherapy)
Gadgeel SM. et al. (80)	Any *KRAS* (n=89), first line chemoimmunotherapy	40.7%	9	21
	KRAS G12C	50%	11	18
West H., et al. (81)	*KRAS (n=80)* (first line chemoimmunotherapy with VEGFR targeted therapy)		8.11	19.81
	With *mutant STK* and/or *mutant KEPA1 (n=34)*		6.03	11.1
	With wild-type *STK* and wild-type *KEPA1 (n=46)*		15.21	26.18
Passiglia F., et al. (82)	*KRAS*, (n=206)	20%	4	11.2
Jeanson A., et al. (83)	*KRAS* (n=162)	18.7%	3.09	14.29

ICIs, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; ORR, overall response rate; mPFS, median progression free survival; mOS, median overall survival, HR, hazard ratio, CI, confidence interval.

patients with *KRAS*, *STK11(LKB1)*, and *KEAP1* mutations (81). Among 920 patients included, *KRAS* mutations were found in 80 patients (24.5%), with 39 patients found to have co-occurring mutations in *STK11* and/or *KEAP1*. The addition of ICI improved mOS and PFS in the *KRAS* mutant patients regardless of STK11 and KEAP1 status (**Table 7**), supporting the use of this regimen in *KRAS* mutant NSCLC.

The correlation between STK11/LKB1 genomic alterations and the efficacy of ICI treatment in KRAS mutant NSCLC was also evaluated using the Stand Up To Cancer (SU2C) dataset (13). Unlike the *post hoc* analysis of the IMpower150 study, this study showed that the concurrent *STK11/LKB1* mutation in *KRAS* mutant NSCLC was associated with an inferior ORR to PD-1 blockade when compared with *KRAS* mutation without *STK11/LKB1* mutation and *KRAS* mutation with *P53* mutations groups (7.4, 28.6 and 35.7% (P <0.001)). The details of the ICI therapy in this dataset were not available, and it is unclear whether this group of patients also received angiogenesis targeted agent treatment.

In a systemic review and metanalysis aiming to investigate the predictive clinicopathological characteristics for the relative efficacy of ICIs vs docetaxel in the second-line setting in NSCLCs, the authors analyzed data from five randomized clinical trials involving 3,025 patients (85). ICIs were associated with prolonged overall survival (HR, 0.69; 95% CI, 0.63–0.75; $P < .001$). The survival benefit was also seen among the 148 *KRAS* mutant patients (HR, 0.65; 95% CI, 0.44–0.97; $P = .03$) but not in the 371 *KRAS* wild-type patients (HR, 0.86; 95% CI, 0.67–1.11; P = .24; interaction, P = .24) (85).

The efficacy of ICI in *KRAS* mutant non-squamous NSCLC in the beyond first-line setting was also investigated in patients who received nivolumab in an Italian expanded access program (EAP) study (82). Among the 530 patients evaluated, 206 (39%) had *KRAS* mutations. No significant differences in OS, PFS or ORR were seen between *KRAS* mutant and *KRAS* wild-type patients in this study, supporting that nivolumab should be considered for patients regardless of *KRAS* mutation status. Interestingly, any significantly higher grade and grade 3–4 treatment related adverse events were seen in the *KRAS* mutant group than the wild-type group, although the underlining mechanism for the finding is unknown.

KRAS mutant NSCLC was also evaluated in the IMMUNOTARGET study. Two hundred and seventy-one patients treated with ICIs were found to have *KRAS* mutations. An encouraging ORR of 26% was found, and the mPFS and mOS were 3.2 and 13.5months, respectively (31).

In a single institution retrospective study conducted in France, a total of 162 *KRAS*-mutant advanced NSCLC were identified among the 282 subjects analyzed. No significant difference was seen in ORR, mPFS or mOS between the *KRAS* mutant and the *KRAS* wild-type groups. The ORR, mPFS, and mOS associated with ICI of *KRAS* mutant NSCLC were 18.7%, 3.09 months and 14.29 months. No significant difference in treamtent outcomes was seen among the KRAS mutation subtypes including G12A, G12C, G12D, G12V, and G13C (83).

HER 2

Human epidermal growth factor 2 (HER2 erbB-2/neu) is one of the four receptor tyrosine kinase members of the human epidermal growth factor receptor family. Upon forming homo- or hetero-dimers with other family members, HER2 becomes activated and signal through the PI3K-AKT and MEK-ERK downstream pathways to activate proliferation (86). In NSCLC, activating *HER2* mutations occur in 2–4% of cases, most commonly in adenocarcinoma histology and never smokers (87). Patients with *HER2* mutant NSCLC have worse OS if treated without HER2 targeted therapy (88). Although there has not been any HER 2 targeted agent approved by NSCLC by the FDA, several agents have showed promising activity. Ado-trastuzumab emtansine, a HER2-targeted antibody-drug conjugate was found to be associated with an ORR of 44% in NSCLC with *HER2* exon 20 insertions and point mutations (89), and another HER2-targeted antibody-drug conjugate, trastuzumab deruxtecan, also showed an encouraging ORR of 55% in patients with metastatic HER2-overexpressing or *HER2*-mutant NSCLC whose disease had relapsed during standard treatment or was refractory to standard treatment (90). Both agents are included as novel therapeutic options for *HER2* mutant NSCLC in the current NCCN guidelines (2). Poziotinib, a tyrosine kinase inhibitor targeting *EGFR/HER2* exon 20 insertion mutation, was found to have an ORR of 27% in HER2 exon 20 mutant NSCLC, gaining fast track designation by FDA (91, 92).

The efficacy of immunotherapy in patients whose cancer harbors *HER2* mutation is largely unknown. The ORR associated with ICI among the 29 patients with exon 20 activating mutations in the IMMUNOTARGET study was only 7%. PFS was 2.5 months, and the 12-month PFS was 13.6 months. The OS was 20.3 months (31). The ORR among the 23 patients with exon 20 insertions included in the IMAD2 study by the French Lung Cancer Group was 27.3%. PFS was similar to the findings in the IMMUNOTARGET study and was 2.2 months, and the 12-month PFS was 22.9%. The mOS was an encouraging 20.4 months (47) (**Table 8**).

TABLE 8 | Efficacy of ICIs in NSCLS with *HER2* mutations.

Reference	Characteristics	ORR, %	mPFS, months	mOS, months since start of ICI
Mazieres J., et al. (31)	*HER2* (n=29)	7%	2.9 (1^{st}-3^{rd} line ICI) 2.0 (>3^{rd} line)	20.3
Guisier F., et al. (47)	*HER2* (n=23), number of lines prior to ICI =one median	27.3%	2.2	20.4

ICIs, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; ORR, overall response rate; mPFS, median progression-free survival; mOS, median overall survival.

DISCUSSION

To delineate the benefit of ICI treatment in NSCLC harboring actional mutations other than *EGFR* alterations, we reviewed the current available data in this area. We found that the ORR, median PFS, and OS with ICPi varied significantly across genetic alteration subgroups. While the ORR observed in the *BRAF*, *c-MET*, and *KRAS* altered NSCLC appeared to be similar to what had been observed in the non-selected NSCLC groups, the ORRs in the *ALK* and *RET* altered NSCLC groups were much lower (2).

Unlike *ALK* and *RET* fusions, *BRAF*, *MET*, and *KRAS* mutations can be seen in both smokers and non-smokers. The higher prevalence of smoking history in these patients could be a potential reason of the higher response rates since smoking has been found to be associated with the benefit derived from ICI treatment in some of the literatures (93, 94), although not confirmed by other studies (95). Other known predictive biomarkers for ICI treatment include PD-L1 expression level, microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR), and TMB (96–98). A higher percentage of PD-L1 TPS high (67%) was reported in *BRAF* non V600E mutant and *MET* mutant NSCLC in some of the reports (46), and a relatively higher response was seen in patients with PD-L1 TPS high *BRAF* mutant NSCLC (43), albeit the sample numbers was too small to draw any firm conclusion.

The current NCCN guidelines support the use of targeted therapy in the first line setting for advanced NSCLC with actionable genomic alterations involving *EGFR*, *ALK*, *ROS1*, *BRAF*, *NTRK1/2/3*, *METex14* skipping, and *RET*. After disease progression, chemoimmunotherapy is recommended for this population based on the guidelines (2). Although most of the chemoimmunotherapy trials excluded the *EGFR* and *ALK* altered NSCLC patients, these groups of patients were evaluated in the IMpower150 study if they have had progression with or unacceptable side effects from treatment with at least one approved tyrosine kinase inhibitor. The data showed improved PFS associated with the additional of atezolizumab to the bevacizumab, carboplatin, and paclitaxel combination in *EGFR* and *ALK* altered NSCLC (9.7 months vs. 6.1 months), providing direct evidence supporting the use of the regimen in this population (38). In our review, while not robust, we see that ICI's do have activity in patients with NSCLC harbouring actionable mutations, and that a response can be seen after progression on targeted therapy, supporting offering chemoimmunotherapy in the post-targeted therapy setting. For *KRAS* G12C NSCLC, ICI monotherapy or chemoimmunotherapy is the current standard of care first line treatment. The data included in this review did confirm the benefits of ICI in the *KRAS* mutant NSCLC, supporting the current treatment approach. The benefit of ICI monotherapy in PD-L1 positive *KRAS* mutant NSLCL was suggested in retrospective studies, warranting further investigation on the selection of ICI monotherapy vs chemoimmunotherapy in this population (78, 79). Furthermore, data from prospective studies will be helpful to identify the best treatment sequence among targeted therapy, ICI, and chemotherapy.

Even in the subgroups where the benefits of ICIs were observed, the ORRs tended to be low. Therefore, developing predictive biomarkers for ICI therapy would be of great importance.

Co-occurring genomic alterations have been reported to be related to responses to immunotherapy through altering the microenvironment. For example, *LKB1/STK11* genomic alterations, a frequent co-occurring mutation in of *KRAS* mutant NSCLC, have been found to be associates with "immune-inert" state (99). This was supported by several studies including a retrospective study conducted in 103 NSCLC patients receiving ICIs. In this study, among the patient with *KRAS* mutations, the presence of concurrent *STK11* mutation or *STK11/TP53* mutations were associated with worse survival with ICI therapy. This association was not observed with chemotherapy, supporting the predictive roles of these co-mutations for ICI therapy in *KRAS* mutant NSCLC (15). The data from a retrospective analysis suggested that co-occurring *LKB1/STK11* mutations in *KRAS* mutant NSCLC may predict lower ORR, while data from another group showed no PFS or OS differences with or without concurrent mutant *LKB1/STK11* and/or mutant *KEPA1* in patients receiving combined chemoimmunotherapy and angiogenesis targeted agent (13, 81), raising the question whether angiogenesis targeted agent may help to overcome the challenge of the immune-inert state. Other co-occurring genomic alterations such as *P53, KEAP1, ATM, PTEN, CDKN2A* are common among *KRAS*-mutant NSCLC, and may play a role in determining response to ICI (100). Furthermore, a recent study showed that co-occurring mutations such as NOTCH and HR pathways were also found to be associated with increased efficacy of immunotherapy in advanced NSCLC (101). Therefore, identifying co-occurring mutations that are responsible for ICI response or resistance could potentially help to identify the candidate for ICI treatments and warrants further investigation in this group of patients.

How to overcome the resistance to ICI therapy is another great challenge. The mechanism of resistance is complex and is a combination of tumor-intrinsic and extrinsic factors. Many factors such as immune contexture and tumor microenvironment, expression of PD-L1 and LAG3, TMB, genetic and epigenetic alterations, antigen-presenting molecules (MHC, HLA) and microbiota may all contribute to the resistance to immunotherapy (102). The tumors with higher initial mutational burdens have been found to be associated with higher sensitivity to ICIs in some studies, although this association may be negated by other factors such as intratumoral heterogeneity and mutations (103). *RET* fusion positive NSCLC was found to have poor response to ICIs, and the alterations appears to be associated with lower TMB. In the analysis by Offin M. et al., the median TMB of *RET* altered NSCLC was significantly lower than that of the *RET* wild-type NSCLCs (1.75 versus 5.27 mutations/Mb, P <.0001) (61). The best outcome in patients in this study was stable disease which only lasted 5.6 months. In the report by Dudnik E. et al, the TBM was low in all 13 patients except one patient who had intermediate TMB, and the ORR in this report was also 0% (46). Nevertheless, an ORR of 37.5% was found among the nine evaluable patients reported by Guisier F. et al. Unfortunately, the TMB information was not available in this

study, and it was unclear if the treatments were ICI monotherapy or chemoimmunotherapy. A prospective study to allow uniform treatment and collection of information on biomarkers such as TMB, PD-L1, MSI/MMR, tumor-infiltrating lymphocytes, whole-exome sequencing analysis on tumor samples and intestinal microbiome composition may be helpful to identify the resistance mechanisms.

ALK fusion positive NSCLC showed poor response to ICI in retrospective studies. However, this group of patients did benefit from ICI in the IMpower 150 trial, raising the question if the inhibition of angiogenesis could sensitize cancer cells to ICI therapy. Tumor angiogenesis can lead to immunosuppression through various mechanisms including maintaining an acidic/hypoxic and immunosuppressive environment, development of dysfunctional blood vessels which limits T cell trafficking, and suppression of dendritic cell maturation. Moreover, the angiogenic factors such as VEGF are also immunosuppressive (104). Therefore, further investigation is warranted in co-inhibition of angiogenic factors in NSCLC harboring actionable driver mutations undergoing ICI treatment.

With regard to the combination of ICIs and targeted therapies, a number of studies had evaluated the combiantion of *ALK* TKIs and different ICIs in NSCLC, including the combination of nivolumab with ceritinib or crizotinib and the combiantion of alectinib plus atezolizumab (105–107). However, significant toxicities were observed without survival benefit. In addition, there has been some concerning safety signals where ICI treatment is followed with targeted therapy (108). Reports showed risk of hepatotoxicity in a series of patients with *ALK*, *ROS1*, or *MET* exon 14 alterations who received ICI before crizotinib. Among the eleven patients treated with crizotinib following ICI, five patients (45.5%) developed grade 3 or 4 hepatotoxicity, whereas only 8% of those patients who received crizotinib alone experienced hepatotoxicity. The increased hepatotoxicity in sequentially treated patients led to permanent discontinuation of crizotinib in four of the five patients (108), highlighting the importance of establishing the presence of actionable mutations prior to initiating ICI therapy in patients with advanced NSCLC. The frequency and severity of toxicities associates with sequential use of ICI followed by targeted therapy may vary among different therapeutic agents. In the CodeBreaK100 phase II study evaluating Sotorasib in the beyond first-line setting, even though 91.3% patients had received ICI treatment prior to Sotorasib, the tolerability remained acceptable. Ongoing clinical trials DESTINY-Lung03 (NCT04686305) and the HUDSON trial (NCT03334617) are investigating the combination of T-DXd with immunotherapy, chemotherapy, novel anticancer agents and will hence shed more light on the approach in *HER2* mutant subgroup NSCLC patients.

Our review certainly has limitations. We were unable to comment on the response of *HER2*, *ROS1* and *NTRK* altered NSCLC to ICI as there were few reports in the literature, and the patient numbers in these reports were often very small. The challenges are obviously associated with the low incidences of these alterations. A recent report from Negrao et al. showed that *RET*, *ROS1* and *ALK* alterations were associated with low sensitivity to ICIs. However, there were only three *ROS1* fusion NSCLC patients included in the study, and the outcome of all three alterations were reported collectively (15). Furthermore, we were also unable to compare the responses to ICIs among different alterations which can be better investigated in prospective studies. Moreover, many studies included in this reivew did not have the biomarker information on all the evaluable patients. The ICI treatments and the number of lines of treament received previously by the patients also varied significantly. Additionally, it was not always clear whether the ICI treament was given as a monotherapy or in combination with cytotoxic chemotherapy. Randomized prospective studies would undoubtly provide more definitive information on this topic.

In conclusion, we see low responses to ICI in *ALK and RET* altered NSCLCs whereas *BRAF, KRAS and c-MET alterations* were associated with benefit from ICIs, and PD-L1 positive *KRAS* mutant NSCLCs may be more responsive to ICI monotherapy. Furthermore, the response to ICIs in *KRAS* mutant NSCLCs may vary depending on co-existing mutations, and responses to ICIs in *HER2*, *ROS1* and *NTRK* altered NSCLCs are less clear and varies significantly across a small number of studies. Ultimately, immunotherapy in the second line after progression on targeted agents can be considered as a treatment option at the discretion of treating physicians, following a mutual discussion with patients about the pros and cons of this approach.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the writing, development, editing, and information gathering of the manuscript.

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Efficacy of Intraoperative Hypertonic Glucose Solution Administration on Persistent Air Leak After Extended Pleurectomy/Decortication for Malignant Pleural Mesothelioma: A Retrospective Case–Control Study

Alberto Testori[1], Gianluca Perroni[2,3], Marco Alloisio[1,4], Emanuele Voulaz[1], Veronica Maria Giudici[1], Umberto Cariboni[1†] and Edoardo Bottoni[1†]*

[1] Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Humanitas Research Hospital, Division of Thoracic Surgery, Milan, Italy, [2] Division of Thoracic Surgery, IRCCS San Raffaele Scientific Institute, Milano, Italy, [3] Department of Thoracic Surgery, Tor Vergata University, Rome, Italy, [4] Department of Biomedical Sciences, Humanitas University, Milan, Italy

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***Correspondence:**
Gianluca Perroni
gianlucaperroni91@gmail.com

†These authors have contributed equally to this work and share last authorship

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Background: Persistent air leak is a common complication occurring from 6% to 23% of cases after extended pleurectomy/decortication for malignant pleural mesothelioma. Treatment options for this complication after major lung resection are well documented in literature; nevertheless, lines of evidence in extended pleurectomy/decortication for malignant pleural mesothelioma are absent. The aim of the study is to evaluate the efficacy of intraoperative administration of 50% hypertonic glucose solution in reducing duration of air leak following extended pleurectomy/decortication for malignant pleural mesothelioma.

Materials and Methods: In this retrospective case–control study, we analyzed our electronic health record and selected those patients with a histological diagnosis of malignant pleural mesothelioma who underwent extended pleurectomy/decortication in the period 2013–2021. From 2018, we introduced a lavage with 500 ml of glucose solution at 50% concentration into the chest cavity at the end of the surgical procedure. Patients operated before 2018 were used as the control group. Postoperative glycemia was measured, and patients were followed after hospital discharge until the air leak resolved and the chest tube was removed. Statistical analysis was performed using R software.

Results: A total of 71 patients met our criteria. Treatment and control groups were similar for age, sex, smoking status, number of comorbidities, tumor histotype, and side of disease. Use of hypertonic glucose solution resulted in shorter chest tube maintenance after hospital discharge ($p = 0.0028$). A statistically significant difference ($p = 0.02$) was also found in postoperative glycemia between the treatment (103 g/dl ± 8.9) and control group (98.8 g/dl ± 8.6). Days of hospitalization and chest tube maintenance during hospitalization did not significantly differ between the groups.

Interpretation: Intraoperative administration of 50% hypertonic glucose solution reduced the duration of air leak after hospital discharge. An increase in postoperative glycemia was found in the treatment group, but with no clinical effect. Hypertonic glucose solution is an effective and safe method to manage persistent air leak after extended pleurectomy/decortication for malignant pleural mesothelioma.

Keywords: malignant pleural mesothelioma (MPM), persistent air leak (PAL), prolonged air leak (PAL), hypertonic glucose solution, thoracic surgery (TS)

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a rare but aggressive tumor arising from pleural surface, with a poor prognosis in most of the cases (1). The main cause of MPM is exposure to asbestos fibers, a silicate mineral largely used in Europe between 1920 and 1970 (2). Use of asbestos fibers is prohibited in most of the countries worldwide; Italy introduced the ban in 1992 (3). Time between inhalation of asbestos fibers and development of MPM can be up to 40 years; therefore, incidence is bound to increase in the next years.

Extra-Pleural Pneumonectomy (EPP) combined with neo-adjuvant chemotherapy and adjuvant radiotherapy represented the standard treatment for carefully selected patients with MPM (4). However, Extended Pleurectomy/Decortication (EPD) has proven to be as effective as EPP in terms of median overall survival and 2-year mortality with lower incidence of death at 30 days (5, 6). Nonetheless, EPD comes with a high rate of postoperative complication, with persistent air leak (PAL) occurring in 6% to 23% of cases (7, 8). As well known, PAL increases hospital stay, intensive care unit readmission, and risk of empyema, and leads to higher in-hospital mortality rate (9).

Data regarding techniques for treatment of PAL comes primarily from lung resective surgery, such as wedge or segmentectomy or lobectomy. Most of the techniques currently available are not applicable to EPD due to the surgical procedure itself: pleural tenting is effective on duration of air leak (2.5 vs 7.2 days; $p < 0.001$) and chest tube maintenance (7.0 vs. 11.2 days; $p < 0.0001$) when performing upper lobectomy and, thus, is unfeasible in EPD since the aim of the procedure is to remove all the pleura (10); transient phrenic nerve paralysis by injecting local anesthetic is useful in reducing pleural space through elevation of diaphragm, but clearly is not possible in EPD since diaphragm is removed and a mesh prosthesis is used in substitution (11); use of intraoperative synthetic sealants is also effective in reducing PAL and chest tube maintenance after lung resection; nonetheless, efficacy in decortication is limited to benign disease (12, 13). As a result, PAL following EPD is usually managed conservatively by applying mild suction to chest tube, then weaning to water seal, and finally using pneumostats for portability if needed (14).

Interestingly, some authors described intraoperative lavage of chest cavity using glucose solution at 50% concentration (GS50) as an effective and inexpensive treatment for reducing PAL and chest tube maintenance. Fujino et al. in 2015 demonstrated safety and efficacy of GS50 in reducing air leak following lung resection and bullectomy for pneumothorax (15). In 2016, Al-Naimi et al. demonstrated a reduction of air leak in 80% of patients at postoperative day (POD) 3 (16). The main limitation of those studies is lack of a control group. In 2008, a prospective randomized controlled trial by Won et al. enrolled 141 patients with primary spontaneous pneumothorax. Patients were divided into three groups based on treatment: thoracoscopy alone, thoracoscopy + glucose solution at 20% concentration, and thoracoscopy + mixture of talc and glucose solution at 20% concentration. The authors found no difference in recurrence rate of pneumothorax and chest tube duration between the three treatment groups (17). Yet, there are two main limitations: concentration of glucose solution was lower in comparison to other studies and criteria for sample size was unclear (β-power unknown).

Until 2018, our department of thoracic surgery routinely used aerosolized fibrin sealant (Tisseel) as the only intraoperative aerostatic agent for EPD. Starting from 2018, we added a lavage of chest cavity with GS50 in EPD for MPM. The aim of this study is to evaluate efficacy of GS50 in reducing duration of air leak and subsequently chest tube maintenance following EPD.

MATERIALS AND METHODS

Selection Criteria

From 2013 to 2021, we retrospectively analyzed our electronical health records and selected those patients with age above 18 years and histological diagnosis of malignant pleural mesothelioma who underwent EPD. Metastatic cancer, severe heart disease, renal impairment, and ASA score > 3 were considered exclusion criteria. Diabetes was not an exclusion criterion. Patients where then divided into two groups: those who did not receive GS50 intraoperatively (before 2018) were used as a control group, whereas those who received GS50 intraoperatively (after 2018) were the treatment group. The study was approved by our hospital ethical committee (MESO1).

Operative Procedure

After placing a thoracic epidural catheter, a naso-gastric tube (NGT), a central venous catheter (CVC), and an arterial line, the patient was positioned in a lateral fashion. An extended posterolateral thoracotomy with section of latissimus dorsi was performed, and one rib was divided when needed.

After entering the extrapleural plane, the parietal pleura was bluntly separated from the chest wall until a satisfactory

mobilization of the lung was reached. Moving towards the mediastinum, the pericardium was removed if macroscopic evidence of invasion was present. Moving downwards, diaphragm was always detached from its insertion. Subsequently, pleura was peeled off from lung starting from the apex to the base. To make the peeling easier, an anesthesiologist was asked to maintain the lung partially inflated.

Once the base was reached, pleura and diaphragm appeared strongly adherent to parenchyma and often the use of a stapler was required to divide and remove the specimen.

After proper hemostasis with gauze compression, the reconstructive phase was initiated by applying a bovine pericardial patch onto the pericardial defect. The patch was fixed using separated stitches, thus preventing pericardial herniation. Suture was not applied in the upper part of the patch in order to avoid cardiac tamponade and adhesion on ascending aorta. A Proceed ® mesh prosthesis was then fixed one or two ribs above the natural insertion of diaphragm, reducing pleural space and allowing the lung to occupy the residual cavity easier.

Once reconstruction was completed, 500 ml of 50% glucose solution was instilled into the chest cavity and aspirated after 2 min of application. Argon beam was then used to improve hemostasis of chest wall. A "cotton-candy like smell" is produced due to high temperature applied on residual glucose. Afterwards, aerosolized Tisseel was applied on lung parenchyma. A satisfactory value of less than 30% in Tidal Volume loss was reached before chest closure. Two chest tubes were positioned into the cavity and connected to a water-sealed chamber with −15 cmH_2O suction applied on it.

Postoperative and Outpatient Care

After monitoring in a post-anesthesia care unit, NGT and arterial line were removed if parameters were stable. The patient was then sent to the ward on Post-Operative Day (POD) 0.

Blood test and a chest x-ray (CXR) were routinely performed every day until POD3, then every 2 days if clinical conditions were stable. Sugar blood level was tested only on POD1 and repeated on the following days only if greater than 125 mg/dl. Chest tube suction was applied continuously until POD2 and removed when a satisfactory lung expansion was confirmed at CXR. Due to the high risk of urinary retention, urinary catheter was maintained until thoracic epidural catheter was removed and satisfactory diuresis (>40 ml/h) was reached. Air leak was evaluated according to the five-grade scale by Sang et al. (18). A positive pressure of 10 cmH_2O was then applied by raising water level in the drainage chamber to test the stability of lung expansion. If no to minimal air leak (grade 0 to 2) and stability of lung expansion at CXR were present after 1 day of positive pressure, discharge was possible. Chest tube was removed if no air leak was evidenced. If minimal air leak persisted, the patient was discharged with a single chest tube and trained to return at our hospital when no air leak was seen coming from Heimlich valve. Confirmation of air leak resolution was confirmed by connecting chest tube to a water-sealed chamber for 30 min, and no suction was applied. If no air leak was noticed, chest tube was removed.

Statistical analysis

Statistical analysis was performed using R statistical software v 4.0.4. The following data were collected: hospitalization days, chest tube maintenance during hospitalization and after discharge expressed as continuous data (days), glucosate administration expressed as categorical variable (yes or no), age (continuous scale), sex (dichotomic) and postoperative glycemia (continuous scale; expressed in mg/dl), number of comorbidities, smoking habits, side of disease (dichotomic), and pathological stage.

The treatment group was defined as those patients who obtained glucosate administration during surgery; otherwise, they were categorized as the control group. The association of each continuous variable with the categorical ones was evaluated by resorting to the nonparametric Kruskal–Wallis test; comparison of two categorical variables was performed applying a chi-squared test from stats R package.

RESULTS

A total of 71 patients met our inclusion criterion and were included in the study. Fifty (70.4%) were male and 21 (29.6%) were female. A prevalence of gender in favor to male was therefore present. Mean age at surgery was 65 ± 8 years; 51 patients (71.8%) underwent neoadjuvant chemotherapy.

Treatment (42 patients) and control (29 patients) groups did not differ when comparing age, sex distribution, smoking status, number of comorbidities, neoadjuvant treatment, and side of disease (**Table 1**).

Comparing treatment (42 patients) and control (29 patients) groups, no differences between groups were found regarding days of hospitalization, days of chest tube maintenance during hospitalization, and pathological stage. Interestingly, a difference was found when comparing days of chest tube maintenance after hospital discharge between treatment (15 patients) and control (16 patients) groups (**Table 2**, **Figure 1**). In depth, chest tube was removed earlier in the treatment group (4.95 ± 7.79 versus 9.14 ± 8.4; $p = 0.0028$), thus meaning an earlier resolution of air leak after discharge. Postoperative glycemia was higher in the treatment group (42 patients;103 g/dl ± 8.9; $p = 0.02$) when compared to control (29 patients), but none of the patients had clinically significant symptoms and therefore pharmacological treatment was not necessary.

DISCUSSION

PAL represents a common complication following EPD; nowadays, no evidence is available regarding treatment to reduce air leak duration. Based on our results, intraoperative instillation of GS50 is an effective method to reduce duration of air leak, leading to earlier removal of chest tube. This efficacy was evident after patients were discharged from the hospital, thus potentially reducing risk of infections and improving the quality of life perceived by patients.

TABLE 1 | Clinicopathological characteristics of patients divided in control and treatment group.

		Control group (*n* = 29)	Treatment group (*n* = 42)	*p*-value
Sex, *n* (%)	Male	22 (75.9)	28 (66.7)	0.41
	Female	7 (24.1)	14 (33.3)	
Smoking status, *n* (%)	Never	7 (24.1)	20 (47.6)	0.51
	Current	7 (24.1)	7 (16.7)	
	Former	15 (51.7)	15 (35.7)	
Histotype, *n* (%)	Epithelioid	25 (86.2)	38 (90.5)	0.58
	Biphasic	3 (10.3)	3 (7.1)	
	Sarcomatoid	1 (3.5)	1 (2.4)	
Comorbidities, *n* (%)	0	15 (51.7)	22 (52.4)	0.9
	1	8 (27.6)	13 (30)	
	2	6 (20.7)	7 (16.7)	
Side, *n* (%)	Left	11 (37.9)	22 (52.4)	0.23
	Right	18 (62.1)	20 (47.6)	
Age, mean (SD), years		65 (9)	65 (7)	0.76

Aerosolized Tisseel was used in both treatment and control groups; based on our result, we can say that there is an effect of hypertonic glucose solution on air leak reduction that is visible in the long term. However, the interaction between Tisseel and glucose solution should be explored in experimental models to better understand the effects of these compounds when separated and in combination.

The treatment group had a higher mean glycemic value in comparison to the control group; however, values remained below the threshold of 125 mg/dl. Glucose is therefore minimally absorbed when administered inside the chest cavity, despite what is observed in a previous study published by Tsuboshima et al. (19). A reduction of either quantity or concentration of hypertonic glucose solution administered may limit this phenomenon, and further studies are needed to find the right minimal effective dose.

The mechanism of action leading to reduction of air leak is still unknown. A possible explanation can be found in the inflammation generated by exposure to hypertonic glucose solution and subsequent generation of fibrous adhesion between lung and chest wall that lead to resolution of air leak. In experimental models, cell exposure to high level of glucose leads to overexpression of transforming growth factor beta (TGF-β) and specific surface receptor type I (TβRI) and type II (TβRII) (20) that are the main actors of fibrotic tissue deposition. TGF-β plays a key role in the genesis of adhesion through binding and activating TβRI and TβRII. Activation of receptors leads to phosphorylation of small mother against decapentaplegic (SMAD) intracellular proteins that translocate into the nucleus and act as transcription factors for the deposition of extracellular matrix (ECM). An unbalanced expression of TGF-β isoforms results in the production of altered ECM and subsequent tissue fibrosis (21). In rabbit models the TGF-ß$_2$ isoform was effective in generating pleural adhesion, resulting in superior to talc pleurodesis (22, 23). Glucose may therefore be able to determine an altered production of TGF-β isoforms, and this hypothesis can be tested to better understand the mechanism of action.

Another possible explanation is the role of osmotic cell injury secondary to hypertonic glucose solution exposure, leading to precipitation of fibrin and lastly to adhesion between chest wall and lung parenchyma (15).

To our knowledge, this is the first evidence in literature describing efficacy of 50GS in reducing duration of air leak after extended pleurectomy/decortication for malignant pleural mesothelioma. Chest tube was removed earlier in the treatment group, and this difference was significant after patient discharge (p = 0.0028). A statistically significant difference was also found in postoperative glycemia (p = 0.02), being higher in the treatment group but still below the threshold of 125 mg/dl and therefore with no clinical effect. The mechanism of action leading to reduction of air leak after administering GS50 is still unknown. Due to the efficacy and inexpensiveness, we suggest the use of GS50 for the management of persistent air leak following EPD.

The two main limitations of this study are the retrospective nature of data and the small sample size of patients available. The former does not allow to control potential confounding factors and to evaluate other parameters that were not routinely

TABLE 2 | Postoperative and pathological characteristics of patients.

		Control group (*n* = 29)	Treatment group (*n* = 42)	*p*-value
Postoperative glycemia, mean (SD), days		98.8 (8.6)	103 (8.9)	0.02
Hospitalization days, mean (SD), days		14.8 (7.1)	17 (6.9)	0.07
Chest tube during hospitalization, mean (SD), days		13.1 (6.9)	14.3 (5.6)	0.13
Chest tube after discharge, mean (SD), days		9.14 (8.4)	4.95 (7.79)	0.0028
pStage, *n* (%)	0	2 (6.9)	2 (4.7)	0.44
	IA	0 (0)	1 (2.4)	
	IB	15 (51.7)	21 (50)	
	IIIA	0 (0)	7 (16.7)	
	IIIB	12 (41.4)	11 (26.2)	

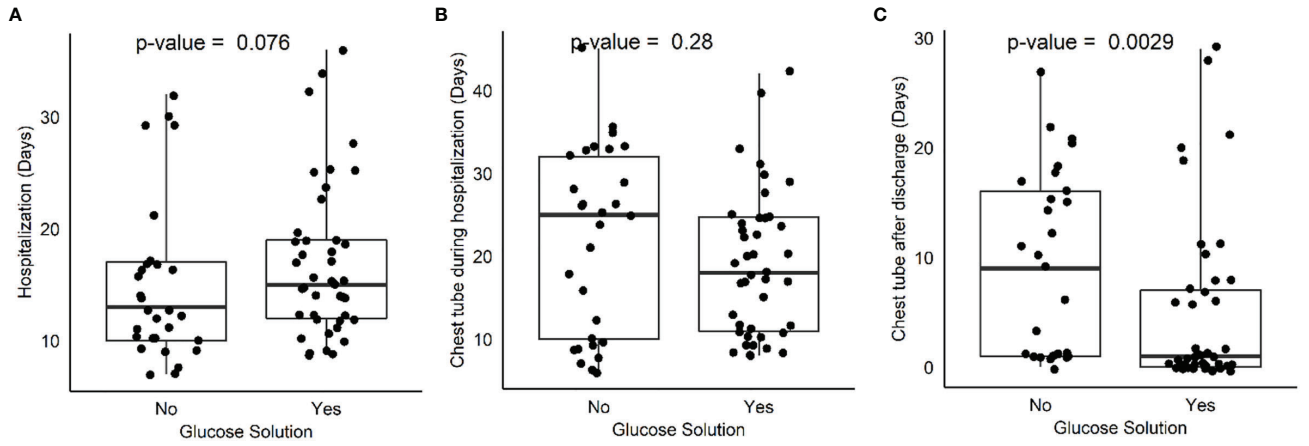

FIGURE 1 | Box plot comparing hospitalization days **(A)**, chest tube maintenance during hospitalization **(B)** and after discharge **(C)** between treatment and control group.

included in the clinical practice of our ward. The latter prevented us from observing minor differences that may exist between treatment and control groups. A future study with a prospective design and a larger sample size has the potential to validate these results, while the creation of an experimental model may explain the mechanism of action that lies behind the use of hypertonic glucose solution for the treatment of air leak.

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 Humanitas Research Hospital ethical committee, study code MESO1. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AT, GP, UC and EB contributed to the conception and design of the study. MA, VMG, EV, and UC organized the database. VMG and GP contributed to data collection. GP wrote the draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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High Circulating Sonic Hedgehog Protein Is Associated With Poor Outcome in EGFR-Mutated Advanced NSCLC Treated With Tyrosine Kinase Inhibitors

*Paul Takam Kamga[1], Aurélie Swalduz[2,3], Adrien Costantini[1,4], Catherine Julié[1,5], Jean-François Emile[1,5], Maurice Pérol[2], Virginie Avrillon[3], Sandra Ortiz-Cuaran[3], Pierre de Saintigny[2,3] and Etienne Giroux-Leprieur[1,4]**

[1] Université Paris-Saclay, UVSQ, EA 4340 BECCOH, Boulogne-Billancourt, France, [2] Department of Medical Oncology, Centre Léon Bérard, Lyon, France, [3] Univ Lyon, Université Claude Bernard Lyon 1, INSERM 1052, CNRS 5286, Centre Léon Bérard, Centre de Recherche en Cancérologie de Lyon, Lyon, France, [4] Department of Respiratory Diseases and Thoracic Oncology, APHP—Hopital Ambroise Pare, Boulogne-Billancourt, France, [5] Department of Pathology, APHP—Hopital Ambroise Pare, Boulogne-Billancourt, France

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Nico van Zandwijk,
Sydney Local Health District, Australia

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Robert Pirker Pirker,
University of Vienna, Austria
Kathy Ann Gately,
Trinity College Dublin, Ireland

***Correspondence:**
Etienne Giroux-Leprieur
Etienne.giroux-leprieur@aphp.fr

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Introduction: Growing preclinical evidence has suggested that the Sonic hedgehog (Shh) pathway is involved in resistance to tyrosine kinase inhibitor (TKI) therapy for EGFR-mutated (EGFRm) non-small cell lung cancer (NSCLC). However, little is known concerning the prognostic value of this pathway in this context.

Materials and Methods: We investigated the relationship between plasma levels of Shh and EGFRm NSCLC patients' outcome with EGFR TKIs. We included 74 consecutive patients from two institutions with EGFRm advanced NSCLC treated by EGFR TKI as first-line therapy. Plasma samples were collected longitudinally for each patient and were analyzed for the expression of Shh using an ELISA assay. The activation of the Shh–Gli1 pathway was assessed through immunohistochemistry (IHC) of Gli1 and RT-qPCR analysis of the transcripts of Gli1 target genes in 14 available tumor biopsies collected at diagnosis (baseline).

Results: Among the 74 patients, only 61 had baseline (diagnosis) plasma samples, while only 49 patients had plasma samples at the first evaluation. Shh protein was detectable in all samples at diagnosis (n = 61, mean = 1,041.2 ± 252.5 pg/ml). Among the 14 available tumor biopsies, nuclear expression of Gli1 was observed in 57.1% (8/14) of patients' biopsies. Shh was significantly ($p < 0.05$) enriched in youth (age < 68), male, nonsmokers, patients with a PS > 1, and patients presenting more than 2 metastatic sites and L858R mutation. Higher levels of Shh correlated with poor objective response to TKI, shorter progression-free survival (PFS), and T790M-independent mechanism of resistance. In addition, the rise of plasma Shh levels along the treatment was associated with the emergence of drug resistance in patients presenting an initial good therapy response.

Conclusion: These data support that higher levels of plasma Shh at diagnosis and increased levels of Shh along the course of the disease are related to the emergence of TKI resistance and poor outcome for EGFR-TKI therapy, suggesting that Shh levels could stand both as a prognostic and as a resistance biomarker for the management of *EGFR*-mutated NSCLC patients treated with EGFR-TKI.

Keywords: non-small cell lung cancer (NSCLC), epidermal growth factor receptor (EGFR), Sonic Hedgehog (Shh), biomarker, tyrosine kinase inhibitor (TKI)

INTRODUCTION

Lung cancer is the main cause of cancer death in the Western world. Non-small cell lung cancer (NSCLC) represents 85%–90% of lung cancer subtypes (1). Growing efforts have led to tremendous advances in early diagnosis and the implementation of more efficient drugs that target key oncogenic events (2). Among others, the introduction of epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (EGFR-TKIs) as first-line therapy for the treatment of *EGFR*-mutated NSCLC patients has led to longer progression-free survival (PFS) and higher response rates compared to platinum-based chemotherapy (3–6). Notwithstanding the clinical advantage observed in patients treated with EGFR-TKI, primary or acquired resistance limits the benefit of TKI treatment (7, 8). Therefore, there is still a continual need of new predictive biomarkers for EGFR-TKI therapy. Studies have addressed many sources of EGFR-TKI resistance, underlining the involvement of EGFR-dependent (i.e., T790M or C797S resistance mutations) or EGFR-independent molecular aberrations (i.e., *MET* amplification or the activation of other oncogenic pathways) (9). In general, primary or acquired molecular aberrations involved in poor clinical outcome collaborate with pro-survival signaling such as Hedgehog, Wnt, Notch, Akt, and Ras/Erk (10–12). Therefore, the expression/activation levels of these pathways have been proposed as predictive biomarker for EGFR-TKI therapy.

Hedgehog (Hh) pathway is a highly conserved pathway involved in developmental processes such as tissue patterning and organogenesis (13). There are three hedgehog ligands, Desert Hedgehog, Indian Hedgehog, and Sonic Hedgehog (Shh). In human, the activation of the pathway occurs when the ligand binds to the receptor Patched (Ptch), activating the Ptch-coreceptor Smoothened (Smo). Smo stabilizes and activates the transcription factors of the Gli proteins family; then, Gli proteins enter into the nucleus to activate genes involved in cell proliferation, self-renewal, and survival during development or in cancer (14, 15). The overexpression or/and the hyperactivity of Shh/Gli signaling are found in several solid and hematological malignancies, being associated with aggressive disease, poor survival, and resistance to many anti-cancer therapies (16–19). In NSCLC, we have observed that the activation of Hedgehog pathway was associated with resistance to platinum-based chemotherapy and to immune checkpoint inhibitors (20, 21). In *EGFR*-mutated NSCLC, inhibition of the Shh pathway sensitizes primary tissues and cancer cell lines both to classic chemotherapy and EGFR-TKIs, suggesting a role for Hh pathway in resistance to EGFR TKI (22). Therefore, we hypothesized that the expression levels of Shh could predict the outcome of *EGFR*-mutated NSCLC patients treated with EGFR-TKIs. In this study, expression levels of Shh in plasma samples collected from patients treated with EGFR-TKIs were correlated with patients' features to demonstrate that high levels of Shh in patient samples is a biomarker of poor prognosis.

MATERIALS AND METHODS

Patients and Samples

Plasma samples and lung biopsies were collected as previously described (21, 23), from consecutive patients with advanced (not irradiable stage IIIb or stage IV) *EGFR*-mutated NSCLC treated by EGFR-TKIs between 08/2011 and 07/2019 at the Department of Respiratory Medicine and Thoracic Oncology (APHP—Ambroise Pare Hospital) and within the framework of the Centre Léon Bérard Cancer Center LIBIL (NCT02511288) (24, 25). All patients were included in the study after written informed consent, as approved by the two Institutional Review Boards (IRB) including the CPP IDF n°8 (ID CRB 2014-A00187-40) and the CPP Ouest 6 (ID-RCB: 2015-A00640-49). Lung biopsies (n = 14) were collected at diagnosis (diagnosis/baseline). Prospective consecutive collection of the plasma was made before the beginning of the TKI treatment, 6–8 weeks after the beginning of the TKI (first evaluation, n = 61), and at disease progression. All other collection points were referred to as follow-up.

Enzyme-Linked Immunosorbent Assay

Plasma levels of Shh were assessed through ELISA assay as previously described (21, 26). Briefly, 50 µl of each plasma sample was loaded in duplicate into the 96-well Shh ELISA plate (ab100639, Abcam Cambridge, UK). After 2.5 h of incubation at room temperature (RT), wells were washed and probed for 1 h at RT with Biotinylated Shh detection antibody. Then, wells were washed and probed with HRP-Streptavidin for 45 min at RT. Wells were washed and the revelation was performed by adding in each well 100 µl of a TMB substrate following by the addition of a Stop solution. Optical densities at 450 nm were determined with a plate reader, the Multiscan GO reader, V.1.01.10 (ThermoFisher Scientific, France). The concentration of Shh in each sample was determined using standard controls (recombinant proteins) and the generated standard curve.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks were sectioned (4 μm) using a microtome, transferred to slides, and allowed to dry overnight. Samples were stained using a LEICA BOND-III automate. Activation of the Hh pathway was assessed through the staining of nuclear Gli1 (anti-Gli1 mouse monoclonal antibody sc-515751; 1:5; Santa Cruz Biotechnology, Santa Cruz, USA) in tumor cells. Stained slides were mounted on an optical microscope Axio ZEISS Scope. They were evaluated and scored by one biologist (PK) and one pathologist (J-FE). Gli1 nuclear staining was evaluated on tumor cells as recently described. A sample of Basal Cell human carcinoma (BCC) was used as a positive control and nonspecific immunoglobulin isotype was used as a negative control (21, 23).

Reverse Transcription and Quantitative Polymerase Chain Reaction

RNA extraction from tumor tissue and reverse transcription using previously described TaqMan PCR primers and probes were performed as previously described (21). Briefly, RNA was extracted from tumor tissue using the AS1480 Maxwell RSC Simply RNA tissue kits (Promega, USA) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed in cDNA using the High-capacity RNA cDNA kit (ThermoFisher Scientific, France) and amplified using the TaqMan Universal PCR Master Mix, No AmpErase UNG (ThermoFisher Scientific, France). Primers and probes specific for Gli1 target genes and control gene β-actin (**Table S1**) were purchased commercially and used according to the manufacturer's instructions (ThermoFisher Scientific, catalog number 4331182). All values were normalized to the control gene β-actin using the ΔΔ Ct method. Here, again, a BCC sample was used as positive control.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 (La Jolla, CA, USA) and XLSTAT 2019.1.3 (Addinsoft, Paris, France). The Wilcoxon-Mann–Whitney and Kruskal–Wallis methods were used to compare two groups or more than two groups, respectively. Pearson's Chi-squared analyses including the parametric Chi-squared and the non-parametric Fisher's exact test were used to test the association between variables. Survival curves including overall survival (OS) and progression-free survival (PFS) were calculated by the Kaplan–Meier method and the application of log-rank test. *p*-values less than 0.05 were considered as significant.

RESULTS

Patients

Clinical characteristics of patients are described in **Table 1**. Seventy-four consecutive patients were included in the study. The median age was 67 years old (range, 35 to 90 years old). Patients were mostly females (79.7%, *n* = 59) and nonsmokers (66.2%, *n* = 49). The main histological type was adenocarcinoma (95.9%, *n* = 71). Most patients had a stage IV disease (78.4%, *n* = 58) including 29 patients with central nervous system (CNS) metastasis (39.2%). Forty-seven (62.2%) NSCLC were Exon 19 mutated, 22 (29.7%) were L858R mutated patients, and 5 cases were found with another EGFR mutation type (**Table 1**). Only 61 patients with available plasma samples at diagnosis were included in correlative studies because 13 were excluded for missing data and/or no available sample at baseline (**Figure S1**). Among them, 20.1% (*n* = 23) were treated with erlotinib, 24.6% (*n* = 15) received gefitinib, 19.7% (*n* = 12) received afatinib, and 14.8% (*n* = 9) received osimertinib. Thirty-six patients (60.7%) responded to the therapy including 2 complete responses and 34 partial responses. On the other hand, 14 (22.9%) patients did not respond to the treatment, including 5 patients with stable disease and 6 patients in progression.

TABLE 1 | Demographic and clinical profile of the study cohort.

Variable	Patients (*n* = 74)
Age	
Median [range]	67 [35–90]
<67	37 (50%)
>67	37 (50%)
Gender	
Female	59 (79.7%)
Male	15 (20.3%)
Smokers	
Never	49 (66.2%)
Former	17 (23.0%)
Current	6 (8.1%)
Unknown	2 (2.7%)
Performance Status	
0	17 (23.0%)
1	44 (59.4%)
2	8 (10.8%)
3	1 (1.4%)
Unknown	4 (5.4%)
Histology	
Adenocarcinoma	71 (96.0%)
Squamous cell carcinoma	2 (2.7%)
Large cell neuroendocrine carcinoma	1 (1.3%)
Number of metastatic sites	
0	4 (5.4%)
1	21 (28.4%)
2	13 (17.6%)
3	9 (12.2%)
4	9 (12.2%)
5	6 (8.1%)
Unknown	12 (16.2%)
CNS metastasis	29 (39.2%)

Shh and Gli1 Expression at Baseline

To determine whether plasma levels of Shh were associated to patient outcomes, we first used the ELISA assay to analyze levels of circulating Shh in the plasma samples from NSCLC patients. Assessed in 61 samples collected before TKI initiation, the protein expression was readily found in plasma samples with a median of 466.185 pg/ml (range: 0.3–11,522.6 pg/ml) (**Figure 1A** and **Table S2**). The Kolmogorov–Smirnov (KS) normality test confirmed that data were normally distributed ($p < 0.001$) (**Table S2**).

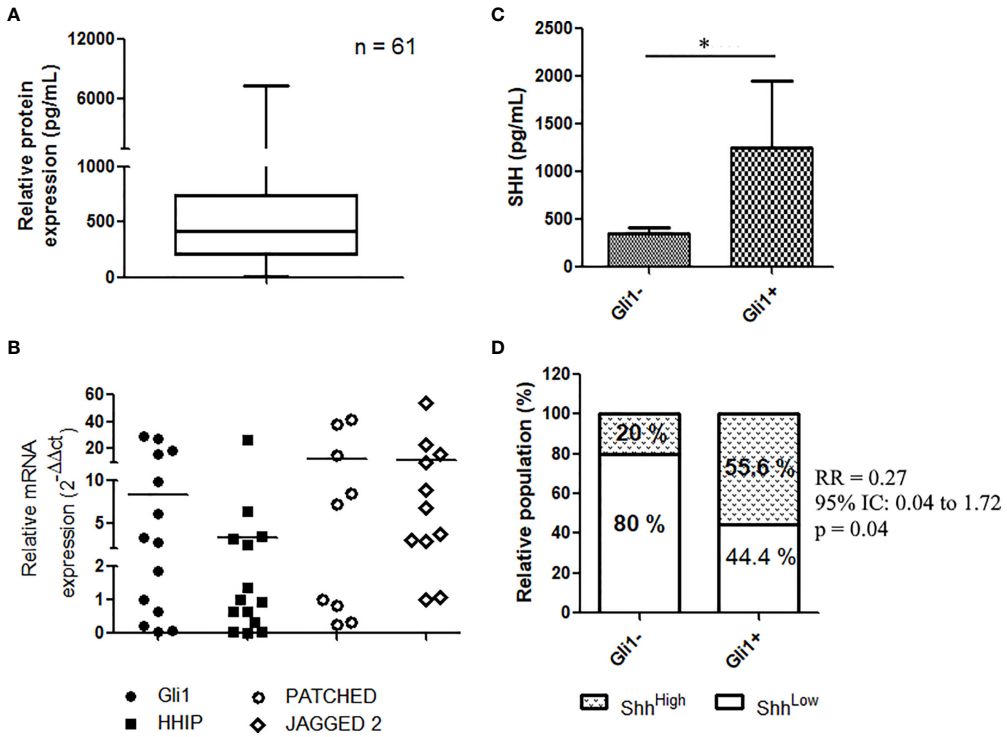

FIGURE 1 | Shh signaling expression and activation in EGFR-mutated NSCLC patients: **(A)** Shh concentration in plasma from patients at diagnostic (n = 61) as assessed by ELISA assays. **(B)** mRNA expression of Gli1 target genes in NSCLC; data are expressed as mean ± SEM of 14 patients analyzed in duplicate. **(C)** Representative, IHC of Gli1 in NSCLC (n = 14). **(C, D)** Expression of Shh in plasma from patients (14) according to Gli1 expression. *p < 0.05.

We recently provided evidence that the Shh–Gli1 signaling axis is operative in NSCLC, demonstrating that primary lung biopsies from NSCLC express *GLI1* transcript and Gli1 target genes including *HHIP, PTCH*, and *JAG2* (20). Similarly, analyzing the mRNA in 14 patients' tissues, we found that NSCLC expressed HHIP, PTCH, and JAG2 transcripts (**Figure 1B**), confirming that the Shh signaling is operative in NSCLC (**Figure 1B**). We then asked whether expression of plasma Shh was related to the pathway activation in NSCLC. Shh pathway activation was assessed by the mean of the nuclear staining through IHC of Gli1, in tumor tissue samples from 14 patients with known Shh plasma concentrations. Nuclear expression of Gli1 (**Figure S2**) was observed in 57.1% (8/14) of patients' biopsies. Patients classified as Gli1-positive patients (Gli1+) showed elevated levels of plasmatic Shh compared to Gli1- patients (1,255 ± 696.1 vs. 352.9 ± 59.8; p = 0.05) (**Figure 1C**), suggesting an association between tumoral activation of the Shh pathway and the plasmatic concentration of its ligand. We applied a cutoff to divide plasma samples into low expressing samples (Shh < median, n = 34) and high expressing samples (Shh > median, n = 27). We used a Fisher's rank test to demonstrate a positive association between the nuclear expression of Gli1 and higher levels of Shh in patients' plasma [relative risk, RR (95% CI, confidence interval), 0.27 (0.041–1.73); p = 0.05] (**Figure 1D**).

Correlation Between Shh Levels and Patients' Characteristics

We then looked for associations between Shh plasma levels and patients' characteristics. In accordance with previous studies, parameters considered were age, gender, smoking status, performance status (PS) at diagnosis, number of metastatic sites, CNS metastasis, and *EGFR* mutation type (**Figure 2** and **Table S3**) (23, 26, 27). Shh was significantly (p < 0.05) enriched in youth (age < 68 years old), males, and nonsmoker patients (**Figure 2**). However, we found no association between Shh levels and any of these three parameters (**Table S3**). A trend for higher Shh plasma levels in patients with a PS > 1 and those with more than 2 metastatic sites was observed (**Figure 2**), without reaching statistical significance (**Table S3**).

Then, we analyzed Shh levels in patients according to their specific *EGFR* mutation (del ex19, L858R and other mutations). Shh expression levels in these three groups were respectively 542.2 ± 94.29 pg/ml, 1,274 ± 440.4 pg/ml, and 524.8 ± 98.30 pg/ml (**Figure 2**). Compared to all other patients, patients with L858R *EGFR*-mutated NSCLC displayed higher Shh levels (**Figure 2**). A Pearson chi-squared analysis revealed a positive association between higher levels of Shh and L858R mutation [RR (95% CI), 0.4 (0.16–0.99); p = 0.02] and between del ex19 mutation and lower levels of plasmatic Shh [RR (95% CI), 1.55 (1.11–2.16); p = 0.02].

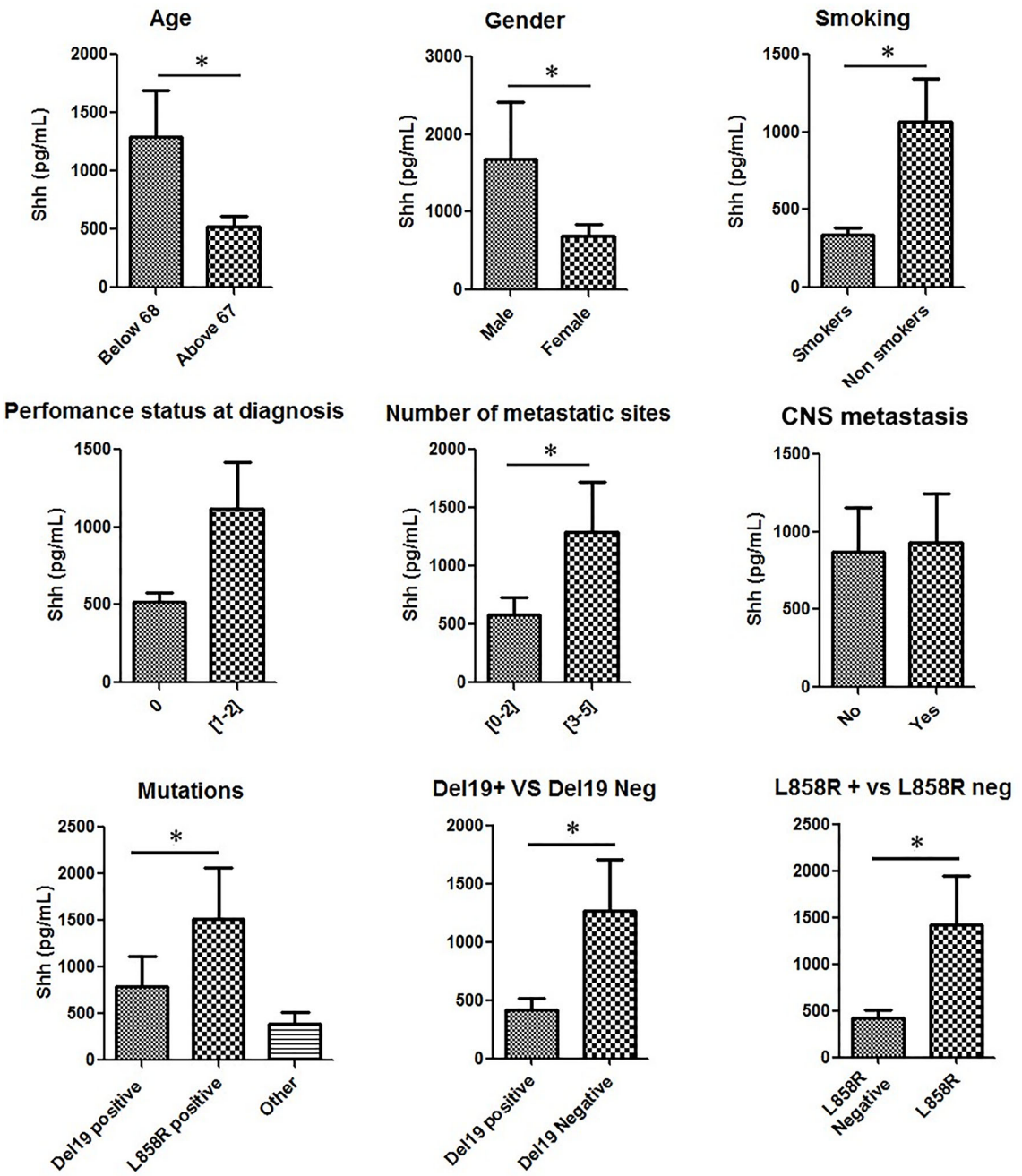

FIGURE 2 | Shh concentrations in plasma from patients at diagnosis according to patients' characteristics: Patient samples analyzed for Shh expression were classified according to age, gender, smoking status, performance status at diagnosis, number of metastatic sites, central nervous system (CNS) metastasis, and EGFR mutation type. A Mann–Whitney test was used to analyze the differences between means. *$p < 0.05$.

Correlation With Treatment Response

Inhibition of Shh–Gli1/2 signaling is known to sensitize NSCLC to TKI treatment (10). Here, we have observed that Shh levels are related to patients' characteristics such as the type of *EGFR* mutation, which is a well-known factor able to influence patients' outcomes (28). It suggested that Shh plasma levels could vary along the disease course, being correlated to clinical response to TKI therapy. To validate this hypothesis, we first tested the relation between baseline Shh and response to TKI treatment. Shh analysis showed that the protein levels at baseline were lower in the plasma of responders compared to non-responders (779.4 pg/ml vs. 1,510 pg/ml; $p = 0.01$) (**Figure 3A**).

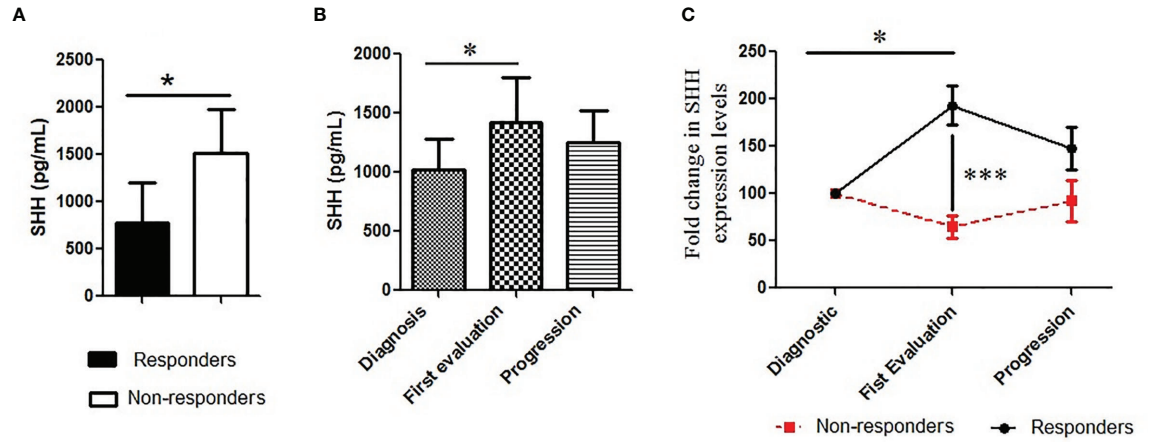

FIGURE 3 | Pattern of plasmatic Shh concentration along the course of the disease: **(A)** Shh was analyzed in plasma collected from patients and classified according to **(A)** treatment response (responders vs. non-responders); **(B, C)** and treatment steps (diagnosis or base line, the first evaluation and the progression). A Mann–Whitney test was used to analyze the differences between means. *$p < 0.05$, ***$p < 0.001$.

Correlation test confirmed a positive association between lower levels of plasmatic Shh at diagnosis and a good response to therapy [RR (95% CI), 0.45 (0.22–0.90); $p = 0.006$]. The changes of Shh levels along the course of the disease were also evaluated. Considering all patients including responders and non-responders, we observed increasing levels of Shh in the plasma of patients at the time of first evaluation, with a median fold increase by 30% (**Figure 3C**). The separation of patients into responders and non-responders revealed that the variation in Shh levels was mainly observed in responders, while the non-responders showed a stable level of Shh, notwithstanding the disease steps (**Figure 3B**).

Correlation With Patients' Survival

Then, we analyzed patients' survival. Shorter OSs were significantly related to the following parameters: the absence of del19 mutations [hazard ratio (HR) (95% CI), 0.28 (0.1–0.79); $p = 0.0156$] and age > 67 years [HR (95% CI), 0.29 (0.11–0.73); $p = 0.01$]. However, while older patients showed shorter PFS [HR (95% CI), 0.515 (0.28–0.95); $p = 0.035$], there was no difference in PFS according to del 19 mutations (**Table S4**). Shorter OS was also associated with the presence of L858R mutations [HR (95% CI), 2.5 (0.87–7.19)] and PS > 0 [HR (95% CI), 0.62 (0.18–2.07); $p = 0.43$] (**Table S4**). To study the influence of Shh levels on patients' survival, patients were classified according to the Shh concentration; Shh^{low} (32/61) and Shh^{high} (29/61) 5/61 patients were excluded for clinical reasons. In accordance with the work of Kim et al., we found that patients with higher Shh levels at diagnosis showed prolonged OS compared to patients with lower concentrations [OS: undefined vs. 884 days; HR (95% CI), 3.12 (1.288–7.561); $p = 0.0271$] (**Figure 4A**) (29). However, preclinical data revealed that inhibition of Shh–Gli1/2 signaling is known to sensitize NSCLC to TKI treatment, supporting the fact that level of Shh–Gli1/2 signaling during TKI therapy could reflect or influence treatment outcome and patient survival (10). Thus, we also analyzed patient survival according to Shh levels in plasma samples at the first evaluation following TKI induction. Interestingly, patients with lower Shh levels at the first evaluation underwent longer PFS (342 vs. 210 days) compared to patients with high Shh levels (HR (95% CI), 0.578 (0.2831–1.180); $p = 0.05$) (**Figure 4B**).

Correlation With Resistance Mechanisms

Finally, we tested how effective Shh levels at baseline could be related with acquired mechanisms of TKI resistance. Among the 61 patients with available Shh concentration at baseline, 35 went in progression. Analysis of the resistance mechanism was available only for 27/35 patients including 17 patients with a known resistance mechanism. The main resistance mechanisms were T790M ($n = 9$), MET amplification ($n = 4$), and other ($n = 4$) (**Figures 5A**, **S1**). Previous studies demonstrated that activation of Hedgehog pathway is a T790M-independent mechanism of EGFR-TKI resistance in *EGFR*-mutated NSCLC (23). Consistently, we observed higher Shh levels at baseline in patients ($n = 19$) with T790M-independent resistance mechanisms at the time of disease progression, whereas lower levels of Shh were observed in patients positive for T790M mutation at the time of progressive disease (with erlotinib, gefitinib, and afatinib treatments). Correlation tests confirmed the association between lower baseline levels of Shh and the acquisition of T790M mutation at the time of progression [RR (95% CI), 11.2 (1.61–78), $p = 0.0013$], supporting that higher Shh levels at baseline may be associated with EGFR-independent resistance mechanisms at the time of progression (**Figure 5B**). Interestingly, when we classified samples according to the presence of a resistance mechanism ($n = 16$) or not (11), we found higher levels of Shh in patients presenting no resistance mechanism (**Figure 5C**). This was confirmed by a positive correlation between high Shh levels at diagnosis and the absence of a resistance mechanism [RR (95% CI), 2.424

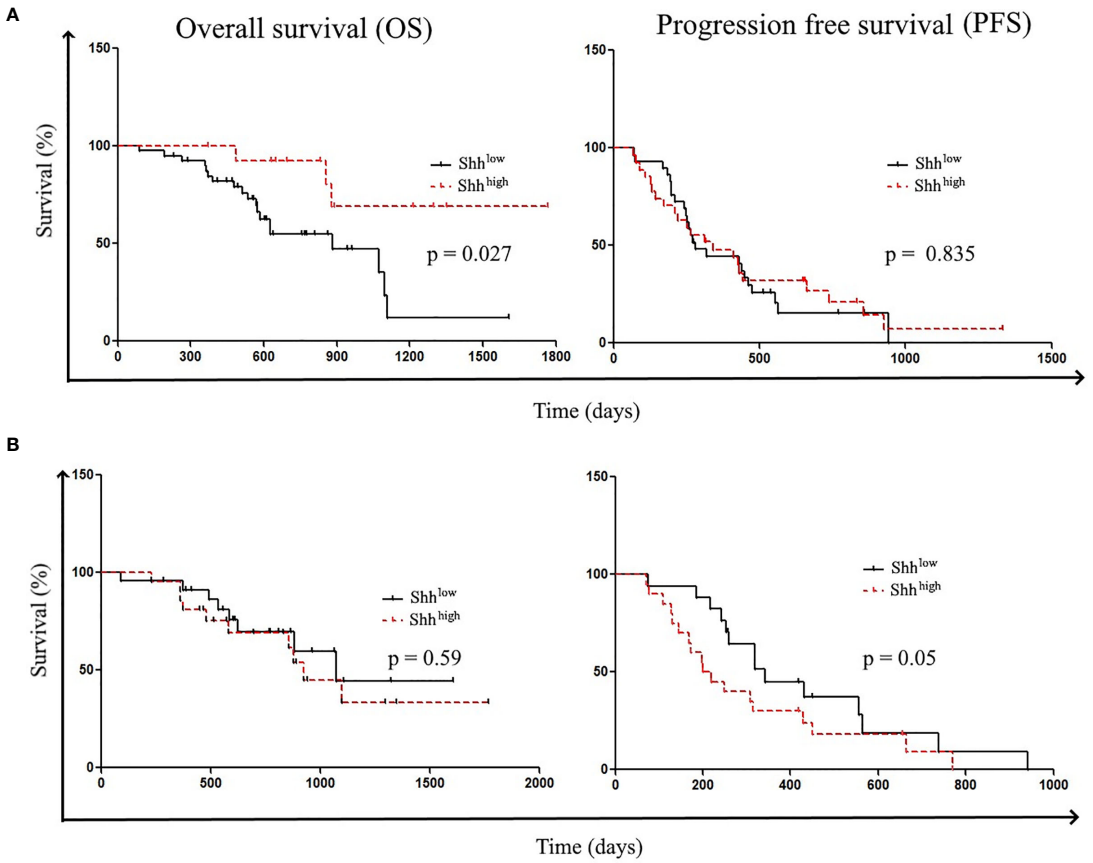

FIGURE 4 | Patient's survival according to Shh levels: Patient survivals were classified according to **(A)** Shh levels at diagnosis (Shh^{low} vs. Shh^{High}) and **(B)** Shh levels at the first evaluation (Shh^{low} vs. Shh^{High}).

(1.68–164.9), $p = 0.0076$], suggesting that activation of hedgehog is independent not only of T790M, but also of other resistance mutations including.

DISCUSSION

This study provides evidence that Shh pathway activation, as assessed by high levels of plasma Shh at diagnosis, is associated with resistance to EGFR TKIs in *EGFR*-mutated NSCLC.

Because blood collection is less invasive, growing efforts have been made these past years to study the prognostic value of blood components, including circulating DNA, exosomes, and soluble proteins (30–32). Noman et al. observed that increased levels of circulating Shh are associated with a worse survival, proposing that blood Shh can stand as cancer biomarker (17). Determining whether the plasma Shh could reflect the molecular dynamics of the Hedgehog pathway within the tumor bulk remains a key challenge. Raz et al. established a strong correlation between Shh expression and downstream Hedgehog pathway effectors including Smo, Gli1, and Gli2 (27). Similarly, we clearly established that plasma levels of Shh are positively correlated with nuclear expression of Gli1 in lung tumor cells, providing evidence that plasma Shh, reflecting nuclear localization of Gli1, can be used to study the activation of Hh pathway in *EGFR*-mutated NSCLC.

Among the clinicopathological parameters influencing the survival of TKI-treated patients, we found an enrichment of Shh in plasma from young patients, male patients, and patients with multiple metastatic sites and with L858R mutations. In *EGFR*-mutated NSCLC, Shh pathway promotes stemness, epithelial-to-mesenchymal transition (EMT), invasion, and resistance to therapies (28). Bermudez et al. reported that while the endogenous Gli1/2 signaling supports autonomous proliferation of NSCLC, the secreted Shh educates the tumor microenvironment including fibroblasts, to produce Shh itself, and proangiogenic and metastatic factors (33). Accordingly, in most cancers, increased levels of Shh are linked to higher invasive capabilities and drug resistance (17, 34). Consistently, we found elevated levels of plasma Shh in patients presenting several metastatic sites ($n > 3$).

We have previously observed that high activation of Shh pathway as assessed through the IHC of Gli proteins correlated

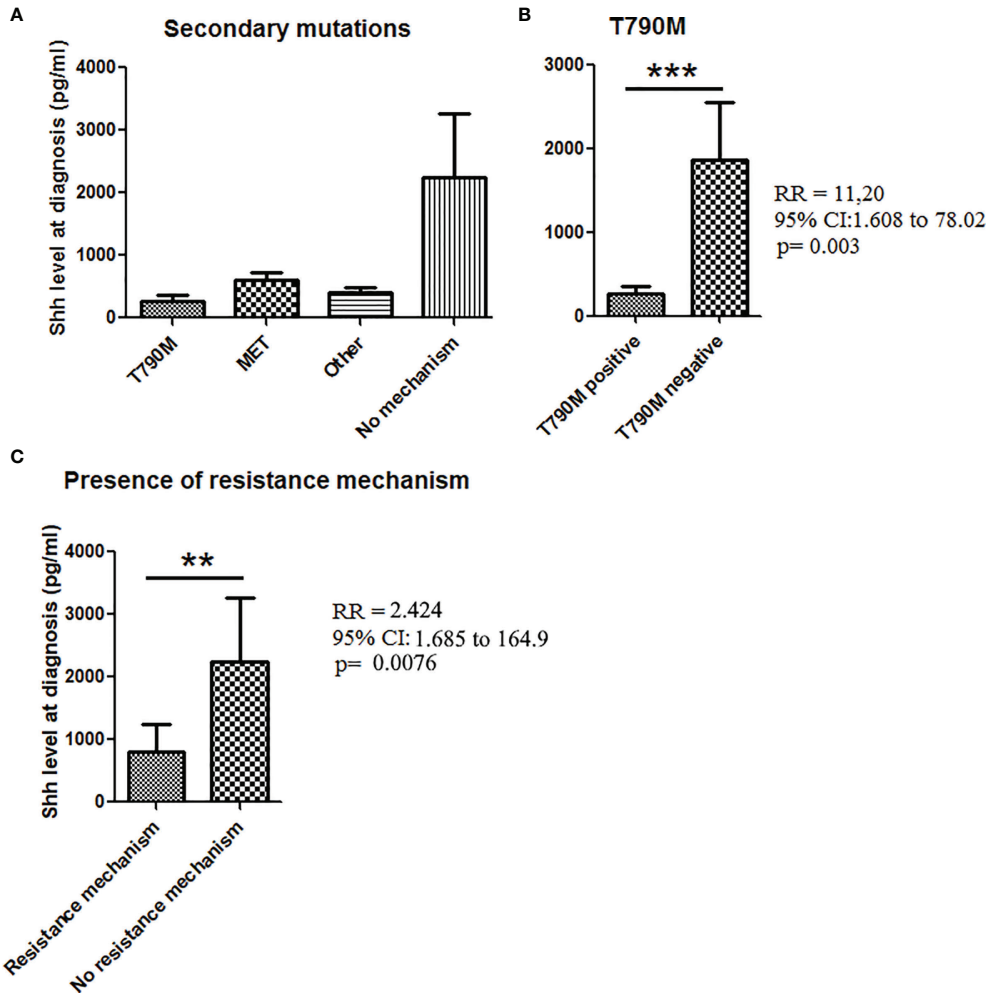

FIGURE 5 | Shh concentrations in plasma from patients at diagnosis according to the presence of secondary mutations. **(A)** Patient samples analyzed for Shh expression at baseline were classified according to the progression mechanism observed at the time of the progression. **(B)** Association between Shh concentrations and the presence of T790M mutation at the time to the progression. **(C)** Shh concentration in plasma from patients according to the presence of a resistance mechanism. A Mann–Whitney test was used to analyze the differences between means. $^{**}p < 0.01$; $^{***}p < 0.001$.

with shorter survival in patients treated with immune checkpoint inhibitors or platinum-based chemotherapy (20, 21). Gialmanis et al. also observed correlations of Hedgehog pathway activation (as assessed by IHC of Gli1 and Gli2) with histological type and unfavorable prognosis parameters in NSCLC (35). Overall, studies support a positive correlation between Hh signaling as assessed by expression of Gli1/2 and shorter OSs (21, 35–37). Bora-Singhal et al. have addressed the relation between Hedgehog signaling and the outcome of patients treated with EGFR-TKI. In their study, the authors used Gli1 expression as the surrogate of Hh activation. Consistently, they demonstrated that Hedgehog activation as assessed by the mean of Gli1 expression correlated with poor OS (36).

Our study showed that Shh was highly enriched in patients unresponsive to the treatment. In the study of Dong et al., the transcript of Gli1 was found to be positively correlated with Gefitinib IC50, when the drug was used to treat different NSCLC cell lines (10). Hh signaling is generally quite inactive in NSCLC cells responsive to EGFR-TKI, and fully operative in EGFR-TKI-resistant NSCLC (22). The role for the synergistic crosstalk between Hh and EGFR signaling in mediating EMT and drug resistance to EGFR inhibition has been established elsewhere (36–38). Hedgehog pathway, through EMT induction, leads to reduced sensitivity to EGFR-TKIs in NSCLC (39). The RAF/MEK/ERK signaling cascade upregulates Gli1 expression/activation to promote cancer cell, proliferation, survival, invasion, and drug resistance. However, even though Shh could be activated downstream EGFR signaling, the two pathways have overlapping roles, favoring EMT, metastasis, and drug resistance (40). Therefore, upon TKI-mediating EGFR inhibition, cancer cells rapidly upregulate Shh signaling to compensate for the absence of EGFR signaling (38).

Concordantly, we have observed an increase of Shh levels in patients after induction therapy. *In vitro* and *in vivo* studies revealed that upon a continuous treatment with EGFR-TKI, sensitive EGFR-mutated NSCLC cells become refractory to EGFR-TKI after a period where the Hh pathway is upregulated (38, 41). Consequently, it has been observed in preclinical studies that the pharmacological interference of Hh signaling keeps cells sensitive to TKI-EGFR treatment including gefitinib, afatinib, or osimertinib. Notably, the selective Smo inhibitors such as Sonidegib, GDC-0449, and LDE225 are capable both to prevent resistance to EGFR-TKI and to restore TKI sensitivity in refractory EGFR-mutated NSCLC cells (38, 39, 42). These provide the basis to test the association of EGFR-TKI with inhibitors of Hedgehog signaling, both to prevent drug resistance and to sensitize refractory EGFR-mutated NSCLC patients (43, 44).

Putting our observations in parallel with preclinical studies, we can suggest a model where insensitive patients have higher levels of Shh that make them refractory to therapy, while responsive patients present lower levels of Shh, which will start to increase upon drug treatment, until a peak of Shh that will correspond to EGFR-independent resistance mechanism and disease progression. Therefore, the increase in Shh levels along the treatment could correspond to the emergence of resistance. Further studies should be done to (i) find the Shh cutoff levels that could accurately define responder and non-responder patients, and (ii) determine the fold increase corresponding to disease progression.

Overall, we provided the evidence that increased levels of Shh could be related to the emergence of TKI resistance, providing the rationale to implement larger studies in EGFR-mutated NSCLC treated with TKIs, with the following aims: to validate plasma levels of Shh as a new criterion of patient stratification, and to use and to validate the efficacy of Hh inhibitors in clinical studies.

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 CPP IDF n°8 and CPP Ouest 6. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PTK: data collection and analyses, statistical analyses, writing of the manuscript, and revision of the manuscript. AS: data collection and analyses, statistical analyses, and revision of the manuscript. AC, CJ, J-FE, MP, and SO-C: data collection and analyses. PS: data collection, data analyses, and revision of the manuscript. EG-L: conception of the project, data analyses, data collection and analyses, statistical analyses, writing revision, and approval of the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.747692/full#supplementary-material

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"How Long Have I Got?" in Stage IV NSCLC Patients With at Least 3 Months Up to 10 Years Survival, Accuracy of Long-, Intermediate-, and Short-Term Survival Prediction Is Not Good Enough to Answer This Question

Huiru Guo[1], *Hegen Li*[1]*, *Lihua Zhu*[1], *Jiali Feng*[1], *Xiange Huang*[1] and *Jan P. A. Baak*[2,3]

[1] Department of Medical Oncology, Longhua University Hospital, Shanghai, China, [2] Department of Pathology, Stavanger University Hospital, Stavanger, Norway, [3] Medical Practice Dr. Med Jan Baak AS, Tananger, Norway

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Edited by:
Marcello Migliore,
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Duke University, United States

***Correspondence:**
Hegen Li
lydia8108@sina.com

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Background: Most lung cancer patients worldwide [stage IV nonsmall cell lung cancer (NSCLC)] have a poor survival: 25%–30% die <3 months. Yet, of those surviving >3 months, 10%–15% (70,000–105,000 new patients worldwide per year) survive (very) long. Surprisingly, little scientific attention has been paid to the question, which factors cause the good prognosis in these NSCLC stage IV long survivors. Therefore, "How long do I still have?" currently cannot be accurately answered. We evaluated in a large group of 737 stage IV NSCLC patients surviving 3.2–120.0 months, the accuracies of short- and long-term survival predictive values of baseline factors, radiotherapy (RT), platinum-based chemotherapy (PBT), and tyrosine kinase inhibitor targeted therapy (TKI-TT).

Methods: This is a noninterventional study of 998 consecutive first-onset stage IV NSCLC patients. A total of 737 (74%) survived 3.2–120.0 months, 47 refused RT, PBT, and TKI-TT. Single and multivariate survival analysis and receiver operating curve (ROC) analysis were used with dead of disease (DOD) or alive with disease (AWD) as endpoints.

Results: The median survival (16.1 months) of 47 patients who refused PBT, RT, and TKI-TT was significantly worse than those with RT, PBT, and/or TKI-TT (23.3 months, HR = 1.60, 95% CI = 1.06–2.42, p = 0.04). Of these latter 690 patients, 42% were females, 58% males, median age 63 years (range 27–85), 1-, 2-, 5-, and 10-year survival rates were 74%, 49%, 16%, and 5%. In total, 16% were alive with disease (AWD) at the last follow-up. Pathology subtype (adenocarcinoma vs. all others), performance score, TNM substage, the number of PBT cycles and TKI-TT had independent predictive value. However, with the multivariate combination of these features, identification results of short-term nonsurvivors and long-term survivors were poor.

Conclusions: In stage IV NSCLC patients with >3 months survival, baseline features, and systemic therapeutic modalities have strong survival predictive value but do not accurately identify short- and long-term survivors. The predictive value of other features and interventions discussed should be investigated in the worldwide very large group of stage IV NSCLC patients with >3 months survival.

Keywords: nonsmall cell lung cancer, stage IV, outcome prediction, long-term survival, baseline features, treatment factors

INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality, and the global overall 5-year survival rate of nonsmall cell lung cancer (NSCLC) is less than 20% (1). Similar rates are found in the People's Republic of China (2). With 2 million new lung cancers worldwide in 2020 (3), about 1.6 million are NSCLC, and of these, 60%–70% are in advanced stage IV at the time of diagnosis. Thus, the annual worldwide number of new NSCLC stage IV patients is close to 1 million.

Patients with metastatic (stages IIIB–IV) NSCLC have such a poor life expectancy that surgery in general is not recommended, leaving platinum-based therapy (PBT), radiotherapy (RT), tyrosine kinase inhibitor target therapy (TKI-TT), or immunotherapy as the treatment options. The response rate of the standard first-line chemotherapy of stages IIIB–IV lung cancers has improved significantly from 4–6 to 8–10 months; 1-year survival rate is ≥30% (4). Moreover, tyrosine kinase inhibitors (TKI) (5) can significantly prolong survival, especially in epidermal growth factor receptor (EGFR) mutation-positive NSCLCs. In Asian patients, EGFR-mutation status was 51% positive (6), but in Western countries, such as Norway, it is much lower (8%) (7).

Of the metastatic NSCLC patients, those with stage IV (8, 9, current patient material) has an especially poor prognosis as 25%–30% are dead of disease (DOD) <3 months after initial diagnosis despite RT, PBT, and/or TKI-TT. As far as we know, large, long-term studies in this stage IV NSCLC with at least 3 months survival are lacking. In the patients to be described in the current article, 10%–15% survived between 60 and 75 months. At 10 years follow-up, still 5% was alive with distant metastases. This latter percentage may seem low, but speaks much more, when it is considered that with worldwide 700,000 new NSCLC stage IV patients who annually survive >3 months, even 5% regards yearly 35,000 new stage IV alive-with-disease (AWD) patients with 10 years follow-up.

The decision for systemic therapy of stage IV NSCLC patients usually depends on performance score (PS) 0–1, adenocarcinoma, presence of EGFR mutation, and age 18–75.

However, surprisingly, truly little scientific attention has been paid to the factors causing the good prognosis in the worldwide 35,000–105,000 stage IV NSCLC long survivors. As scientific support currently is lacking, the question from individual stage IV NSCLC patients surviving at least 3 months: "How long do I still have?," currently cannot be accurately answered.

We evaluated in a large group of 737 stage IV NSCLC patients surviving 3.2–120.0 months whether baseline patient-, tumor-, and treatment factors can accurately predict long- and short-term survival.

Abbreviations: AWD, alive-with-disease; BRAF, B-raf proto-oncogene; BCL11A, *BCL11A* gene; CDK4, cyclin-dependent kinase 4 gene; CGP, cancer gene panel; CT, computer tomography; DNA, desoxyribose nucleic acid; DOD, dead-of-disease; EGFR, epidermal growth factor receptor; FOXA1, Forkhead box *protein* A1 gene; GP, gemcitabine 1,250 mg/m^2, was administered on days 1 and 8 and cisplatin75 mg/m^2 was administered on day 1 of a 4-week cycle; HR, hazard ratio; HG, high glucose; IGF2BP1, insulin-like growth factor 2 mRNA-binding protein 1; LUH, Longhua University Hospital; MET, receptor tyrosine kinase protein coding gene; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSS, microsatelite stable; NCC-GP150, a cancer gene panel called NCC-GP150; netrin-1 (NTN1), protein coding gene, could function as an oncogene; NF1, neurofibromin 1 gene; NP, vinorelbine 25 mg/m^2, was administered on days 1 and 8 and cisplatin 75 mg/m^2 was administered on day 1 of a 4-week cycle; NSCLC, nonsmall cell lung cancer; OS, overall survival; P, probability of no difference; PBT, platinum-based chemotherapy; PD, progressive disease; PD-1, programmed cell death protein 1; PD-L1, programmed cell death-ligand 1; PET, positron emission tomography; PFS, progression-free survival; PIK3CA, phosphatidylinositol 3-kinase gene; PS, *Eastern cooperative oncology group performance score*; RNA, ribonucleic acid; ROC, receiver operating curve; RT, radiotherapy; SI, smoking Index; TILs, tumor-infiltrating lymphocytes; TKI, tyrosine kinase inhibitors; TKI-TT, tyrosine kinase inhibitors targeted therapy; TMB, tumor mutational burden; TNM, eight edition of the TNM classification for lung cancer; TP, paclitaxel 135 mg/m^2 and cisplatin 75 mg/m^2 was administered on day 1. The cycle was repeated every 4 weeks; TP53, TP53 gene; WES, whole-exome sequencing; 95% CI, 95% confidence interval.

MATERIALS AND METHODS

Ethics and Patients

This noninterventional, retrospective observational study on patients with first-time onset stage IV NSCLC was approved by the Institutional Research Board of the Longhua University Hospital (LUH), Xuhui district, Shanghai, China before the study commenced. JB later got permission from the Research Director of the Stavanger University Hospital, Stavanger, Norway, to participate. The study was performed in accordance with the Declaration of Helsinki (2013) for experiments involving humans and in line with the recommendations for the conduct, reporting, editing, and publication of scholarly work in medical journals. The work complies with the principles laid down in the CIOMS International Ethical Guidelines for Biomedical Research Involving Human Subjects (2002). All patients have approved and signed an agreement before they received treatment, to use their data in noninterventional studies

without any changes in their treatment. These signed papers are saved in the patient record of each patient. As this is an observational noninterventional study, it did not add any risk for the patients.

All consecutive patients diagnosed at the LUH between January 1, 2009–December 31, 2018 were considered. There were originally 998 pathologically confirmed first onset stage IV NSCLC, of which 737 survived 3.2–120.0 months.

The data were retrieved from the LUH patients' records, by four well-trained, experienced medical oncologists. Each item in every patient record was carefully evaluated and registered. For quality control, in a random 10% of the records, the items were controlled by another independent medical oncologist and consensus was always obtained.

Dead of disease (DOD) or alive with disease (AWD) were used as endpoints. There were originally 998 pathologically confirmed first-onset stage IV NSCLC, 737 survived 3.2–120.0 months. Patients were treated with/without RT and/or PBT and/or TKI-TT.

Stage was defined according to the Eighth Edition of the TNM Classification for Lung Cancer as IVA or IVB (8).

The Eastern Cooperative Oncology Group PS was used, defined as follows: 0 = Fully active, able to carry on all pre-disease performance without restriction; 1 = Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work; 2 = Ambulatory and capable of all selfcare but unable to carry out any work activities; up and about more than 50% of waking hours; 3 = Capable of only limited selfcare; confined to bed or chair more than 50% of waking hours; 4 = Completely disabled; cannot carry on any selfcare; totally confined to bed or chair; 5 = Dead. As in previous studies, the PS was dichotomized as 0–1 vs. ≥2, as the latter patients have a much worse outcome expectation.

The smoking habit index was defined by the number of cigarettes smoked per day (as indicated by the patient) times the number of years smoked (e.g., with 20 cigarettes per day, for 25 years, results in a Smoking Index = SI is 500). As many patients were heavy smokers, we used ≤ 500 vs. > 500 as the thresholds to distinguish between "Nonsmoker plus Light-smokers" and "Heavy smokers".

Details of Chemotherapy and Pharmaceutical Intervention for Nausea and Vomiting, Radiotherapy, and Targeted Therapy

The patients were randomly treated with one of the following regimens (there were no differences in the outcome of lung cancer with different platinum-based regimens (4) (1): NP: vinorelbine 25 mg/m^2 was administered on days 1 and 8, and cisplatin 75 mg/m^2 was administered on day 1 of a 4-week cycle (2). TP: paclitaxel 135 mg/m^2 and cisplatin 75 mg/m^2 were administered on day 1. The cycle was repeated every 4 weeks (3). GP: gemcitabine 1,250 mg/m^2 was administered on days 1 and 8, and cisplatin 75 mg/m^2 was administered on day 1 of a 4-week cycle. The standard of care for initial treatment of advanced NSCLC has been four to six cycles of platinum-based chemotherapy followed by close observation. This approach was based on studies that suggested increased toxicity with no improved clinical benefit when the platinum doublet was continued until disease progression (9). Patients with grades 1–3 nausea or vomiting routinely received pharmaceutical antiemetic ondansetron (Zofran) treatment.

RT was given for palliative reasons. The dosages for body tumors were 50–55 Gray/25–30 times, and for brain tumors, total dosage was 30 Gray/10–15 times. For stage IV NSCLC, radical palliative thoracic RT with a median dose of 55 Gray was given, as this is safe and might be beneficial for primary lung lesions of metastatic NSCLC patients with controlled extrathoracic diseases (10).

The following first-line TKIs for treating advanced lung cancer with epidermal growth factor receptor (EGFR) (11–14) mutation (+) were used: gefitinib (Iressa®), erlotinib (Tarceva®), and icotinib (Conmana®). The latter is a highly selective, first-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) for use as first-line monotherapy in patients with NSCLC with somatic EGFR mutations. Currently, it is solely approved and marketed in China. When the first-line TKIs failed, any of the following TKIs was given: osimertinib (Tagrisso®), afatinib (Gilotrif®).

Statistical Methods

SPSS version 25 (IBM-SPSS, Armunk, New York, USA) and MedCalc version 20.014 (MedCalc Software Ltd, Ostend, Belgium) were used for statistical analyses. Descriptive statistics were assessed for all features. For survival analyses, continuous features were discretized according to medians, tertiles, quartiles, or the results of receiver operating curve (ROC) analysis. DOD and AWD at the last follow-up were used as endpoints. Clinical impressions suggested that patients ≥75 years more often have a worse survival; this was therefore especially analyzed. Single and multivariate survival (Cox model) and binary logistic regression analysis were used. To evaluate the influence of the duration of the follow-up, as a cofounder in the regression analysis, the quartiles of follow-up duration were used as a covariate.

RESULTS

Univariate Results

Of the total group of 737 stage IV NSCLC patients, 47 refused chemotherapy, radiotherapy, and TKI-TT. Their median survival was 16.1 months, significantly worse than those who received either chemotherapy, TKI-TT, or both (HR = 1.60, 95% CI = 1.06–2.42, p = 0.04). Their median age was 72 (range 41–85), i.e., higher than that of the other 690 patients (which were median 63 years, p = 0.0009). In 28%, their performance scores were >1 (compared with 17% in the other 690 patients, p = 0.07). On the other hand, TNM IVA and IVB were 30% and 70%, which was not different from the other 690 patients (p = 0.39). Thus, they were older, had a (nonsignificant) trend towards higher PS, but TNM stages were not different from the other 690

patients. We therefore assume, that not only their slightly higher age and PS but also fear for side effects of the treatments may have been the major factors for them to refuse treatment.

Of the other 690 patients, 288 were females (42%) and 402 males (58%). Median age was 63 years (range 27–85), median survival was 23.3 months (range 3.2–120.0), and 1-, 2-, 5-, and 10-year survival rates were 74%, 49%, 16%, and 5%; 112/690 = 16% of the patients were AWD at the last follow-up. Median survivals for the dead-DOD and AWD patients were 20.0 and 35.0 months. **Table 1** shows the distribution of the different features and their univariate prognostic value.

Of the patients receiving TKI-TT, 58 of 60 did not get chemotherapy. Note the good survival rate of this no-chemotherapy subgroup.

Several of the TKI-TT (both Iressa and Tarceva) patients received the treatment without EGFR-mutation analysis. Many of these had squamous or large cell pathology subtypes and had the same poor survival as non-TKI-TT patients. These patients should never have received TKI-TT. This explains the somewhat less favorable outcome in the TKI-TT patients than described before in patients with EGFR mutations (5).

The following features had univariable predictive AWD vs. DOD survival value. Age (<75 vs. > 74 years, $p = 0.006$), gender (female vs. male, $p = 0.0002$), smoking habit index (no-plus-light vs. heavy smokers, $p = 0.003$), performance score = PS (≤1 vs. ≥ 2, $p < 0.0001$), TNM substage (IVA vs. IVB, $p < 0.0001$), pathological type (adenocarcinomas vs. all other NSCLC subtypes, $p < 0.0001$), sum of TNM + PS + pathology ($p < 0.0001$), EGFR mutations (no vs. yes, $p = 0.04$), the number of platinum-based-chemotherapy (PBT) cycles ($p < 0.0001$), and TKI-TT ($p = 0.007$).

Multivariate Analysis Results

The shape of the survival graph of the 690 patients (**Figure 1**) is curved. This suggests that the group is heterogeneous, i.e., consists of subgroups with different survival rates.

We therefore studied by multivariate Cox regression analysis (both with Enter and Stepwise models), if short- and long-term survivors could be identified with the patient, tumor, and treatment characteristics. First, all features and then the univariably significant ones were included. These 2 different approaches selected the same features (**Table 2**).

The following features had independent prognostic value: pathology ($p = 0.003$), performance score ($p < 0.0001$), TNM substages ($p = 0.0002$), the number of chemotherapy cycles ($p < 0.0001$), and TKI-TT ($p = 0.04$) to predict survival (Chi-square = 92.9, $p < 0.0001$). None of the other features (age, gender, smoking habits index, radiotherapy) had additional value.

The same procedure was then repeated, but now for the 615 patients who had received the full 4–6 chemocycles (excluding all patients with 0, 2, and 3 chemocycles). The results were comparable. We concluded that the results are robust.

Moreover, including the quartiles of follow-up duration as a confounding covariate in the regression analysis showed that the duration of the follow-up did not influence these results.

ROC analysis (area under the curve 0.66, $p < 0.001$) showed that the optimal prediction threshold for the multivariate classifier with the abovementioned variables and coefficients was < −1.53 vs. ≥ −1.53 (**Figure 2**).

Using this threshold for the multivariate prognostic classifier resulted in sensitivity = 87%, specificity = 27%, overall correctly classified cases 73%, and positive and negative predictive values 80% and 38%. Different thresholds gave higher specificities and negative predictive values, but at the expense of sensitivities, overall correctly classified cases and positive predictive values.

Prediction of Short- and Long-Term Survival

A possible objection against the multivariate survival analysis above could be that the follow-up times in the DOD and AWD subgroups partly overlapped. To make possible differences between short-term nonsurvivors and long-term survivors more clear, binary logistic regression analysis was used to study if the features could distinguish between DOD patients in the first survival duration quartile (<11.7 months, $n = 166$), and those who were AWD at the last follow-up in the fourth quartile (≥39.0 months, $n = 51$). Different methods (Enter, Forward, Backward, Stepwise, with $p < 0.05$ to enter and $p > 0.1$ to remove) were used, with approximately the same results (Chi-square 94.3, $p < 0.0001$). Age < vs. ≥75, pathological type, and the number of PBT cycles had independent prognostic value. Once these were included, none of the other features were significant ($p > 0.10$). ROC analysis gave an area under the curve of 0.70 ($p < 0.001$). However, again the sensitivities, specificities, overall correctly classified cases, and positive and negative predictive values were disappointing. The same holds for all other features. The multivariate classifier gave the best results, as **Table 3** and **Figure 3** show.

At the start of the study of the long-term survival predictors of patients with late-stage NSCLC, the researchers equipped themselves with the knowledge from studies on stages IIIB–IV patients, that PS, TNM stage, and pathological types would accurately predict prognosis. However, in this stage IV with at least 3 months of survival, this was not the case. The prediction results of the baseline and therapeutic features to distinguish DOD nonsurvivors from long-term AWD survivors is too poor overall to be of clinical value and to answer in individual patients the question "How long do I have?".

DISCUSSION

The annual worldwide number of new NSCLC stage IV patients is close to 1 million. In total, 25%–30% die within 3 months, making stage IV NSCLC one of the deadliest cancers. However, little, if any scientific attention has been paid to the question, which factors among the worldwide 700,000 NSCLC stage IV patients, surviving longer than 3 months up to 10 years, cause the good prognosis of the 5%–15% (35,000–105,000) (very) long survivors. It is widely presumed, based on studies in the nearly 1.2 million stages IIIB–IV patients, that age, TNM substage, PS, histopathology, and TKI-TT

TABLE 1 | Univariate survival analysis in 690 stage IV NSCLC patients with at least 3 months survival after primary diagnosis, using AWD or DOD as endpoints.

Characteristic	Dead of disease/at risk	% Censored (alive with disease)	Median survival time (months)	Probability of no difference[a]	Hazard ratio[a]	95% confidence interval[a]
Baseline features						
Total	578/690	16%	23.3			
Age (years)						
<57	168/191	12%	27.0			
57–63	138/171	19%	23.0		1.00	0.81–1.26
64–69	142/181	22%	24.0		1.00	0.80–1.25
≥70	130/147	12%	21.0	0.59	1.14	0.91–1.45
<75	497/601	17%	25.0			
≥75	81/89	9%	17.8	0.006	1.46	1.11–1.91
Gender						
Females	233/288	19%	31.0			
Males	345/402	14%	21.0	0.0002	1.37	1.16–1.61
Smoking habit index						
No plus light	406/488	17%	26.0			
Heavy	172/202	15%	20.0	0.003	1.33	1.10–1.62
Performance score						
0–1	463/571	19%	26.0			
>1	115/119	3%	17.0	<0.0001	1.91	1.49–2.45
TNM						
IVA	197/248	21%	31.2			
IVB	381/442	14%	19.0	<0.0001	1.39	1.18–1.64
Pathology						
Adenocarcinomas	398/490	19%	27.4			
Squamous cell	77/88	13%	18.0		1.43	1.10–1.88
Other NSCLC subtypes	103/112	8%	17.1	<0.0001	1.59	1.24–2.04
Sum of TNM, performance score, and pathology subtypes						
Sum = 3 (TNM = 4A) + (PS = 0–1) + pathology = adenocarcinoma)	109/151	28%	39.0			
Sum = 4 (different other combinations of TNM, PS, and pathology)	286/342	16%	23.0		1.47	1.22–1.78
Sum = 5 (different other combinations of TNM, PS, and pathology)	159/172	8%	17.0		2.25	1.77–2.87
Sum = 6 (TNM = 4B) + (PS = 2–4) + pathology = no adenocarcinoma)	24/25	4%	10.0	<0.0001	3.28	1.79–6.03
EGFR mutations[b]						
No	0/152	14%	23.0			
Mut-19	16/26	38%	28.0			
Mut-20	1/2	50%	14.0		0.72	0.47–1.10
Mut-21	9/20	55%	44.1		0.47	0.30–0.73
Others	14/18	22%	23.3	0.04	0.98	0.57–1.66
Therapeutic modalities						
Radiotherapy						
No	378/462	18%	22.5			
Yes	200/228	12%	25.3	0.69	0.97	0.81–1.15
Number of chemocycles						
0	45/60	25%	28.0			
2	1/1	0%	3.2	–	–	–
3	14/14	0%	4.0	–	–	–
4–6	518/615	16%	24.0	<0.0001	–	–
Targeted therapy						
No	422/494	15%	22.0			
Tarceva	36/40	10%	17.7		0.97	0.69–1.38
Iressa	99/127	22%	34.0		0.74	0.61–0.91
Conmana	16/24	33%	50.0		0.57	0.39–0.85
Second-line TKIs[c]	5/5	0%	9.0	0.007	1.81	0.53–6.20

NSCLC, nonsmall cell lung cancer; PS, Eastern cooperative oncology group performance score; EGFR, epidermal growth factor receptor; Mut, mutation; TKI, tyrosine kinase inhibitor.

[a]*Minus sign (–) means cannot be calculated, divided by zero error.*

TNM, PS, and pathology were coded as follows: TNM 4A = 1, TNM4B = 2, PS = 0–1 = 1, PS >1 = 2; pathology = adenocarcinoma = 1; all other pathology subtypes = 2. The total sum could thus be 3 [(TNM = 4A=1) + (PS = 0–1 = 1) + pathology = adenocarcinoma = 1)], 6 [(TNM = 4B =2) + (PS >1 = 2) + pathology = squamous or other cell types = 2)], 4 (one of the three features = 2, the other two = 1) or 5 (2 of the 3 features were 2, the other one was 1).

[b]*Based on determinations in 218 of the 690 patients.*

[c]*Osimertinib, afatinib.*

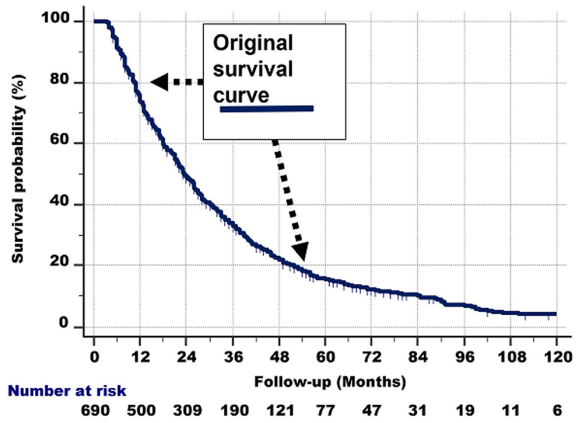

FIGURE 1 | The survival curve of the 690 patients. As it is curved (i.e., not linear), it is probable that the total group consists of subgroups with different survival rates.

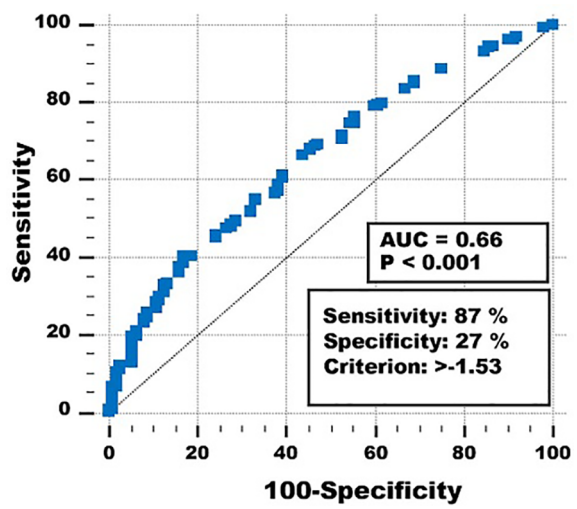

FIGURE 2 | Receiver operating curve of the multivariate classifier to predict short- and long-term survival.

are significant prognostic factors. However, in the absence of scientific data for the large group of stage IV NSCLC patients surviving at least 3 months, the question from many individuals: "How long do I still have?" currently cannot be reliably answered as scientific data are lacking. We therefore evaluated a large group of 998 stage IV NSCLC patients, of which 261 died within 3 months. In the 737 surviving 3.2–120.0 months, it was evaluated which patients, tumor, and therapeutic factors determine long- and short-term survival.

The first interesting result was the much better than expected 1-, 2-, and 5-year survival rates: 74%, 49%, and 16%. Even at 10 years follow-up, 5% of patients were still AWD. None of these patients surviving 10 years was disease free, which is not surprising as all had stage IV disease at the start of the study. However, the metastases were dormant, and one may expect even longer survival than 10 years (which was the maximum follow-up in the current study). Future studies in the surviving patients therefore would be interesting. Secondly, the 47 patients who refused any form of platinum-based chemotherapy (PBT) or TKI-TT had a worse survival than those who received either of the two or both treatments. They were older (although with considerable overlap), did have a nonsignificant trend towards a worse PS ($p = 0.07$) but substages were not different. The fear for serious side effects of PBT and TKI-TT may have been the major argument of these patients to refuse PBT and TKI-TT. In view of the better survival of PBT and/or TKI-TT patients, it may be considered to explain future stage IV NSCLC patients refusing PBT and TKI-TT, that their prognosis is probably worse if not taking these treatments.

Radiotherapy did not improve prognosis, which was not surprising as it was given in a palliative setting. The number of PBT cycles was strongly prognostic. Patients with 4–6 cycles had a much better survival than those with 1–3 cycles. One could ask why. Patients tolerating standard 4–6 cycles may be in a better physical condition, such as younger or with a (lower performance score) and TNM stage. However, TNM stage and age did not differ ($p > 0.20$), but PS was slightly more often higher ($p = 0.04$) in those with 1–3 cycles. Sixty patients who received

TABLE 2 | Multivariate survival analysis results in 690 stage IV nonsmall cell lung cancer patients with at least 3 months survival after primary diagnosis, using dead of disease and alive with disease at the last follow-up as endpoints (Chi-square 92.9, $P < 0.0001$).

Covariate	Beta	Standard error	Wald	Probability of no difference	Exp Beta	95% confidence interval of Exp Beta
Age: *1 = <75, 2 = ≥75*	0.18	0.123	2.07	0.15	1.19	0.95–1.52
Gender: *1 = female, 2 = male*	0.18	0.097	3.35	0.07	1.19	0.98–1.44
Smoking habit index: *1 = no-plus-light, 2 = heavy*	0.07	0.104	0.50	0.48	1.08	0.88–1.32
Pathology: *1 = adenocarcinoma, 2 = All others*	0.29	0.098	8.79	***0.003***	1.34	1.10–1.62
Performance score: *1 = <2, 2 = ≥2*	0.48	0.107	20.45	***<0.0001***	1.62	1.31–2.00
TNM: *1 = stage IVA, 2 = stage IVB*	0.34	0.091	14.00	***0.0002***	1.41	1.18–1.68
Radiotherapy 0 = No, 1 = Yes	–0.11	0.090	1.48	0.22	0.89	0.75–1.07
Chemotherapy cycles: *1 = <4, 2 = 4–6*	–0.92	0.152	36.60	***<0.0001***	0.40	0.30–0.54
TKI-targeted therapy *0 = No, 1 = Yes*	–0.10	0.049	4.27	***0.04***	0.90	0.82-0.99

TKI, tyrosine kinase inhibitor. Bold values is: "P < 0.05".

TABLE 3 | Percentages of overall correctly classified cases, sensitivities, specificities, positive, and negative predictive values of features studied to predict alive with disease vs. dead of disease.

	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Overall correctly classified
Multivariate classifier	87%	27%	80%	38%	73%
TNM	66%	46%	86%	21%	37%
Gender	60%	49%	86%	19%	42%
Sum of TNM, performance score, and pathology subtypes 3 + 4 vs. 5 + 6	32%	88%	93%	20%	59%
Pathology adenocarcinoma vs. all others	31%	82%	90%	19%	61%
Smoking habits index	30%	73%	85%	17%	63%
Performance score	20%	96%	97%	19%	68%
Age <75 vs. ≥75	7%	86%	9%	83%	73%
EGFR mutation (on 218 of 690 cases only)	7%	77%	61%	14%	82%

%	Color
<10%	
10%–25	
30%–49%	
50%–79%	
80%–100%	

EGFR, epidermal growth factor receptor.

TKI-TT but not PBT, had a better prognosis than average (28 rather than 23 months median survival).

With univariate survival analysis, many features were associated with outcome: age, gender, no- or light vs. heavy smoking, PS, TNM substages, pathological type adenocarcinomas vs. others, sum of TNM-PS-pathology, EGFR mutations, the number of PBT cycles and TKI-TT. With multivariate analysis, only baseline features pathological subtype, PS, and TNM IVA/B substages had strong independent survival predictive value, and of the therapy modalities, the number of PBT cycles, and TKI-TT.

We thus could confirm in the stage IV NSCLC patients with more than 3 months survival, the predictive survival significance of the commonly used therapeutic decision criteria. On the other hand, age >75 did NOT have independent multivariate prognostic value. The fact that presence of EGFR mutation also multivariably was not significant can be due to the overshadowing prognostic significance of the pathology adenocarcinoma subtype. Survival analysis showed no survival differences between different locations of the EGFR mutations. However, mutations in 21 were associated with a (just significant) better survival (**Table 1**). Due to the small

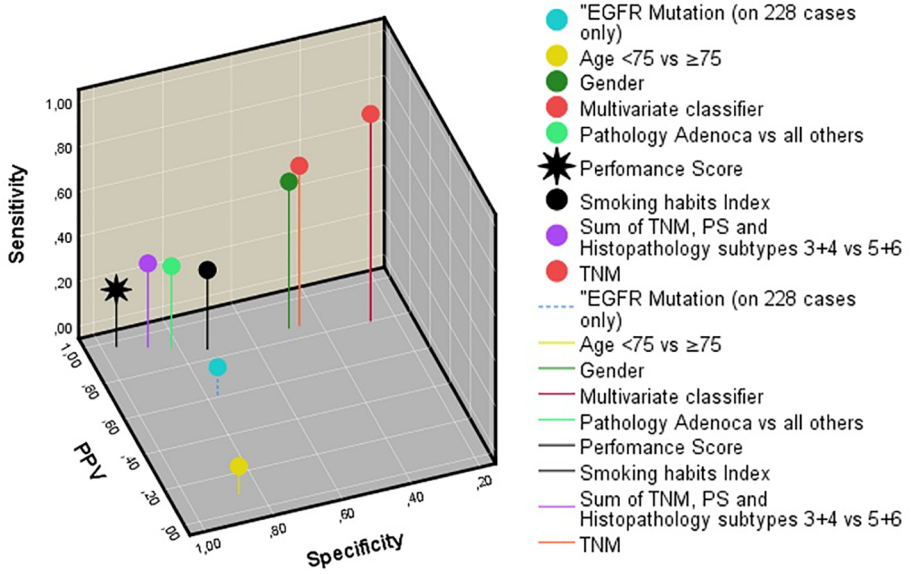

FIGURE 3 | Three-dimensional representation of the sensitivity, specificity, and positive predictive value of all features studied. Note that the multivariate classifier gives the best results. EGFR, epidermal growth factor receptor. PS, Eastern cooperative oncology group performance score.

numbers, we are reluctant to draw strong conclusions. Future large studies are required to confirm this interesting finding.

The survival results of TKI-TT were somewhat worse than expected of previous stages IIIB–IV NSCLC (5, 15, 16). Recently, third-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR TKI), osimertinib, demonstrated superior PFS compared with first-generation EGFR TKI in the first-line setting (18.9 months median vs.10.2 months in the EGFR TKI comparator (gefitinib/erlotinib)) (17). Our own 2011 study on stage IV NSCLC also gave stronger prognostic effect for TKI-treated patients, but follow-up time was maximally 30 months (18). We presume that the less favorable results of TKI-TT are due to the fact that the current stage IV NSCLC study is on both adenocarcinomas, squamous, and other pathological cell types, whereas the earlier study only included stage IV adenocarcinomas. Moreover, the follow-up of the current study is much longer. Interestingly, a large nationwide study from Sweden on stages IIIB–IV NSCLC patients showed a significantly better survival for patients with short follow-up (median overall survival (OS): 18.6 months in years 2014–2015) compared with earlier year of diagnosis and hence longer follow-up (15.3 months in years 2010–2011, $p < 0.05$). Note, that the follow-up in the 2014–2015 group in the Swedish study was maximally 36 months (19).

Several of the TKI-TT patients received the treatment without EGFR-mutation analysis, as was usual in the early years of TKI-TT. In retrospect, many of these had squamous or large cell pathology subtypes and had the same poor survival as non-TKI-TT patients. These patients should never have received TKI-TT. When these were left out, the predictive value of TKI-TT strongly improved. Another explanation for the less good than expected survival rates of TKI-TT patients could be that the patients in the current study have very long follow-up, much longer than in all previously published studies. Most patients with advanced NSCLC with EGFR activating mutations will develop resistance after 6–9 months of treatment with first-generation reversible TKIs such as erlotinib, gefitinib, (5, 16, 20). Moreover, earlier TKI studies on adenocarcinomas evaluated all stages IIIB–IV, whereas the current patients all had stage IV NSCLC with >3 months follow-up. This may have been associated with a larger tumor burden than in stages IIIB–IV patients, have led to increased resistance and consequently less good survival.

Despite the strongly significant predictive value of several of the baseline and treatment modalities, none of the features (neither alone nor in combination) can accurately identify subgroups with a short survival (<11.7 months), and relatively long survival (>38 months). The remaining question therefore is, which other candidate features, or interventions could perhaps help to identify and modify short- and long-term survivors among stage IV NSCLC long survivors with >3 months survival.

Attempts to answer this question should keep in mind, that all stage IV NSCLC patients have metastases at the time of diagnosis. Most stage IV NSCLC patients die from metastases when the total growing load becomes so large that it is not compatible with life. In such cases, there are many hallmarks of cancer which can play a role, but 5 main possible features could be especially important to influence the outcome and survival duration:

1. The total number of viable metastatic cells reaches a lethal threshold;
2. The condition of the patient becomes too poor;
3. The response of the body on the tumor cells diminishes under a critical level;
4. The geno- and phenotype and especially proliferation speed of the tumor cells dramatically increase;
5. Interventions influencing these 4 features become insufficient to support life.

Much less-frequent causes of death are life-threatening locations of in itself nonlethal masses, or sudden development of uncontrollable effusions from massive metastases. Unfortunately, we did not have details of the metastatic status of the patients (M1a, M1b, M1c) or localization sites and eventual effusions as death causes. It would be most interesting to study these in a future study.

Very recently, an excellent series of articles was published on the topic emerging biomarkers for NSCLC (11). None of these articles considered NSCLC stage IV only with >3 months survival. Therefore, we will discuss below potentially important biomarkers and possible interventions for this special group.

Important molecular biological features were already implicitly included in the current study, by the choice of TKI-TT type. Yet, others could be important as well. Microsatellite instability (MSI) is a well-known predictive marker for cancer immunotherapy. MSI-high (MSI-H) colorectal cancer is known to be associated with increased tumor-infiltrating lymphocytes (TILs), elevated host systemic immune response, and a favorable prognosis (12). The immune checkpoint molecules CD274, LAG3, and IDO1 expressions in tumor-infiltrating immune cells showed a better prognosis for patients with MSI-H colon cancer. MSI-H causes a build-up of somatic mutations in tumor cells and leads to a spectrum of molecular and biological changes including high tumor mutational burden, increased expression of neoantigens, and abundant tumor-infiltrating lymphocytes (13). Data about the prevalence of MSI among NSCLC are conflicting, and clinical relevance of MSI in TKI is largely unknown. In a series of 480 pulmonary adenocarcinomas, those with a high amount of MSI-H had a higher proliferative activity (39%) than microsatellite stable (MSS) neoplasms (28%) (14). The abundant TILs in MSI-H cancers are especially interesting, as independent studies have shown that TILs play essential roles in the development and progression of different cancer types (21, 22), also in NSCLC (23–25). However, the occurrence of MSI-H in stage IV NSCLC could be very low (14).

Widespread intratumor heterogeneity for both somatic copy-number alterations and mutations has been found in NSCLC. Intratumor heterogeneity mediated through chromosome instability was associated with an increased risk of recurrence or death, a finding that supports the potential value of chromosome instability as a prognostic predictor (26). Driver mutations in EGFR, the MET proto-oncogene, receptor tyrosine kinase protein coding gene (MET), B-raf proto-oncogene (BRAF), and TP53 genes were almost always clonal. Heterogeneous driver alterations later in evolution were found in more than 75% of the tumors and were common in phosphatidylinositol 3-kinase (PIK3CA) and neurofibromin 1 (NF1) genes and in genes that are involved in

chromatin modification and DNA damage response and repair. Genome doubling and ongoing dynamic chromosomal instability were associated with intratumor heterogeneity and resulted in parallel evolution of driver somatic copy-number alterations, including amplifications in the cyclin-dependent kinase 4 gene (CDK4), Forkhead box protein A1 gene (FOXA1), and BCL11A genes. Elevated copy-number heterogeneity was associated with an increased risk of recurrence or death (hazard ratio, 4.9, $p = 4.4 \times 10^{-4}$), which remained significant in multivariate analysis. In 224 patients with EGFR-mutant lung adenocarcinoma treated with EGFR-TKIs, the tumor burden, expressed as the number of metastatic sites at EGFR-TKI treatment, and rapid tumor progression at progressive disease (PD) were predictive of inferior survival in patients with lung adenocarcinoma with activating EGFR mutations (27). Tumor mutational burden (TMB) can also be measured by whole-exome sequencing (WES) or a cancer gene panel (CGP), and these are associated with immunotherapy responses. The recently established CGP named NCC-GP150 with an optimized gene panel size and algorithm is feasible for TMB estimation. This may serve as a potential biomarker of clinical benefit in patients with NSCLC treated with antiprogrammed cell death protein 1 (PD-1) and antiprogrammed cell death-ligand 1 (PD-L1) agents (28). It is unknown if TMB as measured by WES or a CGP, is associated with TKI treatment response. It is also not known if TMB in stage IV NSCLC, with at least 3 months survival, has stronger prognostic value than TNM IVB stage alone.

The large-scale genetic profiling of tumors can identify potentially actionable molecular variants for which approved anticancer drugs are available. However, when patients with such variants are treated with drugs outside of their approved label, successes and failures of targeted therapy are not systematically collected or shared. A highly interesting new approach to test larger gene panels, the drug rediscovery protocol has recently been suggested. This is an adaptive, precision-oncology trial that aims to identify signals of activity in cohorts of patients, with defined tumor types and molecular variants, who are being treated with anticancer drugs outside of their approved label (29). This certainly could be of value for stage IV NSCLC patients with at least 3 months follow-up.

In a very recent study, loss of IL-34 expression is associated with poor prognosis and negative regulation of the immune system of patients with pulmonary adenocarcinoma (30). However, only 18 patients were stage IV, and in these, IL-34 loss and nonloss occurred equally (7/18 and 11/18 patients). Yet, it would be interesting to study IL-34 loss in a much larger group of stage IV NSCLCs with at least 3 months survival.

It is important to consider that all patients in the NSCLC group studied had metastases at the time of diagnosis. The survival differences of the hypothetical prognostic subgroups in **Figure 1** could therefore be explained by differences in proliferation. Proliferation should preferably be measured both in the primary tumor and its metastases. A noninvasive method would be preferable. Recently, a computer tomography (CT) signature consisting of 12 CT features in stage IV EGFR-mutant NSCLC patients with EGFR-TKI therapy, demonstrated good accuracy for discriminating patients with rapid and slow progression to EGFR-TKI therapy (31). Decision curve analysis revealed that the proposed model significantly improved the clinical benefit compared with the clinicopathologic-based characteristics model ($p < 0.0001$). Although the CT signature was developed in one group of patients, and validated in an independent one, multicenter testing of the predictive value and reproducibility of the CT signature remains mandatory.

Can positron emission tomography (PET) scanning be used to measure proliferation in lung cancer? Fluorodeoxyglucose-F18 (18F-FDG) is an indicator of tumor activity *via* glucose metabolism and the most commonly and widely used PET imaging radiotracer. A recent study reported on a new PET tracer 18F-MPG (*N*-(3-chloro-4-fluorophenyl)-7-(2-(2-(2-(2-^{18}F-fluoroethoxy) ethoxy) ethoxy) ethoxy)-6-methoxyquinazolin-4-amine), with high specificity to activating EGFR mutant kinase showed significant correlation between tracer uptake and the EGFR mutation status in both preclinical animal models and in patients with NSCLC. The study aimed to identify patients that are sensitive to EGFR-TKIs and to monitor the efficiency of EGFR-TKI therapy. 18F-MPG uptake positively correlated with median progression-free survival. These results are still preliminary, but when confirmed could become valuable (32).

The relationship between proliferation of cancer cells and glucose loading is historically well known since the pioneering work of Otto Warburg. In 1931, he received the Nobel Prize for his work. Today, the correlation between cancer growth and glucose is used worldwide in scanning. *In vitro* studies of NSCLC cells treated with high glucose dosages showed that the RNA-binding protein insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) was highly expressed in high-glucose-treated NSCLC cells. Knockdown of IGF2BP1 inhibited cancer cell proliferation, migration, and invasion, and also induced cell cycle arrest and apoptosis. Also, IGF2BP1 silencing decreased the netrin-1 level in high-glucose (HG)-treated NSCLC cells. Reintroduction of netrin-1 expression rescued IGF2BP1 deficiency-induced cell proliferation reduction, migration suppression, cell cycle arrest, and apoptosis. These findings suggest that IGF2BP1 silencing inhibits the occurrence of tumor events through downregulating netrin-1 expression, indicating that the IGF2BP1/netrin-1 axis exerts an oncogenic role in high-glucose-treated NSCLC cells (33). A recent prospective long-term breast cancer study found that perioperative high intake of glucose was not only associated with high insulin blood levels but also with an extremely poor prognosis (34). This finding could be further correlated with significant metabolomics changes in the blood shortly after the operation (35). A prospective glucose intervention study, or intervention with medicines which can reduce insulin blood levels in stage IV NSCLC patients, therefore would be interesting.

CONCLUSIONS

With 2 million new lung cancers worldwide annually in 2018 (1–3), about 1.6 million are NSCLC and of these 60%–70% (1

million) are in advanced stage IV at the time of diagnosis; 25%–30% of these die within 3 months of diagnosis. The >70% group or worldwide 700,000 stage IV patients per year who survive 3–120 months is very seriously understudied. By extrapolation from all stages IIIB and IV NSCLCs together, it is regarded as having an extremely poor prognosis, but 2- and 5-year survival rates are nearly 50% and 20%. Even at 10 years, still nearly 5% is alive with disease. This percentage may seem low, but speaks much more, when it is considered that with worldwide 5%, regards yearly 35,000 new stage IV AWD patients at 10 years follow-up.

In this study, we have undertaken detailed statistical analysis and quantitative modelling of the survival of a very large group of stage IV NSCLC patients with 3.2–120 months follow-up. The patients seem to comprise different subgroups with widely different survival rates. Multivariable analysis shows that performance score, TNM substage, pathological type, the number of chemocycles, and targeted therapy each have strong independent survival predictive value. However, the multivariate classifier, comprising some or all of these features, could not accurately predict short- and long-term survivors.

We conclude that the question from stage IV NSCLC patients with >3 months survival "Doctor, how long do I have?" cannot yet be answered reliably. To this end, the predictive value of other features and interventions described in detail in the *Discussion*, should be investigated.

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

This noninterventional, retrospective observational study on patients with first-time onset stage IV NSCLC was approved by the Institutional Research Board of the Longhua University Hospital (LUH), Xuhui district, Shanghai, China before the study commenced. JB later got permission from the Research Director of the Stavanger University Hospital, Stavanger, Norway, to participate. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed extensively to the work presented in this paper. HG: acquisition of data, analysis and analysis support, interpretation of data, drafting the article or revising it critically for important intellectual content, and final approval. HL: conception and design of the study, acquisition of data, revising the manuscript critically for important intellectual content, and final approval. LZ: acquisition of data, analysis and analysis support, and interpretation of data. JF: acquisition of data, analysis and analysis support, and interpretation of data. XH: acquisition of data, analysis and analysis support, and interpretation of data. JB: advice for analysis, interpretation of results, drafting the article and revising it critically for important intellectual content, and final approval. All authors contributed to the article and approved the submitted version.

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The Impact of Comprehensive Genomic Profiling (CGP) on the Decision-Making Process in the Treatment of ALK-Rearranged Advanced Non-Small Cell Lung Cancer (aNSCLC) After Failure of 2nd/3rd-Generation ALK Tyrosine Kinase Inhibitors (TKIs)

Ari Raphael[1,2†], Amir Onn[2,3†], Liran Holtzman[2], Julia Dudnik[4], Damien Urban[3], Waleed Kian[5], Aharon Y. Cohen[4], Mor Moskovitz[6], Alona Zer[6], Jair Bar[2,3], Natalie Maimon Rabinovich[7], Shirly Grynberg[3], Cecilie Oedegaard[3], Abed Agbarya[8], Nir Peled[5,9], Tzippy Shochat[10] and Elizabeth Dudnik[9,11,12] on behalf of Israel Lung Cancer Group on behalf of Israel Lung Cancer Group*

[1] Department of Oncology, Tel-Aviv Sourasky Medical Center, Tel-Aviv, Israel, [2] Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel, [3] Thoracic Oncology Service, Institute of Oncology, Sheba Medical Center, Ramat Gan, Israel, [4] Thoracic Oncology Service, Cancer Institute, Soroka University Medical Center, Beer-Sheva, Israel, [5] Department of Oncology, Shaare Zedek Medical Center, Jerusalem, Israel, [6] Thoracic Cancer Service, Rambam Health Care Campus, Haifa, Israel, [7] Thoracic Cancer Service, Meir Medical Center, Kfar Sava, Israel, [8] Department of Oncology, Bnai Zion Medical Center, Haifa, Israel, [9] Faculty of Health Sciences, Ben Gurion University of Negev, Beer-Sheva, Israel, [10] Statistical Consulting Unit, Rabin Medical Center, Petah Tikva, Israel, [11] Thoracic Oncology Service, Assuta Medical Centers, Tel-Aviv, Israel, [12] Thoracic Oncology Service, Rabin Medical Center, Petah Tikva, Israel

Background: The use of CGP in guiding treatment decisions in aNSCLC with acquired resistance to ALK TKIs is questionable.

Methods: We prospectively assessed the impact of CGP on the decision-making process in ALK-rearranged aNSCLC patients following progression on 2nd/3rd-generation ALK TKIs. Physician's choice of the most recommended next-line systemic treatment (NLST) was captured before and after receival of CGP results; the percentage of cases in which the NLST recommendation has changed was assessed along with the CGP turnaround time (TAT). Patients were divided into groups: patients in whom the NLST was initiated after (group 1) and before (group 2) receival of the CGP results. Time-to-treatment discontinuation (TTD) and overall survival (OS) with NLST were compared between the groups.

Results: In 20 eligible patients (median [m]age 63 years [range, 40-89], females 75%, adenocarcinoma 100%, failure of alectinib 90%, FoundationOne Liquid CDx 80%), CGP has altered NLST recommendation in 30% of cases. CGP findings were as follows: ALK

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***Correspondence:**
Elizabeth Dudnik
elizabeth.dudnik1603@gmail.com

[†]These authors have contributed equally to this work

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mutations 30% (I1171X 10%, G1202R, L1196M, G1269A, G1202R+I1171N+E1210K 5% each), CDKN2A/B mutation/loss 10%, c-met amplification 5%. CGP mTAT was 2.9 weeks [IQR, 2.4-4.4]. mTTD was 11.3 months (95% CI, 2.1-not reached [NR]) and 5.4 months (95% CI, 2.0-NR) in groups 1 and 2, respectively (p-0.34). mOS was 13.2 months (95% CI, 2.9-NR) and 13.0 months (95% CI, 6.0-NR) in groups 1 and 2, respectively (p-0.86).

Conclusion: CGP has a significant impact on the decision-making process in ALK-rearranged aNSCLC following progression on 2nd/3rd-generation ALK TKIs.

Keywords: comprehensive genomic profiling, next-generation sequencing, ALK, failure of ALK TKI, acquired resistance, decision impact

BACKGROUND

Approximately 3-5% of tumors in patients with advanced non-small cell lung cancer (aNSCLC) harbor rearrangements of the anaplastic kinase lymphoma gene (ALK) (1, 2). This is a unique aNSCLC subpopulation that mostly consists of young individuals, with no or limited history of smoking, and an adenocarcinoma histology. Although ALK fusion is a rare phenomenon, it shouldn't be neglected considering the high prevalence of lung cancer overall, and the availability of several effective targeted treatment options (3–5).

The presence of ALK rearrangement results in tumor susceptibility to ALK tyrosine kinase inhibitors (ALK TKIs) (6). Crizotinib, a TKI of ALK, tyrosine-protein kinase Met (c-met), and ROS proto-oncogene 1 (ROS1) kinases (7), was the first ALK inhibitor to replace the standard chemotherapy in the 1st-line treatment of aNSCLC harboring an ALK fusion, providing the significant advantage of this therapy in terms of the progression-free survival - according to the results of the PROFILE 1014 trial (8). Since then, newer 2nd- and 3rd-generation ALK TKIs (e.g., alectinib, ceritinib, brigatinib, ensartinib and lorlatinib) were implemented into the management of ALK- rearranged aNSCLC - first in the post-progression setting (9–11), and later on - in the 1st- line setting - that based on the results of the ALEX trial (12), the ALTA-1L trial (13), the ASCEND-4 trial (14), and the CROWN trial (15).

The questions of ALK TKIs sequencing and optimal treatment strategy following the disease progression on specific ALK TKIs remain open, since these have never been evaluated in a randomized controlled clinical trial (16). Treatment decisions, however, can be guided by the acquired resistance mechanisms responsible for the disease progression during systemic treatment. The mechanisms of acquired resistance to ALK TKIs primarily include development of secondary resistant mutations in the ALK kinase domain occurring in 25-66% of patients (17–21). Of those, G1202R/del mutations predominate (42-53% of cases), while other ALK mutation types responsible for the development of secondary resistance to ALK TKIs are: L1196M, F1174X, G1269A, L1196M, and I1171X (18, 21). Moreover, sequential treatment with increasingly potent ALK TKIs may promote acquisition of treatment-refractory compound ALK mutations (21, 22). Off-target mechanisms of resistance to ALK TKIs involve up-regulation of bypass signaling pathways, such as epidermal growth factor receptor (EGFR), c-met, KIT proto-oncogene (KIT), insulin-like growth factor 1 receptor (IGF-1R), proto-oncogene SRC (SRC), MEK/ERK and others (17, 18, 23, 24). SCLC transformation has been described as a resistance mechanism to ALK TKIs as well (25, 26).

Since ALK resistance mutations appear to be the predominant mechanism of resistance to ALK TKIs, there is a clear rationale for its targeting. Moreover, it has been demonstrated that the presence of an ALK resistant mutation following the progression on 1st- and 2nd-generation ALK TKIs in ALK-rearranged aNSCLC is associated with better lorlatinib efficacy (19). However, different 2nd- and 3rd-generation ALK TKIs appear to have different *in vitro* activity against specific ALK resistant mutations, which, therefore, represents the rationale for identifying the underlying ALK resistant mutation subtype before making the decision regarding the next line of systemic treatment.

In our study, we prospectively assessed the impact of comprehensive genomic profiling (CGP) on the decision-making process in patients with ALK-rearranged aNSCLC following progression on 2nd- and 3rd-generation ALK TKIs.

Abbreviations: Adenoca, adenocarcinoma; ALK, anaplastic kinase lymphoma; (a) NSCLC, advanced non-small cell lung cancer; CDK4/6, cyclin-dependent kinase 4/6; CDKN2A/B, cyclin dependent kinase inhibitor 2A/B; CGP, comprehensive genomic profiling; CI, confidence interval; c-met, tyrosine-protein kinase Met; ECOG PS - Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; gen., generation; IASLC, International Association for the Study of Lung Cancer; IGF-1R, insulin-like growth factor 1 receptor; IQR, Interquartile range; KIT, KIT proto-oncogene; (m)OS, (median) overall survival; (m)TTD, (median) time-to-treatment discontinuation; NA, not available/not applicable; NGS, next-generation sequencing, NLST, next-line systemic treatment; NR, not reached; ROS1, ROS proto-oncogene 1; SRC, proto-oncogene SRC; TAT, turnaround time; TKI, tyrosine kinase inhibitor.

METHODS

Patient Selection, Study Design and Assessments

ALK-rearranged aNSCLC patients following failure of a 2nd/3rd-generation ALK TKI, regardless of prior crizotinib or platinum-based chemotherapy, treated in one of the participating Israeli

oncological centres were selected for this prospective multicentre non-interventional clinical study. CGP [either in the form of FoundationOne CDx or FoundationOne Liquid CDx using algorithm as previously described in detail (27)] was performed, and the results were captured. A questionnaire was filled by the treating oncologist twice: before and after receival of the CGP results. The questionnaire (**Supplementary Document S1**) included the de-identified clinical patient data, the de-identified next-generation sequencing (NGS) results, and the physician's choice of the most recommended next-line systemic treatment (NLST) captured before and after receival of CGP results. The percentage of cases in which the treatment recommendation has changed upon the receival of CGP results was assessed - reflecting the impact of the molecular testing on the decision-making process (**Figure 1**). We hypothesized that change in the treatment recommendation will occur in at least 30% of cases (the minimal clinically meaningful rate according to our perception, the cut-off was chosen arbitrarily).

We prospectively gathered an information regarding the CGP turnaround time (TAT). The number of patients with adverse outcomes while waiting for the NGS results was collected as well. Additional demographic and clinical patient data were retrospectively retrieved from the patient medical records at each of the participating Israeli oncological centers.

The decision regarding the NLST type and initiation was done by the treating oncologist and was not specified by the protocol (**Figure 1**). Therefore, there were patients in whom the NLST was initiated after receival of the CGP results and in accordance with the NGS findings (group 1), and patients in whom the treatment was initiated before the CGP results became available (group 2). Time-to-treatment discontinuation (TTD) and overall survival (OS) with the NLST were retrospectively assessed and compared between the groups.

Next, we selected ALK-rearranged aNSCLC patients following failure of alectinib or ceritinib (the most commonly used 1st line ALK TKIs), regardless of prior platinum-based chemotherapy, and retrospectively assessed TTD with brigatinib and lorlatinib (the drugs typically used in this clinical scenario) in correlation with the NGS findings.

Statistical Analysis

The sample size was determined by the available patients meeting the inclusion criteria and referred for CGP. The statistical analysis was generated using SAS Software, version 9.4 (28). Categorical variables were presented by numbers and percentiles, medians and ranges were reported for continuous variables. TTD and OS were assessed by the Kaplan-Meier method, with the log-rank test for the comparison. Two-sided p values less than 0.05 were considered statistically significant.

Ethical Aspects

Institutional review board approval has been received before study initiation. No patient-identifying data was included in the central data collection.

RESULTS

Patient Baseline and Treatment Characteristics

Twenty-two ALK-rearranged aNSCLC patients performed CGP within the study. One patient did not meet the eligibility criteria (failure of crizotinib and no administration of 2nd/3rd-generation ALK TKIs before enrolment), and the questionnaire was not filled by the treating oncologist in one additional patient – these two were excluded from the analysis. The baseline and treatment characteristics of the selected cohort (n=20) are presented in **Table 1**.

The median age of the patients in the cohort was 63 (range, 40-89); females and never smoking patients with adenocarcinoma histology predominated - as expected for the enrolled population. The majority of patients received alectinib (with or without crizotinib) before enrolment; four, three, three, and one patient, respectively, were treated by brigatinib,

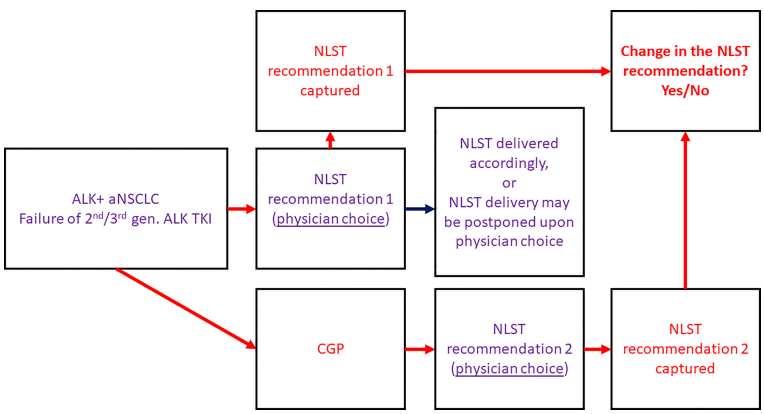

FIGURE 1 | Study design. ALK, anaplastic lymphoma kinase; aNSCLC, advanced non-small cell lung cancer; CGP, comprehensive genomic profiling; gen., generation; NLST, next-line systemic treatment; TKI, tyrosine kinase inhibitor.

TABLE 1 | Patient baseline and treatment characteristics in the whole study population and according to whether NLST was initiated before (group 1) or after (group 2) receival of CGP results.

	Patients according to group assignment (n = 16)*			All patients (n = 20)
	Group 1 (n = 8)	Group 2 (n = 8)	p value	
Age, years – median (range)	62 (40-68)	62 (50-84)	0.17	63 (40-89)
Sex, n (%)			1.00	
Female	5 (63)	6 (75)		15 (75)
Male	3 (37)	2 (25)		5 (25)
Smoking history, n (%)			1.00	
Current/past smoker	4 (50)	4 (50)		8 (40)
Never smoker	4 (50)	4 (50)		10 (50)
NA				2 (10)
Tumor histology, n (%)				
Adenoca	8 (100)	8 (100)	1.00	20 (100)
ECOG PS, n (%)			1.00	
0/1	5 (62.5)	5 (62.5)		11 (55)
2/3/4	2 (25)	2 (25)		4 (20)
NA	1 (12.5)	1 (12.5)		5 (25)
Brain metastases, n (%)	6 (75)	2 (25)	0.13	8 (40)
Previous ALK TKIs, n (%)			0.51	
Alectinib	6 (75)	8 (100)		18 (90)
Ceritinib	2 (25)	1 (12.5)		3 (15)
Brigatinib	3 (37)	1 (12.5)		4 (20)
Ensartinib	1 (12.5)	0 (0)		1 (5)
Lorlatinib	1 (12.5)	1 (12.5)		3 (15)
Crizotinib	3 (37)	4 (50)		7 (35)
Number of previous lines of ALK TKIs - median (range)	1 (1-4)	1 (1-4)	0.83	1 (1-4)
Previous platinum-based chemotherapy, n (%)	2 (25)	2 (25)	1.00	4 (20)
CGP type, n (%)			1.00	
FoundationOne Liquid CDx	7 (87.5)	7 (87.5)		16 (80)
FoundationOne CDx	1 (12.5)	1 (12.5)		4 (20)
ALK mutation, n (%)			0.43	6 (30)
G1202R	0 (0)	1 (12.5)		1 (5)
I1171X	0 (0)	1 (12.5)		2 (10)
L1196M	0 (0)	1 (12.5)		1 (5)
G1269A	0 (0)	1 (12.5)		1 (5)
Complex ALK mutation (G1202R, I1171N, E1210K)	1 (12.5)	0 (0)		1 (5)
Other potentially targetable aberrations	2 (25)	0 (0)		3 (15)
Presence of original ALK fusion, n (%)	5 (63)	4 (50)	1.00	11 (55)
NLST, n (%)			0.19	
Brigatinib	2 (25)	4 (50)		6 (30)
Lorlatinib	4 (50)	1 (12.5)		5 (25)
Platinum-based chemotherapy	1 (12.5)	2 (25)		3 (15)
Other	1 (12.5)	1 (12.5)		2 (10)
NA				4 (20)*
Reason for stopping NLST, n (%)			0.44	
Disease progression	1 (12.5)	2 (25)		3 (15)
Death	4 (50)	3 (37.5)		7 (35)
NA				4 (20)*
NLST ongoing, n (%)	3 (37.5)	3 (37.5)		6 (30)

**One patient did not initiate NLST at the time of this report, one patient died before getting any further systemic treatment, and the information regarding NLST is missing for two additional patients.*

Adenoca, adenocarcinoma; ALK, anaplastic kinase lymphoma; CGP, comprehensive genomic profiling; ECOG PS, Eastern Cooperative Oncology Group performance status; NA, not available/not applicable; NLST, next-line systemic treatment; TKIs, tyrosine kinase inhibitor(s).

ceritinib, lorlatinib and ensartinib; four patients received platinum-based chemotherapy before enrolment.

CGP Results and Change in Treatment Recommendation Upon Their Receival

FoundationOne Liquid CDx was the predominant CGP type performed. The molecular alterations diagnosed by NGS are presented in **Table 1**. ALK resistant mutations were present in 6 (30%) of cases (of those, G1202R in 1 case, l1171X in 2 cases, L1196M in 1 case, G1269A in 1 case, and a complex mutation in ALK gene combining G1202R, l1171N, and E1210K mutations in 1 case). With regards to another potentially targetable genomic aberrations, high level of c-met amplification was present in 1 case, and a cyclin dependent kinase inhibitor 2A/B (CDKN2A/B) mutation or loss was present in 2 additional cases. The original ALK fusion was presented in 11 (55%) of cases.

Overall, the change in NLST recommendation upon receival of the CGP results was registered in 6 patients (30% of the patients in the cohort). The initial physician's choice of the most recommended NLST captured before receival of NGS results was as follows: brigatinib, n=9 (45%); lorlatinib, n=5 (25%); platinum-based chemotherapy, n=4 (20%), alectinib, n=1 (5%);

pemetrexed, n=1 (5%) (**Figure 2A**). The physician's choice of the most recommended NLST captured after receival of NGS results was as follows: brigatinib, n=5 (25%); lorlatinib, n=7 (35%); platinum-based chemotherapy, n=5 (25%), alectinib, n=1 (5%); crizotinib, n=2 (10%) (**Figure 2A**).

The change in the physician's recommendation occurred upon the diagnosis of the following molecular alterations: absence of ALK resistant mutation and presence of original ALK fusion, n=2 (which drove the switch from brigatinib to platinum-based chemotherapy in once case and the switch from pemetrexed to lorlatinib in another case); CDKN2A/B mutation, absence of ALK resistant mutation and presence of original ALK fusion, n=1 (which drove the switch from brigatinib to lorlatinib); CDKN2A/B loss, absence of ALK resistant mutation or original ALK fusion, n=1 (which drove the switch from lorlatinib to crizotinib); presence of ALK G1202R and presence of original ALK fusion, n=1 (which drove the switch from brigatinib to lorlatinib); high level of c-met amplification, n=1 (which drove the switch from brigatinib to crizotinib) (**Figure 2B**).

CGP TAT

The median NGS testing TAT was 2.9 weeks [Interquartile range (IQR), 2.4-4.4]. One patient has died while waiting for the NGS results.

Time-To-Treatment Discontinuation and Overall Survival With the NLST

The NLST was initiated after receival of the CGP results and in accordance with the NGS findings in 8 patients (group 1), and included: brigatinib, n=2; lorlatinib, n=4; crizotinib, n=1; and platinum-based chemotherapy, n=1. The NLST was initiated before the NGS results became available in 8 patients (group 2), and included: brigatinib, n=4; lorlatinib, n=1; alectinib, n=1; and platinum-based chemotherapy, n=2 (**Table 1**). In addition, one patient did not initiate NLST at the time of this report, one

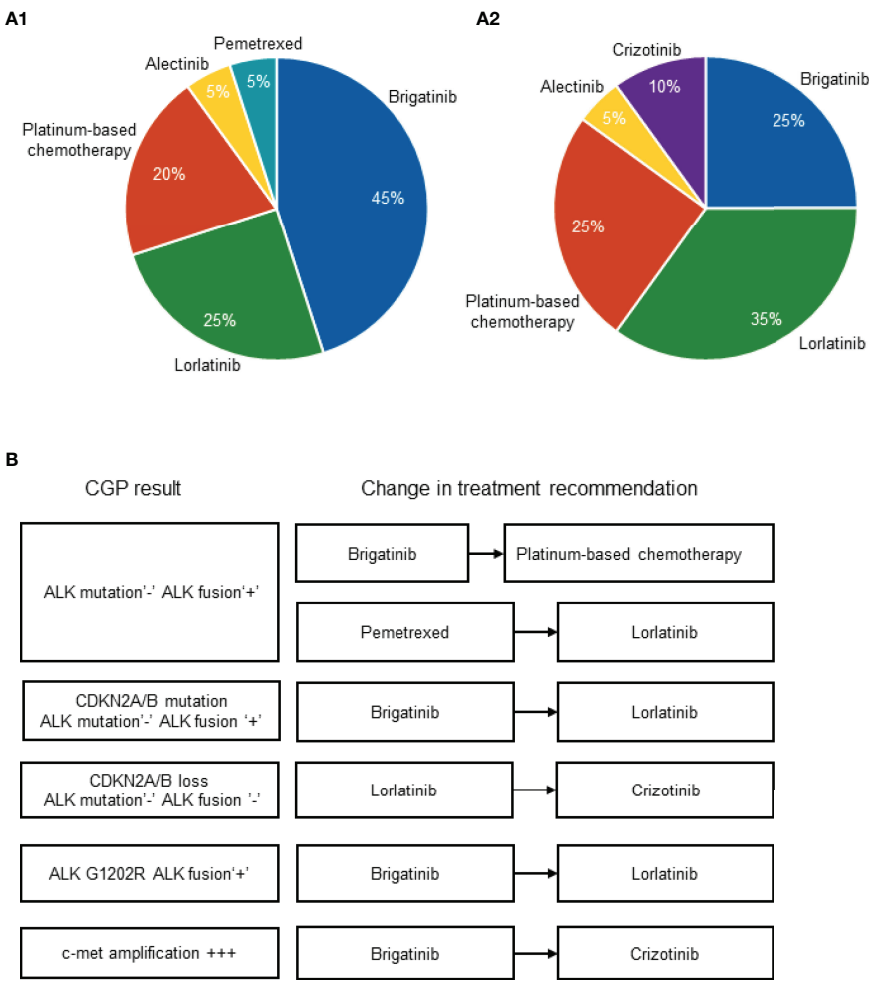

FIGURE 2 | Physician's choice of the most recommended NLST captured before **(A1)** and after **(A2)** the receival of CGP results. Change in treatment recommendation upon the receival of CGP results **(B)**. *ALK,* anaplastic lymphoma *kinase;* CDKN2A/B, cyclin dependent kinase inhibitor 2A/B; CGP, comprehensive genomic profiling; c-met, tyrosine-protein kinase Met; NLST, next-line systemic treatment.

patient died before getting any further systemic treatment (the same patient described in the previous section), and the information regarding the NLST is missing for two additional patients.

The baseline and treatment characteristics according to group assignment are presented in **Table 1**. Higher proportion of patients in group 1 had brain metastases and had previous exposure to novel 2nd-generation ALK TKIs (e.g., brigatinib and ensartinib). Patients in group 2 were less frequently approached with lorlatinib; higher proportion of patients in group 2 appeared to harbor an ALK resistant mutation. Those differences were not statistically significant.

Importantly, the change in NLST recommendation upon receival of NGS results was registered in 4 out of 8 patients included in group 1 (50%); these included cases #1, #2, #3, and #4 (**Figure 2B**). Case #5 was included in group 2, and the information regarding the NLST is missing in case #6 (**Figure 2B**).

The median follow-up was 11.3 mounts [IQR, 5.3-15.1] and 7.8 months [IQR, 6.4-11.9] in groups 1 and 2, respectively. Five (62.5%) patients in each group discontinued the NLST at the time of the last follow-up. Four (50%) patients in group 1, and 3 (37.5%) patients in group 2, respectively, have died (**Table 1**). Median TTD was 11.3 months (95% CI, 2.1-not reached [NR]) and 5.4 months (95% CI, 2.0-NR) in groups 1 and 2, respectively (p-0.34). Median OS was similar in both groups, and comprised 13.2 months (95% CI, 2.9-NR) and 13.0 months (95% CI, 6.0-NR) in groups 1 and 2, respectively (p-0.86). The Kaplan-Meyer curves for the TTD and OS with the next-line systemic treatment according to group assignment are presented in **Figure 3**.

Time-To-Treatment Discontinuation and Overall Survival With Brigatinib and Lorlatinib in Correlation With the NGS Findings

In one patient diagnosed with a complex G1202R, l1171N, and E1210K ALK mutation, TTD and OS with lorlatinib were 13.0 months and 13.0 months, respectively. In one patient diagnosed with a G1202R ALK mutation, TTD and OS with brigatinib were 2.0 months and 6.0 months, respectively. In one patient diagnosed with an l1171T ALK mutation, TTD and OS with brigatinib were 4.5 months and 8.0 months, respectively. One patient diagnosed with an L1196M ALK mutation, continues lorlatinib at the time of the report for 8.0 month since treatment initiation.

DISCUSSION

This is the first prospective study illustrating the value of CGP and molecular assessment of acquired resistance mechanisms in treatment decision-making process in ALK-rearranged aNSCLC patients.

According to our observation, CGP performed at the time of progression on 2nd- and 3rd-generation ALK TKIs, has altered treatment recommendation in one third of cases - which confirmed the initial hypothesis and, overall, appeared to be a clinically meaningful result. In cases the initiation of the NLST was postponed until getting NGS results, the proportion of patients in whom the treatment recommendation has changed was even higher (50%) - pointing to potentially larger effect of genomic assessment on the decision-making process.

Importantly, the median CGP TAT was only 2.9 weeks [IQR, 2.4-4.4] which seems acceptable considering the CGP impact on treatment decision. In our cohort, only one patient has died while waiting for the CGP results. This fact emphasizes the need to assess further the phenomenon of clinical deterioration attributable to rapid disease progression, and the potential adverse effect of the CGP in this association.

Looking into the specific treatment recommendation changes following the receival of the CGP results, we observed increase in the proportion of recommendations on lorlatinib, platinum-based

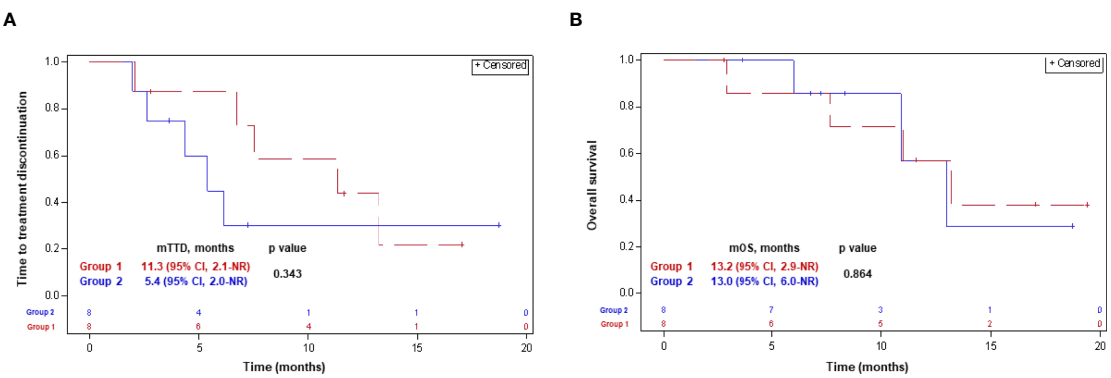

FIGURE 3 | Time-to-treatment discontinuation **(A)** and overall survival **(B)** with the NLST in patients in whom the treatment decision was made before (group 2) and after (group 1) getting the CGP results. CGP, comprehensive genomic profiling; CI, confidence interval; NLST, next-line systemic treatment; NR, not reached; (m)OS, (median) overall survival; (m)TTD, (median) time-to-treatment discontinuation.

chemotherapy, and crizotinib. There were three clinical scenarios which seemed to have a biologic rationale behind the treatment decision alteration. The 1st clinical scenario was switching to a different targeted treatment following the diagnosis of another potentially targetable molecular aberration, or a bypass pathway activation, such as switching from ALK TKI to c-met TKI following the diagnosis of c-met amplification. Indeed, c-met alterations represent one of the most common mechanisms responsible for acquired resistance to osimertinib in EGFR mutant aNSCLC (10-25%) (29), and to ALK TKIs in ALK-rearranged aNSCLC (15%) (24). Moreover, c-met inhibition has been associated with objective response rate of 30% and median duration of response of 7.9 months in c-met-amplified aNSCLC patients following progression on 3rd-generation EGFR TKIs (30). Additionally, there are several case reports suggesting that met-inhibition may overcome c-met-driven resistance in ALK-positive aNSCLC (24, 31, 32). The 2nd clinical scenario of treatment decision alteration in our cohort was switching from brigatinib to lorlatinib following the diagnosis of ALK G1202R mutation – which is justified by the high activity of lorlatinib in tumors harboring this ALK resistant mutation (19). The 3rd clinical scenario in our study included switching from ALK TKIs to platinum-based chemotherapy in the absence of ALK resistant mutation - which, again, seems reasonable considering modest next-generation ALK TKI activity in patients without ALK resistant mutations following progression on prior ALK TKIs (19). Specifically, a positive correlation between presence of ALK resistant mutations, their type, and outcomes with lorlatinib have been reported in ALK-rearranged aNSCLC patients following failure of a 2nd-generation ALK TKI. It remains unknown, however, whether similar correlation is true for brigatinib. Moreover, no comparative clinical studies have been done or planned to be done in order to explore the comparative efficacy of the two agents in correlation with the molecular biomarkers in this clinical setting.

In three additional clinical scenarios which prompted treatment decision changes in our study, it was hard to explain the physician's decision from the biologic perspective. For instance, presence of CDKN2A/B loss or mutation was anticipated to alter the decision towards cyclin-dependent kinase 4/6 (CDK4/6) inhibitors, however, it was not the case. Having said that, it should be emphasized that CDKN2A/B alterations are only rarely seen following progression on 2nd/3rd-generation ALK TKIs (17, 23, 33), and the majority of CDK4/6 inhibitors did not demonstrate a significant antitumor activity in aNSCLC (34–39). Although CDK4/6 inhibitors have demonstrated a myelo-preserving effect in conjunction with chemotherapy in advanced small-cell lung cancer, it did not appear to improve tumor control (40).

The prevalence of ALK resistant mutations (30%) and their distribution in our study were in line with the previously reported data in ALK-positive aNSCLC following treatment with 2nd/3rd-generation ALK TKIs (19, 23). Only one case of high-level c-met amplification was present in our cohort, while higher prevalence of c-met alterations (12-22%) has been reported in the literature (24). This discrepancy might be attributable to the lower proportion of patients progressing on lorlatinib in our cohort, and less so - to the technical limitations of the liquid biopsy assay. For instance, Dagogo-Jack et al. reported on higher prevalence of c-met amplification following treatment with 3rd-generation ALK TKI, on one hand, and on the other hand - on high overall accuracy of liquid NGS as compared to tissue genotyping (24). The detection of CDKN2A/B alterations was not unique to our cohort either (33). Another interesting observation in our study was tissue versus liquid biopsy referral patterns. For instance, liquid biopsy was the preferred method of assessment - probably due to its simplicity and high patient advocacy, which reflected real-world physician and patient preferences. This pattern was also in line with the recently updated International Association for the Study of Lung Cancer (IASLC) guideline on liquid biopsy to discover molecular resistance mechanisms (41).

CGP performed at the time of progression on 2nd/3rd-generation ALK TKIs demonstrated a positive impact on NLST duration but did not affect the OS. Several factors might have attributed to that. First, some of the most expedient alterations in treatment recommendations were not implemented. Second, OS was the subject for the lead-time bias: i.e., those patients in whom the NLST was initiated following the receival of the CGP results, initiated the treatment later as opposed to patients in whom the NLST was started before the CGP results became available. Finally, higher proportion of patients in whom the treatment was initiated before receival of the CGP results appeared to harbor an ALK resistant mutation, which might have an impact on outcomes as well. The lack of the ability to demonstrate an impact of CGP on oncological outcomes remains the most significant limitation of our study, along with the small sample size. Another important study limitation is its non-randomized design allowing patient selection for immediate versus postponed treatment initiation based on the tempo of the disease.

Overall, the study has demonstrated the feasibility and the significant impact of the CGP on the decision-making process in ALK-rearranged aNSCLC following failure of 2nd/3rd-generation ALK TKIs. It remains to be seen whether such strategy affects oncological outcomes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Helsinky ethics committee of the Rabin Medical Center (RMC), approval number: 0561-19-RMC. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Credit statement conceptualization: ED, AR, and AO. Methodology: ED, AR, and AO. Validation: ED. Formal analysis: TS. Investigation: ED. Resources: ED, AR, AO, LH, JD, DU, WK, AC, MM, AZ, JB, NR, SG, CO, AA, NP, and TS. Data Curation: ED. Writing - original draft: ED, AR, AO, and LH. Writing - review and editing: ED, AR, AO, LH, JD, DU, WK, AC, MM, AZ, JB, NR, SG, CO, AA, NP, and TS. Visualization: ED and LH. Supervision: ED. Project administration: ED. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.874712/full#supplementary-material

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