REAL-WORLD EVIDENCE IN LUNG CANCER

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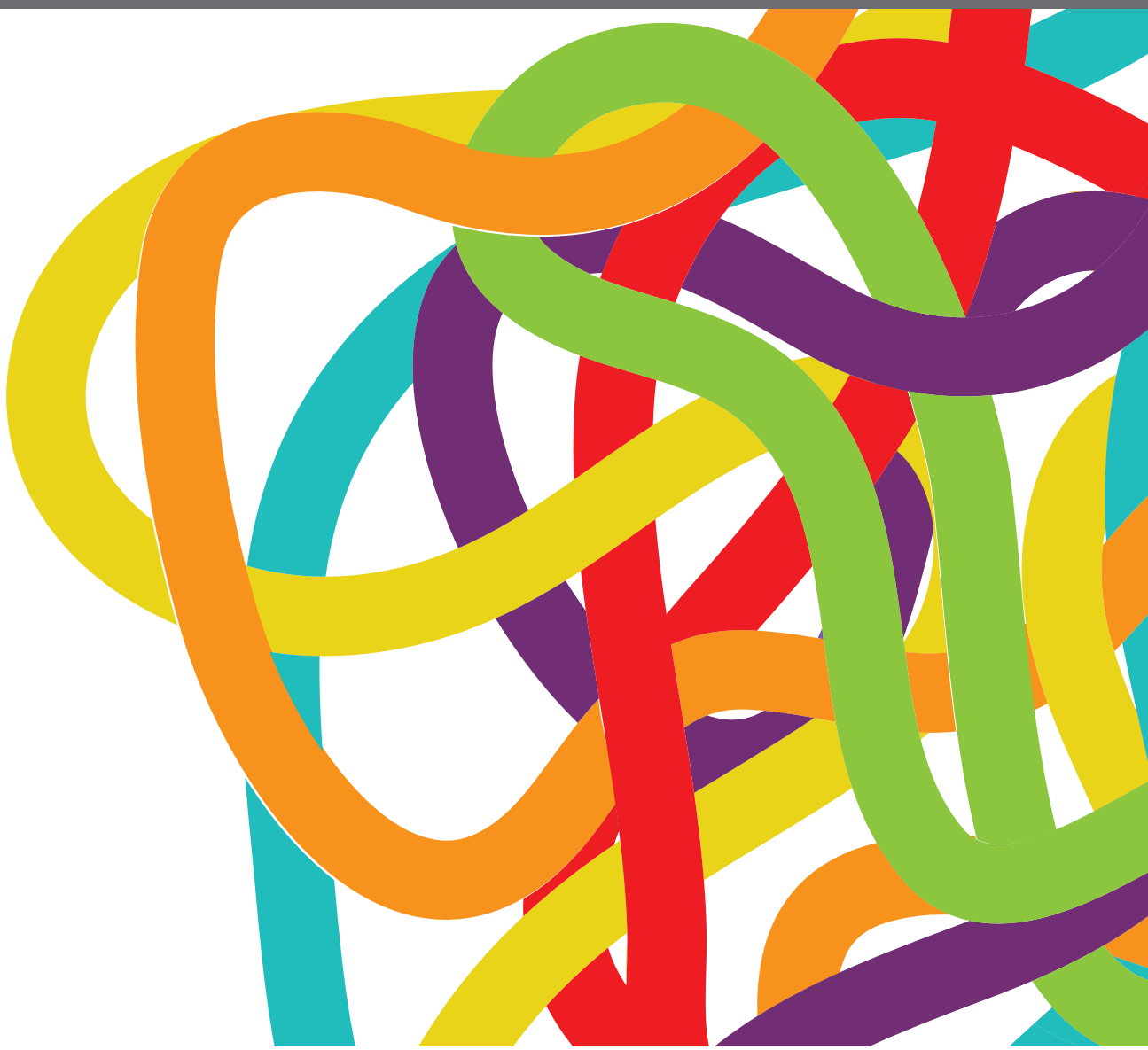

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REAL-WORLD EVIDENCE IN LUNG CANCER

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Table of Contents

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CASE REPORT
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Cecal Volvulus as a Rare Complication of Osimertinib Dosed at 160 mg in Patients With *EGFR*-Mutant Non-small Cell Lung Cancer

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Background: Osimertinib is a 3rd-generation tyrosine kinase inhibitor (TKI) that blocks the epidermal growth factor receptor (EGFR) in non-small lung cancer (NSCLC) and has dramatically improved outcomes for patients with EGFR mutations. While gastrointestinal complications such as diarrhea have been reported with EGFR inhibitors (due to off-target interactions with EGFR receptors within the gut lining), cecal volvulus is an extremely rare complication in advanced malignancy. To date, there are no reported cases associating cecal volvulus with any EGFR TKIs.

Case Presentation: In this case series, we present three cases of cecal volvulus among patients with EGFR-positive NSCLC patients treated with osimertinib dosed at double the standard 80 mg dose (160 mg daily). No patient was receiving concurrent chemotherapy or bevacizumab at the time of this described complication. In two cases where pathology was available for review, peritoneal carcinomatosis or intra-abdominal spread was not observed. In a retrospective evaluation of 101 patients treated with osimertinib in our institution, there was a statistically significant difference in the incidence of cecal volvulus among patients receiving osimertinib at 160 mg vs. patients receiving the 80 mg dose (27 vs. 0%; $p < 0.001$).

Conclusions: To our knowledge, these are the first cases to highlight a potentially important and serious gastrointestinal complication associated with the 160 mg dose of osimertinib.

Keywords: EGFR, NSCLC, tyrosine kinase inhibitor, osimertinib, volvulus

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INTRODUCTION

The identification of the epidermal growth factor receptor (EGFR) in non-small lung cancer (NSCLC) has led to the development of tyrosine kinase inhibitors (TKIs) that inhibit these mutations with a high degree of specificity. Patients with activating *EGFR* mutations demonstrate improved outcomes with respect to objective response rate (ORR), progression free survival (PFS), and quality of life (QoL) due to the availability of selective and effective TKIs (1–6). Osimertinib is a third-generation irreversible TKI that overcomes T790M, the most common acquired resistance

mutation to first generation EGFR inhibitors (1, 5, 7). Gastrointestinal complications such as diarrhea have been reported with EGFR inhibitors due to off-target interactions with EGFR receptors within the gut lining. While malignant bowel obstruction and bowel perforation are commonly seen in advanced cancer (especially in gastrointestinal and gynecological malignancies), volvulus is an extremely rare complication overall (8). To date, there are no reports of a relationship between EGFR inhibition and the development of volvulus. We report three cases of patients with EGFR mutant NSCLC who developed cecal volvulus after being treated with osimertinib at double the standard 80 mg dose (160 mg daily).

CASE VIGNETTE 1

A 53 year old Caucasian female never smoker presented to her primary care physician with shoulder pain and was subsequently diagnosed with metastatic lung adenocarcinoma. Computed tomography (CT) of the chest, abdomen, and pelvis revealed a right middle lobe lung mass (5.2 × 4.5 cm), contralateral mediastinal lymphadenopathy, and numerous osseous metastases involving the vertebrae without epidural extension or cord compression. Brain magnetic resonance imaging (MRI) did not find metastatic disease at the time of diagnosis. Endobronchial biopsy of the right middle lobe lung mass was positive for poorly differentiated lung adenocarcinoma. Next-generation sequencing (NGS) from this sample revealed an *EGFR* L858R point mutation. She was started on erlotinib 150 mg PO daily with an excellent partial response to therapy. She did not receive chemotherapy or bevacizumab prior to starting erlotinib. Approximately 8 months after receiving erlotinib, she received intensity-modulated radiotherapy (5,000 cGy over 10 fractions) to three oligoprogressive lung lesions. After 10 months from radiotherapy, she progressed in the brain and left ulna. She received stereotactic radiotherapy (2,000 cGy over one fraction) to her left cerebellar vermis and switched to rociletinib, a third generation EGFR TKI, in the context of a clinical trial. She had a partial response to this therapy for 5 months before developing worsening headache, gait ataxia, and vision changes secondary to leptomeningeal progression. A CT abdomen and pelvis at time of progression on rociletinib found no peritoneal carcinomatosis or intraabdominal disease. She was switched to osimertinib dosed at 160 mg PO daily for increased intracranial penetrance. She had rapid resolution of her neurological symptoms. She remained on this therapy for 1 year, before being admitted to the hospital for acute right-sided lower quadrant abdominal pain associated with obstipation. Abdominal exam was notable for distension, rebound tenderness along the right upper quadrant, and involuntary guarding. Of note, her admission vitals were notable for bradycardia. A CT abdomen obtained in the emergency department demonstrated cecal interposition between the liver and the anterior peritoneum with mild dilatation of the cecum and swirling of the distal ileum about the ileocolic vasculature. She was taken emergently to the operating room where an exploratory laparotomy, right hemicolectomy, and end ileostomy were performed. There was no evidence of peritoneal carcinomatosis or malignant bowel obstruction by visual inspection of abdomen. Examination of the resected right colon demonstrated serosal adhesions, tortuous contour, and vascular congestion consistent with cecal volvulus. Osimertinib was discontinued after surgery. One month later, she was treated with IV carboplatin (AUC 6), pemetrexed 500 mg/m2, and pembrolizumab 200 mg with ongoing response.

CASE VIGNETTE 2

A 66 year old Caucasian male never smoker developed a non-productive cough for 1 month that failed to respond to outpatient antibiotics, inhaled bronchodilators, and short courses of prednisone. His primary care physician obtained a CT chest that demonstrated a right upper lobe mass (5.2 × 4.7 cm) along with numerous satellite nodules in the right lower lung. He was admitted to an outside hospital where a CT-guided biopsy of the right upper lobe lung mass was performed. Biopsy from this specimen revealed nests of mucinous tumor cells with an immunophenotype negative for cytokeratin 20 (CK20), positive for cytokeratin 7 (CK7), and positive for thyroid transcription factor 1 (TTF-1) consistent with lung adenocarcinoma. Real-time polymerase chain reaction (RT-PCR) testing revealed an *EGFR* L858R point mutation. Staging positron emission tomography–computed tomography (PET/CT) identified a large fluorodeoxyglucose avid (FDG) lesion in the right upper lobe of lung along with subcarinal and ipsilateral mediastinal, paratracheal, and supraclavicular lymph nodes, but did not identify any extrathoracic disease. An MRI of the brain demonstrated a single right parietal lesion measuring 7 mm with surrounding vasogenic edema. This lesion was treated with stereotactic radiosurgery (2,000 cGy over one fraction). He received erlotinib 150 mg PO daily with an excellent partial response to therapy. He tolerated therapy well with Grade 1 diarrhea and acneiform rash as the principal adverse effects. After 2 years on therapy, he progressed in the liver and pancreas. Re-staging imaging with PET/CT did not demonstrate any peritoneal carcinomatosis. A CT-guided biopsy of the liver metastasis demonstrated an *EGFR T790M* mutation and he was switched to osimertinib 80 mg PO daily. His osimertinib dose was increased to 160 mg PO daily after 8 months for progressive CNS disease. He continued this therapy for ∼10 months before being admitted to an outside hospital for acute right sided abdominal pain. On admission, he was found to have cecal volvulus requiring a hemicolectomy. Pathology from the resected specimen was not available for our review. Due to ongoing symptomatic CNS disease, he was restarted on osimertinib 160 mg PO daily 1 week after his hemicolectomy. Three weeks after restarting osimertinib at 160 mg, he developed progressive CNS disease. Assessment of circulating tumor DNA (ctDNA) identified *EGFR* L792V *in trans* with T790M. Based on this acquired resistance mutation, he was switched to afatinib with a partial response in the CNS.

CASE VIGNETTE 3

A 44 year old Caucasian male never smoker was diagnosed with metastatic lung adenocarcinoma after presenting to an emergency room with severe low back pain where he was found

to have a lytic L2 lesion. CT chest imaging demonstrated a large pulmonary mass with irregular borders (3.2 × 3.2 cm) along the posterior basilar segment of the right lower lobe. Staging PET/CT demonstrated widespread metastatic disease including innumerable pulmonary nodules, ipsilateral pleural effusion, liver metastases, osseous metastases, and omental nodularity suspicious for peritoneal carcinomatosis. An MRI of the brain demonstrated two intracranial lesions involving the left occipital and left parietal lobe without vasogenic edema or midline shift. A core needle biopsy of the L2 vertebral lesion demonstrated malignant cells with immunohistochemistry negative for CK20, positive for CK7, and positive for TTF-1 consistent with lung adenocarcinoma. NGS of the decalcified bone sample revealed an *EGFR* Exon 19 deletion and a *TP53 L114** mutation. The patient received erlotinib 150 mg PO daily along with bevacizumab 15 mg/kg IV every 3 weeks with rapid resolution of his back pain. His first on-treatment PET/CT scan demonstrated marked response in all metastatic sites. He continued with this treatment regimen for 6 months before an on-treatment PET/CT scan demonstrated progression in T11, L5, S1 and the left acetabulum. A repeat bone biopsy from his L5 lesion demonstrated evidence of an *EGFR* T790M mutation and high-level *MET* amplification. *MET* copy number analysis was performed by fluorescence *in-situ* hybridization (FISH) testing and demonstrated a mean *MET*-per-cell of 13.87 and *MET*-to-centromeric enumeration probe for chromosome 7 (CEP 7) ratio of 5.04. Given the presence of both T790M and high *MET* amplification, he was switched to osimertinib 80 mg PO daily and crizotinib 250 mg PO BID (an FDA-approved ALK inhibitor with strong *MET* inhibition). He tolerated this combination well with improvement of his osseous metastases. He continued this combination for 8 months before developing left arm weakness and numbness. He was found to have new C3-C4 metastases with epidural extension and leptomeningeal disease on MRI of the brain and spine. Of note, the patient had no radiographic evidence of peritoneal carcinomatosis at time of leptomeningeal progression based on PET/CT. He was given a short course of dexamethasone 4 mg PO every 6 h and his osimertinib dose was increased to 160 mg PO daily. Palliative radiotherapy (3,000 cGy × 10 fractions) using three-dimensional conformal radiotherapy to C3-C4 was administered. He remained on the increased dose of osimertinib with crizotinib for 1 month before presenting to the emergency room with sudden onset of severe lower abdominal pain and distension. An abdominal CT scan on admission demonstrated swirling of the mesentery within the right central hemiabdomen at the level of the cecum/terminal ileum junction with significant gaseous distension of adjacent transverse colon (**Figure 1**). On evaluation in the emergency department, he was noted to be bradycardic (heart rate of 37) with a QTc of 429. He was emergently taken to the operating room where an exploratory laparotomy and right hemicolectomy with ileocolic anastomosis was performed; he was found to have partial malrotation, with the duodenum not crossing the midline, but fixed in the retroperitoneum and a mobile right colon. Pathological review of the resected right colon demonstrated edematous benign colonic mucosa with submucosal hemorrhage, vascular congestion, and vascular dilation. Despite initial concern for peritoneal carcinomatosis grossly, the mucosa was negative for dysplasia or invasive carcinoma. Multiple intra-abdominal nodes examined did not reveal any evidence of pulmonary adenocarcinoma. On post-operative day 1, he developed altered mental status and was found to have hemorrhagic conversion of a new brain metastasis. He was re-challenged with osimertinib 160 mg PO daily and crizotinib 250 mg PO BID 1 week after recovery from his surgical procedure given known leptomeningeal disease and the development of a new brain metastasis. Unfortunately, he went on to have a complicated post-operative course: almost 4 weeks after ileocolic resection with primary anastomosis and returning to work, he had anastomotic dehiscence for which he received an ileostomy and transverse colon mucus fistula in staged procedures. Two months after re-challenging with osimertinib 160 mg PO daily and crizotinib 250 m PO BID, he progressed in the pleura and multiple extrathoracic lymph nodes and was switched to IV carboplatin (AUC 6) and pemetrexed 500 mg/m2. He remained steadfast in his desire for stoma takedown after numerous discussions regarding the associated risks, which was performed 20 months after the stoma was performed; 4 weeks after takedown, he developed a fistula from the anastomosis. After his wound care was optimized, he was discharged with hospice care.

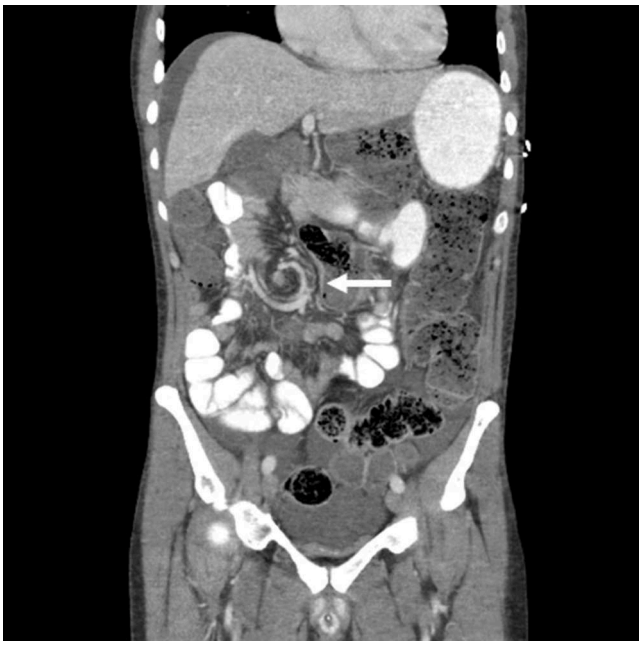

FIGURE 1 | A CT scan of the abdomen demonstrates a "whirl sign" which is when the bowel rotates arounds its mesentery leading to whirls of the mesenteric vessels.

DISCUSSION

The etiology of cecal volvulus is likely related to late embryogenesis; the cecum rotates counterclockwise from the

left side of the abdomen to the right lower quadrant. As this occurs, the mesentery of the right colon fixates to retroperitoneal structures (8). If the patient has incomplete fixation, there is risk of cecal volvulus formation. Based on the autopsy of 125 cadavers, 11.2% of right colons examined were freely mobile with complete common ileocolonic mesenterium, 25.6% were found to have cecum capable of "folding," adding up to 36.8% of cadavers potentially at risk for cecal volvulus (8, 9). Known risk factors for cecal volvulus include chronic constipation, distal colon obstruction, high-fiber diets, ileus, prior colonoscopy, and late pregnancy (8). While malignant large bowel obstruction is a common complication of advanced gastrointestinal and ovarian malignancies, (10, 11) reports of malignancy-associated cecal volvulus are extremely rare and limited to case reports (12, 13). In an older case series of 37 patients with cecal volvulus seen during a 20 year period at surgical departments in Sweden, only two patients were found to have underlying gastrointestinal cancer at the time of surgical resetion (14).

Osimertinib received approval for use in NSCLC using an accelerated approval process. Common side effects of EGFR inhibitors include rash and diarrhea and are related to off-target inhibition of wild-type EGFR receptors distributed along the cutaneous and mucosal lining. Gastrointestinal complications with osimertinib dosed at 80 mg have been reported in two Phase 3 trials that resulted in osimertinib FDA approval (1, 5). Diarrhea was the most common Grade $\geq$3 gastrointestinal complication reported with 2 cases (1%) documented in the AURA3 trial and 6 cases (2%) documented in the FLAURA3 trial. Constipation, a known risk factor for volvulus, was only seen in 7 cases in the AURA3 trial, though neither trial reported any cases of Grade $\geq$3 constipation. In the phase 1 dose escalation study of osimertinib in patients with CNS metastases, there were increasing percentages of Grade 1–2 gastrointestinal toxicities observed at 200 mg (57%; 4/7), 300 mg (100%; 7/7), and 500 mg doses (100%; 3/3) (15). Only one patient had intolerable Grade 2 mucosal inflammation resulting in discontinuation of drug.

Numerous chemotherapeutic agents have been associated with bowel perforation and impaired wound healing, most notably anti-vascular endothelial growth factor (VEGF) inhibitors such as bevacizumab. The third patient in our series had previously been treated with bevacizumab, which could confound our interpretation. A retrospective series of 208 patients with NSCLC who received bevacizumab identified grade 3 diarrhea, febrile neutropenia, and stomatitis as risk factors for bevacizumab-associated perforation (16). Gastrointestinal perforation secondary to erlotinib, a first-generation EGFR inhibitor, is reported in two case reports (17, 18), but there have been no reports of bowel perforation associated with osimertinib.

To date, there has been no association with cecal volvulus and EGFR inhibition. In a review of 3540 osimertinib-treated cases from the FDA Adverse Events Reporting System (FAERS) database, only one case of gastric volvulus was described. A limitation of this database is that there are no data on dose and temporal association of drug delivery and adverse event. We performed a retrospective review of 101 patients treated with osimertinib in our institution. Group comparisons were performed using Fisher's exact test for categorical variables using $p < 0.05$ as cut-off for statistical significance. Cecal volvulus was more common among patients receiving osimertinib at the 160 mg dose ($n = 11$) vs. patients receiving the 80 mg dose ($n = 90$), a finding that was statistically significant (27 vs. 0%; $p < 0.001$).

There are two additional important observations in this series. The delayed wound healing described for the third patient in our series may be related to concurrent *MET* inhibition with crizotinib. The c-MET pathway has been identified as important for the process of wound healing (19). *MET* amplification is a described mechanism of resistance to EGFR inhibition, (20) and the addition of crizotinib, a *MET* inhibitor, is a strategy that is increasingly being utilized (21). While the concurrent use of crizotinib could influence the risk of volvulus, we have been unable to identify any cases of volvulus as a complication with crizotinib. Another important observation in this series was the presence of bradycardia at time of presentation among cases of volvulus, suggesting a potentially autonomic link with higher doses of osimertinib. In anatomically predisposed patients, higher doses of osimertinib may be contribute to a "second hit" resulting in the development of volvulus.

CONCLUSION

To our knowledge, this is the first report associating cecal volvulus with the 160 mg dose of osimertinib. The correlation between three occurrences of a very rare event with all patients receiving the same drug at the same uncommon dosage seems unlikely to be a coincidence. The exact mechanism for this side effect is unclear and warrants further study. These cases highlight a potentially important surgical complication associated with the 160 mg dose of osimertinib.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Colorado Multiple Institutional Review Board (COMIRB). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

TP, DC, RD, and LF were involved with conception, design, and writing of the manuscript. All authors played a critical role in the appraisal of the final manuscript.

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Conflict of Interest: TP has received honoraria from PRIME Oncology and Genetech/Roche. JP has received honoraria or consulting fees Roche/Genentech, Takeda, AstraZeneca, GLG, Novartis, and Pfizer. PB received personal fees from AstraZeneca and Guardant Health. DC has received honoraria or consulting fees from Ariad, Takeda, Ignyta, and Roche/Genentech, and a sponsored clinical trial research agreement with Takeda. RD has consulting fees from Rain Therapeutics, Roche/Genentech, Takeda, AstraZeneca; a sponsored research agreement from Ignyta; licensing fees for patents from Abbott Molecular and Rain Therapeutics; licensing fees for biologic materials from Black Diamond, Pearl River, Ariad, Foundation Medicine, and Genentech; and stock ownership in Rain Therapeutics.

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

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SYSTEMATIC REVIEW
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A Systematic Review of the Efficacy of Preclinical Models of Lung Cancer Drugs

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Background: Preclinical cell models are the mainstay in the early stages of drug development. We sought to explore the preclinical data that differentiated successful from failed therapeutic agents in lung cancer.

Methods: One hundred thirty-four failed lung cancer drugs and twenty seven successful lung cancer drugs were identified. Preclinical data were evaluated. The independent variable for cell model experiments was the half maximal inhibitory concentration (IC50), and for murine model experiments was tumor growth inhibition (TGI). A logistic regression was performed on quartiles (Q) of IC50s and TGIs.

Results: We compared odds of approval among drugs defined by IC50 and TGI quartile. Compared to drugs with preclinical cell experiments in highest IC50 quartile (Q4, IC50 345.01–100,000 nM), those in Q3 differed little, but those in the lower two quartiles had better odds of being approved. However, there was no significant monotonic trend identified (P-trend 0.4). For preclinical murine models, TGI values ranged from −0.3119 to 1.0000, with a tendency for approved drugs to demonstrate poorer inhibition than failed drugs. Analyses comparing success of drugs according to TGI quartile produced interval estimates too wide to be statistically meaningful, although all point estimates accord with drugs in Q2-Q4 (TGI 0.5576–0.7600, 0.7601–0.9364, 0.9365–1.0000) having lower odds of success than those in Q1 (−0.3119–0.5575).

Conclusion: There does not appear to be a significant linear trend between preclinical success and drug approval, and therefore published preclinical data does not predict success of therapeutics in lung cancer. Newer models with predictive power would be beneficial to drug development efforts.

Keywords: lung cancer, preclinical studies, mouse models, lung cancer therapies, cell models

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BACKGROUND

Preclinical data guide the identification of oncology agents that have clinical promise (1). However, the vast majority of agents with favorable preclinical data subsequently fail in human clinical trials. The cost to develop a new cancer drug ranges from $0.5 billion to $2 billion, and about 12 years typically elapse between selection of a candidate compound for human investigation to approval for clinical use. Drugs that enter human research use are met with a low ultimate FDA approval rate

of 5–7% (2), and there is a paucity of studies on whether satisfaction of preclinical criteria predicts eventual regulatory clearance. In regards to lung cancer drug development, specifically, a scholarly review published in 2014 (3) indicated that since 1998, only 10 drugs were approved for lung cancer treatment, while 167 other therapies failed in clinical trials (3).

While mouse and cell models have elucidated pathophysiologic mechanisms of lung cancer, providing a biological framework for identification of therapeutic targets, new understanding that emerges from these efforts rarely translates into human therapeutics. Advantages of preclinical models include the far greater simplicity of both cell culture assays and animal model testing. By comparison, trials in humans are complicated by variability in patient factors such as genetic abnormalities, tumor microenvironment, metastatic potential *in vivo*, drug metabolism, and host immune responses. In addition, dosing schedules, drug delivery methods, and interactions between combination therapies vary significantly in humans compared to cell lines and murine models. These factors may account, at least in part, for failure of cancer therapies to achieve efficacy in clinical phase II and III trials. A recent study on oncolytic viral therapy illustrates the difficulty of applying *in vitro* success to clinical efficacy in humans. NTX-010, a picornavirus with selective tropism for small cell lung cancer tumor cell lines, and excellent preclinical data, was evaluated in a phase II study performed on 90 patients randomized to placebo vs. treatment, and showed no benefit in progression-free survival in patients with small cell lung cancer (4).

Similarly, many cancers have been cured in murine models but not humans (5), illustrating limitations of preclinical testing in mice. It may be tempting to attribute these failures to the complexity and diverse evolutionary etiology of human cancers. However, even advanced cell-line derived xenografts and genetically engineered mouse models that produce tumors with great similarity to human diseases are not accurately and reproducibly translated to human applications.

In the work described here, we conducted in-depth review of design and results of preclinical cell and murine model experiments used in the development of lung cancer drugs, quantitatively comparing 27 drugs that are now FDA approved for treatment of lung cancer with 167 drugs that failed to be approved for this purpose. The goal was to identify features of preclinical experiments or values of efficacy parameters that might predict a drug's success in clinical testing. Whether tested cells were of lung cancer origin was of particular interest, but any feature or efficacy measure found to be predictive could be emphasized to improve future preclinical testing in cells or animals. We recognized that should no such feature be identified, the analysis would underscore a need for alternate approaches.

MATERIALS AND METHODS

Inclusion and Exclusion Criteria

We studied only drugs that had exhibited statistically significant efficacy in preclinical testing and subsequently entered the human testing phase of the United States Food and Drug Administration (FDA) approval process as candidates for single agent lung cancer therapy. From this set, we excluded any drug for which we could not determine specific model used in preclinical studies.

Search Strategy

We identified drugs that failed human testing using a PhRMA review of lung cancer medications that were unsuccessful in clinical trials from 1996 to 2014 (3). We identified approved drugs using the National Cancer Institute's 2017 summary of medications approved by the FDA for treatment of lung cancer. We identified a corresponding set of preclinical studies, conducted either in cell lines, or murine models, by systematically searching Pubmed through May 2018 using as search terms drug names taken from the lists described above together with the keywords, "lung cancer," "preclinical mouse models," "preclinical cell," and "IC50."

Independent Variables

For cell line experiments, the independent variable was the half maximal inhibitory concentration (IC50) expressed in nanomoles/liter (nM). This measure of efficacy is defined as the amount of drug needed to inhibit by half a specified biological process, which in these studies was cell growth.

The independent variable for mouse model experiments was tumor growth inhibition (TGI) calculated as (tumor volume or weight of treated mice in mm^3–tumor volume or weight of control mice in mm^3)/tumor volume or weight of control mice in mm^3 at the end of the follow-up period. TGI is 0 when the final size of tumors does not differ between drug-treated and vehicle-treated groups, <0 when drug-treated tumors are smaller, and >0 when drug-treated tumors are larger. For studies that used this definition of TGI, we used the reported value; if an alternative definition was used, we calculated the TGI according to the above formula from reported tumor volume and weight. For this purpose, we used Engauge Digitizer Version 10.4 application to estimate tumor volume or weight in treated and control mice.

We identified whether each drug was categorized as a nucleic acid damaging agent, cell signal-interrupting agent, tumor microenvironment, and VEGF agent (categorized together based on similarity in mechanism and for purposes of statistical analysis), immunotherapeutic agent (including vaccines and monoclonal antibodies), or miscellaneous (other). **Supplemental Table 1** illustrates all categorized drugs used in the study. For cell culture models, we noted whether cells had been derived from lung cancer or non-lung cancer cell type. For animal models, we noted mouse strain categorized as athymic nude and immunocompetent, athymic nude only, or immunocompetent only; and coded tumor origin as xenograft, spontaneous, orthotopic implantation, induced, or murine vector.

Outcome Variables

The outcome variable for each analysis was drug approval status, scored as approved or failed.

Statistical Analysis

To compare distributions of independent variables between failed and approved drugs, we created box-plots stratified by approval status. When raw data were highly skewed, we log transformed IC50 values and created a second set of box-plots on this scale. To test for differences in central tendency, we used *T*-tests for normally distributed data and the Mann-Whitney procedure for skewed data, and reported *p*-value results of each.

We used logistic regression to estimate associations between drug approval status and quartile of IC50 (cell studies) or TGI (animal studies), and calculated trend *P*-values based on IC50 or TGI value of midpoint of each quartile. We estimated conventional standard errors of TGI. Since there were numerous cell studies of some drugs, we recognized that there could be dependence between measures and thus employed generalized estimating equations to estimate robust standard errors of IC50 to accommodate this apparent non-independence.

Finally, we created empirical receiver operator characteristic (ROC) curves displaying sensitivity and specificity of each value of the independent variable to predict a drug's success. We created a single ROC curve for TGI values; for IC50 values, we created one curve for all measures, and separate curves for studies that employed cell lines derived from lung cancer or from other tissues.

All analyses were conducted using R 3.5.1 (6).

RESULTS

Our search identified reports on preclinical studies of 155 drugs that had been carried forward to human testing as part of the FDA approval process. Of these, 27 had been approved as monotherapy for lung cancer, but 128 had failed at some stage of human testing.

Preclinical Cell Models

Our search identified reports on 378 cell culture experiments (**Supplemental Table 2**) reported from 308 data sources from peer-reviewed articles and public drug libraries (**Supplemental Figure 1**). IC50 values were not reported for 55 of these, precluding their use in the analyses. **Table 1** summarizes the remaining 323 experiments according to type of drug and cell line used, and provides of IC50 values that define each quartile of this variable for failed and approved drugs. Cell lines derived from lung cancer were used in only 25% of experiments that tested approved drugs and 17.3% of studies of drugs that failed.

Reported IC50 values range from 1 to 100,000 nM, with substantial overlap in distributions within the set of drugs that were approved and those that failed. Values for approved drugs were slightly lower than values for drugs that failed, but the difference did not achieve statistical significance (means of $\log_{(IC50)}$, $p = 0.22$; medians of IC50, $p = 0.09$; **Figure 1**). Accordingly, estimated areas under the ROC (AUC) values were only slightly >0.5, consistent with IC50 predicting success barely better than chance, whether the ROC represented data from all preclinical cell experiments (AUC = 0.59, **Figure 2A**) or from subsets (**Figure 2B**) defined by whether the cell line originated from lung cancer (LCLine, AUC = 0.56) or some other source (non-LCLine, AUC = 0.60).

TABLE 1 | Descriptive distributions on initial sample.

Variable	Approved		Failed	
	n	%	*n*	%
Drug type				
Immunotherapy	0	0.0	20	5.8
Nucleic acid damaging agents	21	58.3	125	36.5
Cell signaling interrupting agents	15	41.7	173	50.6
Tumor microenvironment and VEGF agent	0	0.0	23	6.7
Missing	0	0.0	1	0.3
Total	36	100.0	342	100.0
Cell line type				
Lung cancer cell line	9	25.0	59	17.3
Non-lung cancer cell line	27	75.0	248	72.5
Missing	0	0.0	35	10.2
Total	36	100.0	342	100.0
Quartile of IC50 value				
Q1 [0–3.91 nM]	10	27.8	71	20.8
Q2 [3.92–30.00 nM]	15	41.7	66	19.3
Q3 [30.01–345.00 nM]	5	13.9	75	21.9
Q4 [345.01–100,000.00 nM]	6	16.7	75	21.9
Missing*	0	0.0	55	16.1
Total	36	100.0	342	100.0

Distributions of drug type and type of cell line used and IC50 results of preclinical cell experiments identified in the search.
Q, quartile; nM, concentration in nanomoles.
**Studies with missing values of IC50 were not included in analyses.*

In a final set of analyses of these data, we compared odds of approval among ordinal categories of drugs defined by IC50 quartile. Compared to drugs in the highest quartile (Q4, IC50 345.01–100,000 nM), those in the third quartile differed little, but those in the lower two quartiles had somewhat better odds of being approved. Most favorable results were for drugs in the second quartile (Q2, IC50 3.92–30 nM) for which the estimate from conventional logistic regression was OR = 2.84 (95%CI 1.04–7.74). However, results from the more conservative GEE analysis—which accounts for possible non-independence of results from multiple experiments using the same drug—do not achieve statistical significance (OR = 2.84 [95%CI 0.60–13.54]). Neither analysis identified a statistically significant monotonic trend in effect size (**Figures 2C,D**).

Preclinical Murine Models

The search identified 144 preclinical studies using murine models of lung cancer drugs that satisfied inclusion criteria, with all published reports providing sufficient experimental data to use in our analyses (**Supplemental Table 2**). The measure of efficacy used in these experiments was TGI. **Table 2** summarizes the studies according to type of drug and mouse model, TGI measure employed, and quartile of TGI efficacy among results of all studies.

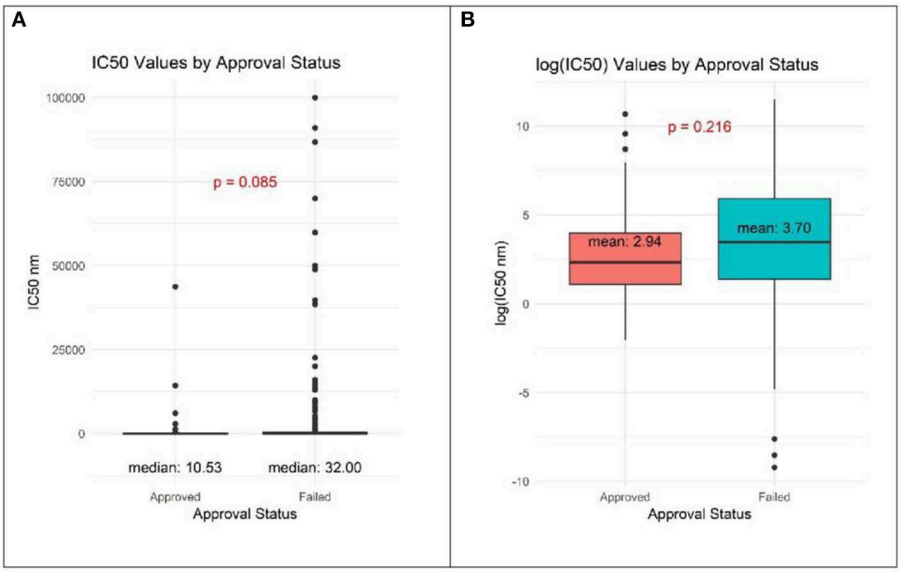

FIGURE 1 | Distributions of IC50 values in preclinical cell line experiments among drugs that were subsequently approved or failed. **(A)** IC50 values and **(B)** $\log_{(IC50)}$.

TGI values ranged from −0.3119 to 1.0000, with a tendency for approved drugs to demonstrate slightly poorer inhibition than drugs that failed to be approved. The respective medians were 0.74 and 0.77, a small difference that does not achieve statistical significance ($P = 0.375$, **Figure 3A**). Analyses comparing success of drugs according to quartile of TGI produced interval estimates too wide to be statistically meaningful, although all point estimates accord with drugs in each of the three highest quartiles (Q2-Q4, TGI 0.5576–0.7600, 0.7601–0.9364, 0.9365–1.0000) having lower odds of success than those in lowest quartile (Q1, –0.3119–0.5575) (**Figure 3B**). In accordance with these results, the AUC estimate was 0.45, (**Figure 3C**), corresponding to TGI value performing slightly worse than chance for predicting eventual success of a drug.

DISCUSSION

We endeavored to quantitatively investigate predictive value of publicly available results from preclinical studies of lung cancer drugs, conducted over nearly two decades. This novel effort identified no value of efficacy parameters that predicted approval of lung cancer drugs.

The current FDA guidelines require animal testing prior to human exposure (7), with the hope that preclinical results may be mimicked in human subjects. Unfortunately, most successful preclinical testing falls short of expectations, with only a third of preclinically approved drugs entering clinical trials (8) at a failure rate of 85% (all phases included), and a 50% success rate in the fraction of therapeutic agents that make it past phase III (9). Anti-cancer agents account for the largest proportion of these failures (10). Flawed methodologies in clinical trial testing may be contributing to the disparity in preclinical and clinical success. Clinical factors such as variability in tumor response to different drug classes, may affect approval status. Pseudoprogression, described in clinical trials of immunotherapy agents as the appearance of new lesions or increase in primary tumor size followed by tumor regression, is an atypical tumor response seen with certain drugs that may have performed differently in preclinical experiments; this phenomenon has not been well-described in cell or murine models. Pseudoprogression may affect progression-free survival as the primary endpoint of immunotherapy trials, and development of immune-specific response criteria such as irRECIST (immune-related RECIST) are being incorporated into more recent studies (11). Another trial design flaw has been discussed in studies of chemotherapy agents in patients with CNS metastases from solid tumors. Several clinical trials exclude patients with brain metastases due to lack of drug activity in the CNS shown in prior studies. This exclusion criteria eliminates up to two-thirds of patients with stage IV disease. However, including such patients may reduce reported efficacy endpoints (progression-free survival and overall response rate) if patients develop early CNS progression, and thus prevent drugs from obtaining approval status (12). Additionally, it is estimated that animal studies overestimate by 30 percent the likelihood of treatment efficacy due to unpublished negative results (13). The poor positive predictive value of successful preclinical testing has been attributed largely to disparity between disease conditions in mice and humans. The nature of the animal model and laboratory conditions, which are currently not standardized, may also contribute to variations in animal responses to therapeutic agents (14).

There have been several published examples of successful cancer drug testing in animal models leading to failed clinical trials. A notable failed targeted therapy is saridegib (IPI-926), a Hedgehog pathway antagonist that increased survival in mouse models with malignant solid brain tumors (15), but had no

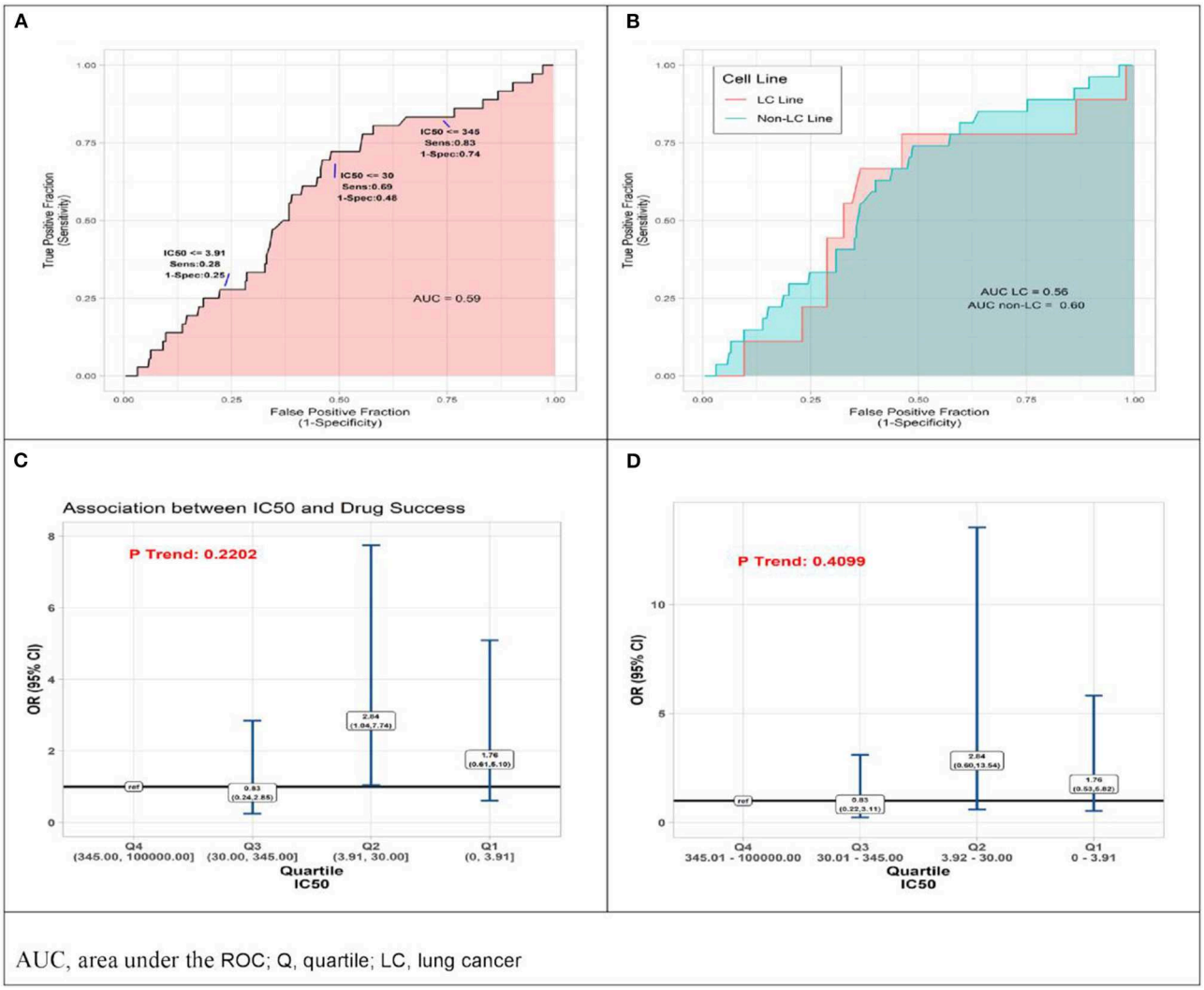

FIGURE 2 | Results of quantitative analyses of preclinical cell line experiments. **(A)** Receiver operator curves (ROC) displaying accuracy of IC50 value as predictor of drug approval for all cell line experiments combined, and **(B)** within subsets defined by type of cell line, lung cancer cell lines (pink) non-lung cancer cell lines (aqua). **(C)** Odds Ratio (OR) associations between drug success and quartile of IC50 result of preclinical cell model experiment, drugs with IC50 in lower quartiles (Q) Q1, Q2, Q3 compared to those with IC50 in highest quartile, Q4 (reference) by two analytic methods, conventional logistic regression, and **(D)** General Estimating Equation (GEE), allowing for non-independence of multiple experiments using the same drug.

significant effect compared to placebo in patients with advanced chondrosarcoma participating in a Phase II randomized clinical trial (16). Another immunomodulatory agent, TGN1412, was tested for safety in preclinical mice models and did not lead to toxicities in doses up to 100 times higher than the therapeutic dose in humans (17). However, when the drug advanced to Phase I testing, trial participants experienced multisystem organ failure and cytokine storm even with subclinical doses (18). Anti-cancer vaccines have had similar issues in translating efficacy to human clinical trials. While therapeutic vaccines have successfully raised an immune response in mice, their effects in humans have been circumvented by immunological checkpoints and immunosuppressive cytokines that are absent in mice (19). Examples of failed vaccines include Stimuvax, which had failed a non-small cell lung cancer phase III trial (20), and Telovac, which failed in a pancreatic cancer phase III trial (21).

The results of our study underscore the need for alternatives to classic cell culture and animal-based preclinical experiments. Human autopsy models have been used to test drugs in their early stages of development to mimic human physiological responses. *In silico* computer modeling may be a more accurate replacement to *in vitro* models, and involves implantation of cells onto silicon chips and using computer models to manipulate the cells' physiologic response to agents and various parameters in the microenvironment (22).

Given the track record of successful preclinical testing leading to failed clinical trials, efforts have been made to push forward direct testing in humans. In 2007, the European Medicines Agency and FDA proposed guidelines for bypassing preclinical

TABLE 2 | Distributions of preclinical animal study data ($n = 213$).

	Approved		Failed	
	n	%	*n*	%
TGI* measure types:				
Tumor volume	28	66.7	89	52.0
Tumor weight	5	11.9	12	7.0
Other	3	7.1	11	6.4
Missing	6	14.3	59	34.5
Total	42	100.0	171	100.0
Quartile of TGI				
Q1 [−0.3119, 0.5575]	10	23.8	26	15.2
Q2 [0.5576, 0.7600]	9	21.4	28	16.4
Q3 [0.7601, 0.9364]	9	21.4	26	15.2
Q4 [0.9365, 1.0000]	8	19.0	28	16.4
Missing	6	14.3	63	36.8
Total	42	100.0	171	100.0
Drug type				
Immunotherapy (including vaccines)	0	0.0	11	3.7
Nucleic acid damaging agents	18	42.9	46	28.7
Cell signaling interrupting agents	24	57.1	84	50.0
Tumor microenvironment and VEGF agents	0	0.0	29	17.6
Missing/NA	0	0.0	1	0.0
Total	42	100.0	171	100.0
Mouse type				
Xenograft on nude mouse	42	100.0	148	94.4
Spontaneous tumor model	0	0.0	4	2.8
Orthotopic model (same origin site of tumor)	0	0.0	5	2.8
Induced tumor model (chemical, radiation, genetic, etc)	0	0.0	1	0.0
Missing/NA	0	0.0	13	0.0
Total	42	100.0	171	100.0

**TGI, tumor growth inhibition, a measure of efficacy estimated as described in Methods.*

testing and using micro-doses of therapeutic agents in humans (23). The doses used in these "phase 0" studies are only a small fraction of the therapeutic dose, which are considered safe enough to bypass the usual testing required prior to phase I testing. Administering these micro-doses would help elucidate characteristics in drug distribution, pharmacokinetics, metabolism, and excretion in humans. Ideally, any new model that seeks to predict drug efficacy in cancer should be evaluated on the basis of its ability to predict clinical success and clinical failure. The widespread adoption of new preclinical models should ideally be accompanied by some measure of the model's ability to predict clinical success as well as failure.

There were limitations to our study that should be acknowledged. Despite the large number of preclinical studies of lung cancer in the public domain, data on features of study design were inadequate. Analyses of cell culture data stratified on whether cells originated in lung cancer provided no indication that lung cancer cells constitute more predictive models; however, only nine studies of approved drugs were conducted in cell lines of this type. Data on other features of cell and mouse models were too sparse to support even exploratory analysis of their predictive value. Another limitation is that some studies could not be included in the analysis owing to missing efficacy values. All of these were studies of failed drugs, and if efficacy values in the missing studies differed notably from those in studies included in our analysis, our results could obscure some true predictive value of the IC50 or TGI. However, notably different distributions of this nature seem unlikely, because all drugs—whether included or excluded for missing values—demonstrated a degree of preclinical efficacy that allowed them to advance to human studies. Regarding TGI efficacies, there were limitations in determining a standardized measure of efficacy for mouse models given the lack of standardized criteria on calculating drug effects in mice. The reported TGI values are based on raw tumor volumes extracted from tumor growth inhibition curves (if provided by articles) and applied to the equation as stated in the Methods, or reported TGI values derived from the same equation. A portion of articles used increase in life span as the measure of efficacy or a quantifiable effect on a molecular target, which were difficult to incorporate into the regression analysis used in this study and were thus excluded. While we attempted to maximally standardize the TGI measure, our reported ability of TGI to predict clinical trial success was lower than chance; this was likely a result of artifact given how variable the TGI measure was across all studies reported in the literature. Due to the naturally low proportion of approved compared to failed drugs, there is a sparse amount of data available for the former drug category, and thus any comparisons between the two drug classes may not be as robust. In addition, the approved drug category was lacking in immunotherapy agents as this study evaluated drugs in the pre-immunotherapy era. It is also important to recognize that there are other preclinical factors, such as drug toxicity, that play a major role in determining a drug's approval or failure status and were not accounted for in the preclinical efficacy endpoints of our study. Therefore, the conclusion that existing preclinical models lack value in predictability of drug approval must be interpreted with these limitations and variability across drug classes in mind.

It is important to note that when not accounting for the multiple studies per drug, we observed a significant association between efficacy values in Q3 and approval status, relative to values in Q1. There are three important points to note with these IC50 results. (1) In the cell experiments, we analyzed the data using two methods, one that accounts for the multiple studies per drug and one that ignores this characteristic of the data. Both methods have their limitations in this context and the truth likely lies between these two measures. (2) We would expect the relationship between drug approval and IC50 values to be characteristic of a monotonic relationship, meaning lower IC50 values correspond to greater odds of approval. In contrast to the individual quartile estimates, the trend statistics best capture the presence of this monotonic relationship, and

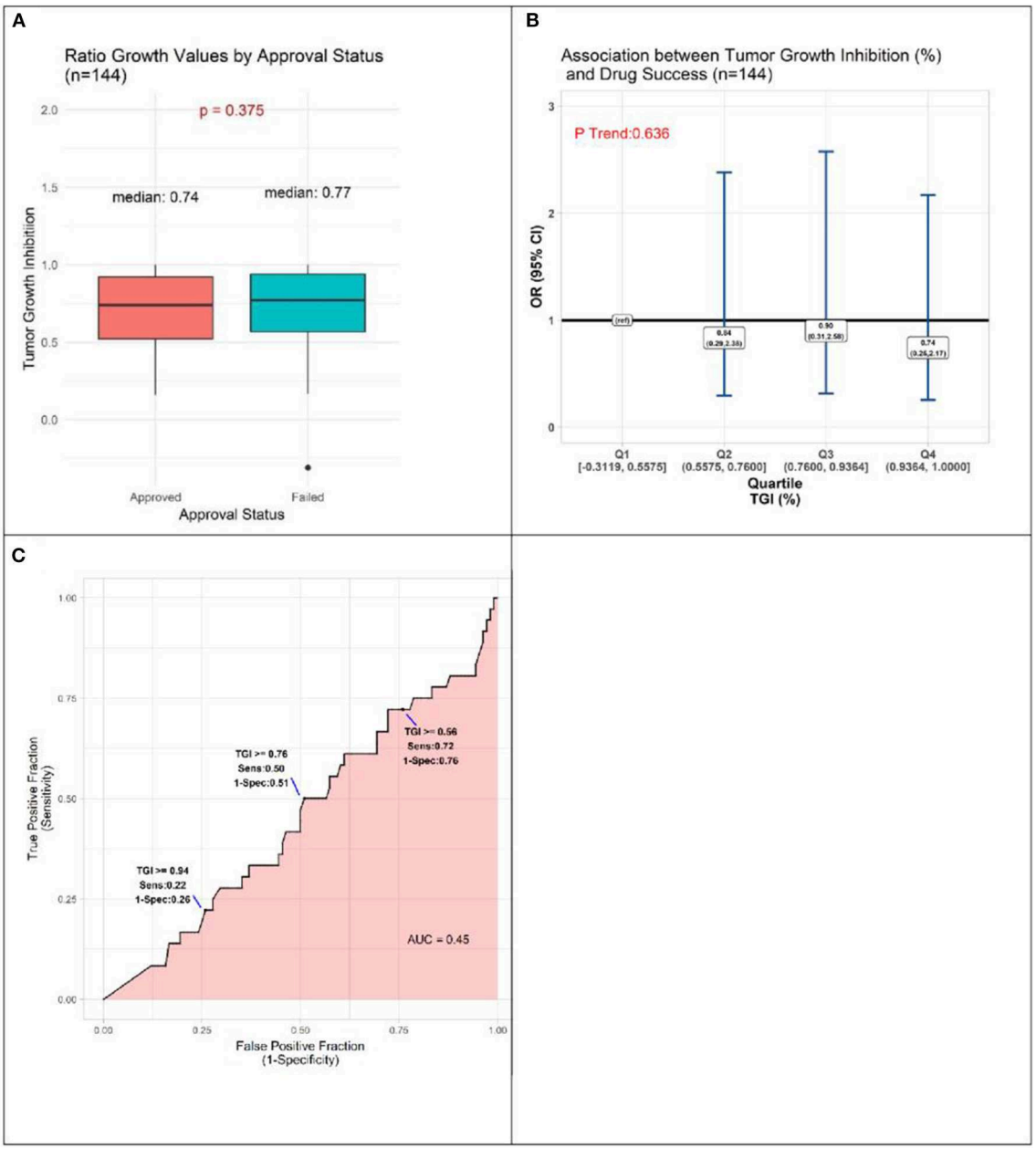

FIGURE 3 | Results of quantitative analyses of preclinical studies in murine models. **(A)** Box-plots displaying distributions of tumor growth inhibition (TGI) in preclinical murine models of subsequently approved and failed drugs. **(B)** Odds Ratio (OR) estimates of association between drug success and TGI result of preclinical animal model experiments, drugs with TGI in each of quartiles (Q) Q1, Q2, Q3 compared to those with TGI in Q4 (reference). **(C)** Receiver operator curve displaying accuracy of TGI value as predictor of drug approval.

in this study we should more heavily weigh the evidence from these statistics relative to the quartile measures. Both p trend statistics show the absence of a significant relationship between IC50 values and odds of drug approval. (3) **Figures 1**, **2A,B**, agree with the absence of (or a weak) relationship between IC50 and approval status.

In conclusion, the findings of this study on preclinical testing of lung cancer therapies are consistent with prior concerns that cell and animal models are inadequate for identifying drugs that warrant human testing. Unfortunately, we found no evidence that either limiting *in vitro* models to cell lines derived from lung cancer or accepting narrower ranges of efficacy parameters is likely to improve performance of these conventional approaches. New models backed by evidence of their ability to predict clinical success and failure are needed.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary material**.

AUTHOR CONTRIBUTIONS

EP: project conception, data collection, data analysis, and manuscript writing. DB: data analysis and manuscript editing. VC: data analysis, manuscript editing, and supervision of analysis. SY: data collection. JN: project conception, project supervision, data analysis, and manuscript editing.

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This article has been released as a pre-print at bioRxiv (24).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00591/full#supplementary-material

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Clinical Characteristics and Treatment Outcomes of 65 Patients With BRAF-Mutated Non-small Cell Lung Cancer

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BRAF mutation is an oncogenic driver gene in non-small cell lung cancer (NSCLC) with low frequency. The data of patients with NSCLC harboring BRAF mutations is rare. We conducted a retrospective multicenter study in Chinese patients with NSCLC harboring BRAF mutations between Jan 2017 and Jul 2019. A total of 65 patients treated in 22 centers were included, 54 harbored BRAF-V600E mutation and 11 had non-V600E mutations, including K601E, G469S, G469V, G469A, G596R, G466R, and T599dup. Of 18 patients with early-stage disease at diagnosis and underwent a resection, the median disease-free survival (DFS) was 43.2, 18.7, and 10.1 months of stage I, II, and IIIA patients, respectively. In 46 patients with advanced-stage disease at data cutoff, disease control rate (DCR), and progression-free survival (PFS) of first-line anti-BRAF targeted therapy was superior than chemotherapy in patients harboring BRAF-V600E mutation (DCR, 100.0 vs. 70.0%, $P = 0.027$; median PFS, 9.8 vs. 5.4 months, $P = 0.149$). Of 30 V600E-mutated patients who received anti-BRAF therapy during the course of disease, median PFS of vemurafenib, dabrafenib, and dabrafenib plus trametinib was 7.8, 5.8, and 6.0 months, respectively ($P = 0.970$). Median PFS were similar between V600E and non-V600E patients (5.4 vs. 5.4 months, $P = 0.825$) to first-line chemotherapy. Nine patients were treated with checkpoint inhibitors, with median PFS of 3.0 months. Our data demonstrated the clinical benefit of anti-BRAF targeted therapy in Chinese NSCLC patients harboring BRAF-V600E mutation. The value of immunotherapy and treatment selection among non-V600E population needs further study.

Keywords: BRAF mutation, chemotherapy, immunotherapy, non-small cell lung cancer, targeted therapy

INTRODUCTION

Lung cancer is one of the most common cancers and remains the leading cause of cancer-related death worldwide (1). The successful applying of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) in non-small cell lung cancer (NSCLC) patients who harbored EGFR mutations has dramatically changed the therapeutic approach of lung cancer and led to a more individualized treatment era. Patients with oncogenic driver mutations may benefited in driver

gene inhibitors rather than cytotoxic chemotherapy. V-raf murine sarcoma viral oncogene homolog B1 (BRAF) mutation is one of oncogenic driver mutation in NSCLC, which phosphorylates the downstream effectors MEK and ERK to promote cell proliferation and survival (2). BRAF mutations occur with a low prevalence of only 2–5% in Caucasian lung cancers, and V600 mutations (amino acid substitution for valine at position 600) accounted for ∼50%, with the rest of cases harbor non-V600 mutations (3–5). BRAF inhibitors (BRAFi) and MEK inhibitors (MEKi) have been demonstrated impressive efficacy in patients with advanced stage NSCLC harboring BRAF V600E mutation. Monotherapy BRAFi vemurafenib showed an objective response rate (ORR) of 43% in patients with refractory BRAF V600E-mutated NSCLC in the "MyPathyway" basket study (6). In an open-label, phase 2 trial, BRAFi dabrafenib plus MEKi trametinib performed an ORR of 64%, and median progression-free survival (PFS) of 10.9 months (95% confidence interval [CI] 7.0–16.6) in patients with previously untreated BRAF V600E-mutant metastatic NSCLC (7). Unlike the definite and promising efficacy of targeted therapies to BRAF V600E-mutant cases, the benefit of targeted agents on various non-V600 mutations were questionable, as each specific non-V600 mutation occurred in a much smaller population thus there were few studies on this topic. In EURAF cohort, five of six patients who harbored non-V600E mutations appeared to be resistant to BRAFi therapies (8). The prevalence of BRAF mutation was even lower in Chinese NSCLC patients with reported of 0.5–2% (9, 10). Considering the difference in genetic background between Caucasians and Asians, studying the BRAF mutation of NSCLC in Asians is of great significance. Clinical efficacy of chemotherapy and targeted therapy in Chinese patients with NSCLC harboring BRAF mutations are not well-explored due to their low prevalence, especially for those with non-V600 mutations, thus none of BRAFi has been approved for BRAF-mutated NSCLC in China. In addition, BRAFi plus MEKi was theoretically efficient in patients progressed of BRAFi monotherapy, but with fewer actual clinical data. Moreover, immune checkpoint inhibitors were increasingly used in clinical practice in China as monotherapy or in combination with chemotherapy, while its efficacy in BRAF-mutated patients is still an unmet area. Therefore, we performed this retrospective study to evaluate the association of BRAF mutations with clinical characteristics and treatment outcomes in Chinese NSCLC patients in the real-world.

MATERIALS AND METHODS

Patient Recruitment and Data Collection

Patients were retrospectively recruited through a patient community. Potential subjects could contact study recruiter individually for more details about the study and eligibility screening. The inclusion criteria included (i) patients were histologically or cytologically diagnosed with NSCLC and were detected harboring BRAF mutation between Jan 2017 and Jul 2019. (ii) BRAF mutation was detected using a next-generation sequencing (NGS) technique, which also provided molecular profile of EGFR, KRAS, ALK, MET, ROS1, HER2, RET, PIK3CA, and NTRK status as well. Patients with a BRAF mutation that never received a treatment for stage IV disease were also included in our study for baseline characteristics analysis. Patients who tested positive for EGFR, ALK, MET, ROS1, or RET, and those who acquired BRAF mutation after resistance to therapies targeting another oncogenic driver gene were ineligible. After receiving study subjects' oral consent, qualified patients were asked to provide their medical records for data collection. To ensure the quality of study data, all medical data were reviewed, and entered by a board-certified oncologist with thoracic expertise from Cancer Hospital of Chinese Academy of Medical Sciences. By the end of July 2019, a total of 65 NSCLC patients with BRAF mutation treated in 22 hospitals in China were included in our analysis. Medical data of age, gender, smoking history, Eastern Cooperative Oncology Group performance status (ECOG PS), histology, stage, BRAF mutation type, and treatment history were retrospectively recorded. Age, smoking status, and ECOG PS were recorded at initial diagnosis. Stage of disease was determined according to the American Joint Committee on Cancer (AJCC) staging system, 8th edition. The study was approved by the Ethics Committee at Cancer Hospital of Chinese Academy of Medical Sciences, and was conducted in accordance with the Helsinki Declaration.

Assessments

The primary objective of our study was to evaluate the efficacy of chemotherapy, anti-BRAF targeted therapy, and immunotherapy in patients with BRAF-mutated NSCLC. The primary endpoints were disease control rate (DCR) and PFS. Tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1). DCR was defined as the percentage of patients who achieved complete response (CR), partial response (PR), or stable disease (SD), while ORR referred to CR and PR. PFS was defined as the time from the date of a systemic treatment regimen (chemotherapy, targeted therapy, or immunotherapy) initiation till date of progressive disease (PD) or death from any causes whichever occurred first. Secondary endpoints were DFS of patients who was diagnosed with early-stage disease at initiation and safety profile of anti-BRAF targeted therapy. DFS was measured from the date of resection to recurrent or metastases.

Statistical Analysis

The distribution of patients' baseline characteristics was described. Difference of ORR and DCR between groups were compared using Fisher's exact tests and chi-square tests. Survival analysis was performed using the Kaplan–Meier method, and compared by the log rank test. Two-sided $p < 0.05$ was indicated statistically significant. All statistical analysis was carried out using the SPSS statistical software, version 23.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Patients Characteristics

A total of 65 patients with BRAF mutation were included in our study. All patients were Chinese, 31 were male

TABLE 1 | Baseline characteristics of BRAF mutated NSCLC patients ($n = 65$).

Characteristics	No. of patients (%)		
	All ***N* = 65**	**V600E** ***n* = 54**	**Non-V600E** ***n* = 11**
Age, years			
Median	58	57.5	58
Range	33–79	33–78	46–79
Sex			
Male	31 (47.7)	23 (42.6)	8 (72.7)
Female	34 (52.3)	31 (57.4)	3 (27.3)
ECOG PS			
0–1	56 (86.2)	49 (90.7)	7 (63.6)
≥2	9 (13.8)	5 (9.3)	4 (36.4)
Smoking status			
Non-smoker	30 (46.2)	28 (51.9)	2 (18.2)
Former/current smoker	35 (53.8)	26 (48.1)	9 (81.8)
Histology			
Adenocarcinoma	64 (98.5)	53 (98.1)	11 (100.0)
Others	1 (1.5)	1 (1.9)	0 (0.0)
Stage at diagnosis			
0	1 (1.5)	0 (0.0)	1 (9.1)
I	10 (15.4)	9 (16.7)	1 (9.1)
II	5 (7.7)	4 (7.4)	1 (9.1)
IIIA	3 (4.6)	3 (5.6)	0 (0.0)
IIIB-IV	46 (70.8)	38 (70.4)	8 (72.7)
Co-occurring mutation			
TP53	4	2	2
PIK3CA	6	6	0
KRAS	1	0	1
NTRK1	1	1	0

and 34 were female with a median age of 58 (range, 33–79). Thirty-five patients (53.8%) were former or current smokers. Most patients had ECOG PS of 0 or 1 (86.2%) and stage IIIB to IV disease (46/65, 70.8%) at diagnosis. Sixty-four were adenocarcinomas and one was squamous cell carcinoma. In 18 early-stage patients who underwent pulmonary surgery, micropapillary component was observed in five patients (27.8%), and these micropapillary feature was only observed in V600E mutated patients. Patient characteristics are summarized in **Table 1**.

Eight BRAF mutation genotypes were identified, 54 patients had BRAF-V600E mutation (83.1%) and 11 (16.9%) had non-V600E mutations, including K601E (6.2%, $n = 4$), G469S (1.5%, $n = 1$), G469V (1.5%, $n = 1$), G469A (1.5%, $n = 1$), G596R (1.5%, $n = 1$), G466R (1.5%, $n = 1$), and T599dup (3.1%, $n = 2$). Nine of 54 patients with a BRAF-V600E mutation had concomitant mutation in TP53 ($n = 2$), PIK3CA ($n = 6$) or NTRK1 ($n = 1$), and concurrent TP53 ($n = 2$) or KRAS mutation ($n = 1$) were identified in 3 of 11 patients with BRAF non-V600E mutations (**Table 1**). The frequency of co-alterations was similar in BRAF-V600E mutated patients and in non-V600E mutated population (16.7 vs. 27.3%, $P = 0.689$).

Eleven patients harbored non-V600E mutations, with median age of 58. Twenty-three (42.6%) of 54 BRAF-V600E patients and 8 of 11 (72.7%) non-V600E patients were male, respectively ($P = 0.068$). Twenty-six (48.1%) of 54 BRAF-V600E patients and 9 of 11 (81.8%) non-V600E patients were smokers, respectively ($P = 0.041$). There was no significant difference in age and histology distribution between patients with BRAF-V600E and non-V600E mutations.

Clinical Outcomes

DFS in Early-Stage Patients

Among overall 65 patients in our study, 1 was stage 0, 10 were stage I, 5 were stage II, 3 were stage IIIA, and 46 were advanced stage (IIIB-IV) at diagnosis, the median follow-up time was 9.2 months. At data cutoff (Jul 31, 2019), 8 of 18 recurrences (44.4%) had occurred in patients who had early-stage disease at diagnosis and underwent a resection, among whom seven had distant metastasis while only one performed locoregional recurrence. The site of relapse included lung ($n = 2$), brain ($n = 2$), bone ($n = 2$), mediastinal lymph nodes ($n = 2$), supraclavicular lymph nodes ($n = 2$), pleura ($n = 1$), and adrenal gland ($n = 1$). The median DFS after surgery of early-stage cancers was 43.2 months of stage I, 18.7 months of stage II, and 10.1 months of stage IIIA patients ($P = 0.07$), respectively (**Figure 1A**). One patient with stage II disease was excluded as he did not undergo resection.

Clinical Outcomes of First-Line Treatment

In 46 patients with advanced stage BRAF-V600E mutated NSCLC at data cutoff, 25 patients received chemotherapy in the first-line (19 with pemetrexed-contained regimen, 5 with paclitaxel-contained regimen, 1 with gemcitabine-contained regimen), while only 16 patients received anti-BRAF targeted therapy as the first-line choice (9 with vemurafenib, 2 with dabrafenib, 5 with dabrafenib plus trametinib). Twenty and 15 patients were evaluable for response analysis in chemotherapy and targeted therapy subgroups, respectively. Of patients who received chemotherapy in response analysis set, 5 patients had PR, 9 had SD, and 6 had PD, with ORR of 25.0%. Among patients treated with targeted therapy, 10 patients had PR, 5 had SD, and ORR was 66.7%. DCR of first-line targeted therapy was higher than that of chemotherapy in patients with BRAF-V600E mutated NSCLC (100.0 vs. 70.0%, $P = 0.027$). The median PFS of patients with BRAF-V600E mutation who received first-line targeted therapy was also longer than chemotherapy, but the difference did not achieve statistical significance (9.8 months [95%CI, 0.4, 19.2] vs. 5.4 months [95%CI, 0.0, 14.1], $P = 0.149$) (**Figure 1B**).

Within BRAF non-V600E subgroup, pemetrexed-contained regimen was the most widely used first-line treatment regimen (7/9, 77.8%). Five of seven (71.4%) measurable patients had SD, and 2 had PD. None of them received targeted therapy in the first-line. No significant differences of ORR and DCR were observed in patients with V600E and non-V600E mutation who were treated with first-line chemotherapy (ORR, 25.0 vs. 0.0%, $P = 0.283$; DCR, 70.0 vs. 71.4%, $P = 1.000$). The median PFS of first-line chemotherapy was also similar between patients with V600E mutation vs. those with non-V600E mutation (5.4 months

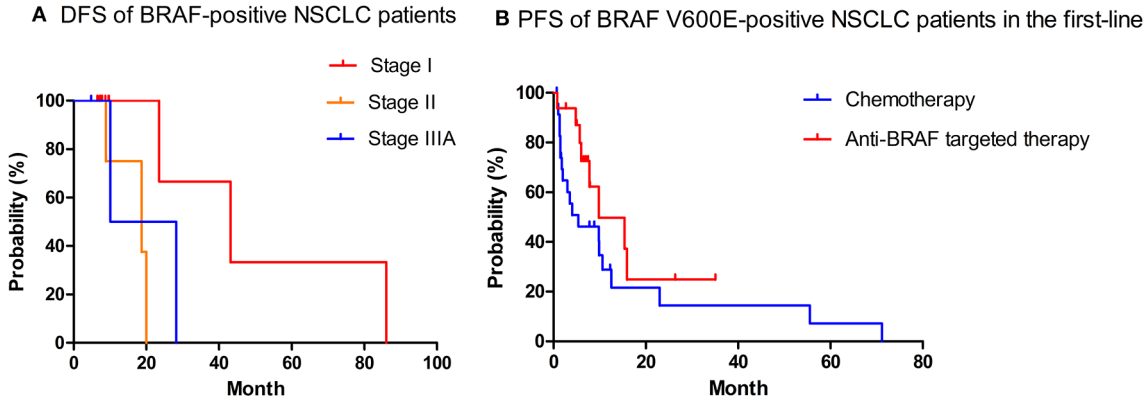

FIGURE 1 | DFS of early-stage BRAF-positive NSCLC patients **(A)**, PFS of first-line regimens in patients with BRAF-positive NSCLC **(B)**. DFS, disease-free survival; PFS, progression-free survival. Tick marks indicate censored observations.

TABLE 2 | Efficacy of first-line treatment strategies in patients with BRAF mutation.

Treatment strategies in first-line	V600E		Non-V600E	
	DCR	PFS months, (95%CI)	DCR	PFS months, (95%CI)
Pemetrexed-contained chemotherapy	11/14, 78.6%	5.4 (1.7, 9.1)	5/7, 71.4%	5.4 (1.3, 9.5)
Paclitaxel-contained chemotherapy	2/5, 40.0%	1.5 (1.1, 1.9)	–	–
Vemurafenib	9/9, 100.0%	9.8 (0.7, 18.9)	–	–
Dabrafenib	1/1, 100.0%	–	–	–
Dabrafenib + Trametinib	5/5, 100.0%	NR	–	–

DCR, disease control rate; PFS, progression-free survival; NR, not reached.

[95%CI, 0.0, 14.1] vs. 5.4 months [95%CI, 1.3, 9.5], $P = 0.825$). The efficacy of first-line regimens in patients with BRAF mutated advanced NSCLC was shown in **Table 2**, **Figure 2**.

For patients who performed multiple mutations, patients with co-occurring mutations in TP53 had a trend of shorter PFS of first-line treatment compared with those without TP53 mutation (median PFS, 3.5 months [95%CI, 0.5, 6.5] vs. 9.8 months [95%CI, 4.9, 14.7], $P = 0.106$). Median PFS of patients with co-occurring PIK3CA mutations was 12.5 months (95%CI, 5.4, 19.6), as compared to 7.2 months (95%CI, 3.7, 10.7) in patients without PIK3CA mutation ($P = 0.823$).

Targeted Therapy

Thirty-two of the 55 patients with advanced stage BRAF mutated NSCLC cases were treated with anti-BRAF targeted therapy during their treatment course, among whom 30 harbored V600E mutation, 1 harbored K601E mutation and 1 harbored T599dup. The only 2 non-V600E mutated patients received dabrafenib plus trametinib after failure of pemetrexed-platinum based chemotherapy and the T599dup case performed SD while the K601E patient had PD as the best response. In 30 patients with V600E mutation, 17 patients received BRAF inhibitor as first-line treatment and 13 had anti-BRAF therapy in further lines. Thirteen patients received vemurafenib, 6 patients received dabrafenib and 9 were treated by a combination of dabrafenib and trametinib as the primary targeted therapy. The median PFS of patients receiving vemurafenib, dabrafenib, and dabrafenib plus trametinib was 7.8, 5.8, and 6.0 months, respectively ($P = 0.970$) (**Table 3**). Five patients received two different targeted regimens, including four patients treated with vemurafenib followed by dabrafenib plus trametinib, and one patient treated with dabrafenib followed by dabrafenib plus trametinib. Efficacy of BRAFi plus MEKi after the failure of BRAFi monotherapy was generally very poor. Four of five patients showed PD, aside from 1 had a SD of dabrafenib plus trametinib after vemurafenib, with PFS of only 2.9 months.

The safety analysis was conducted in patients who received anti-BRAF targeted therapy in the treatment course. For patients treated with vemurafenib, the most common adverse events (AEs) were arthralgia and rash. Four events of grade 3 AEs were observed, including arthralgia, rash and hand-foot syndrome. Dose reductions or interruptions of vemurafenib occurred in 6 (46.2%) patients. AEs of dabrafenib observed including fatigue, pyrexia, rash, mucositis oral, and anemia. One (16.7%) of 6 patients had AEs that led to dabrafenib dose reduction and subsequent dose interruption (grade 2 pyrexia and grade 3 rash). The most common AE among patients receiving dabrafenib plus trametinib regimen was pyrexia, and 4 (36.4%) patients had AEs that led to dose reductions or interruptions. No anti-BRAF targeted therapy-related deaths was observed in our study. AEs of each targeted regimen were shown in **Table 4**.

Immunotherapy

Nine patients were treated with checkpoint inhibitors monotherapy or in combination with chemotherapy or anti-angiogenic treatment (6 of V600E, 3 of non-V600E). Two (25.0%) of 8 patients with measurable disease by RECIST 1.1 had PR, 3 (37.5%) had SD, and 3 (37.5%) had PD. Seven (77.8%)

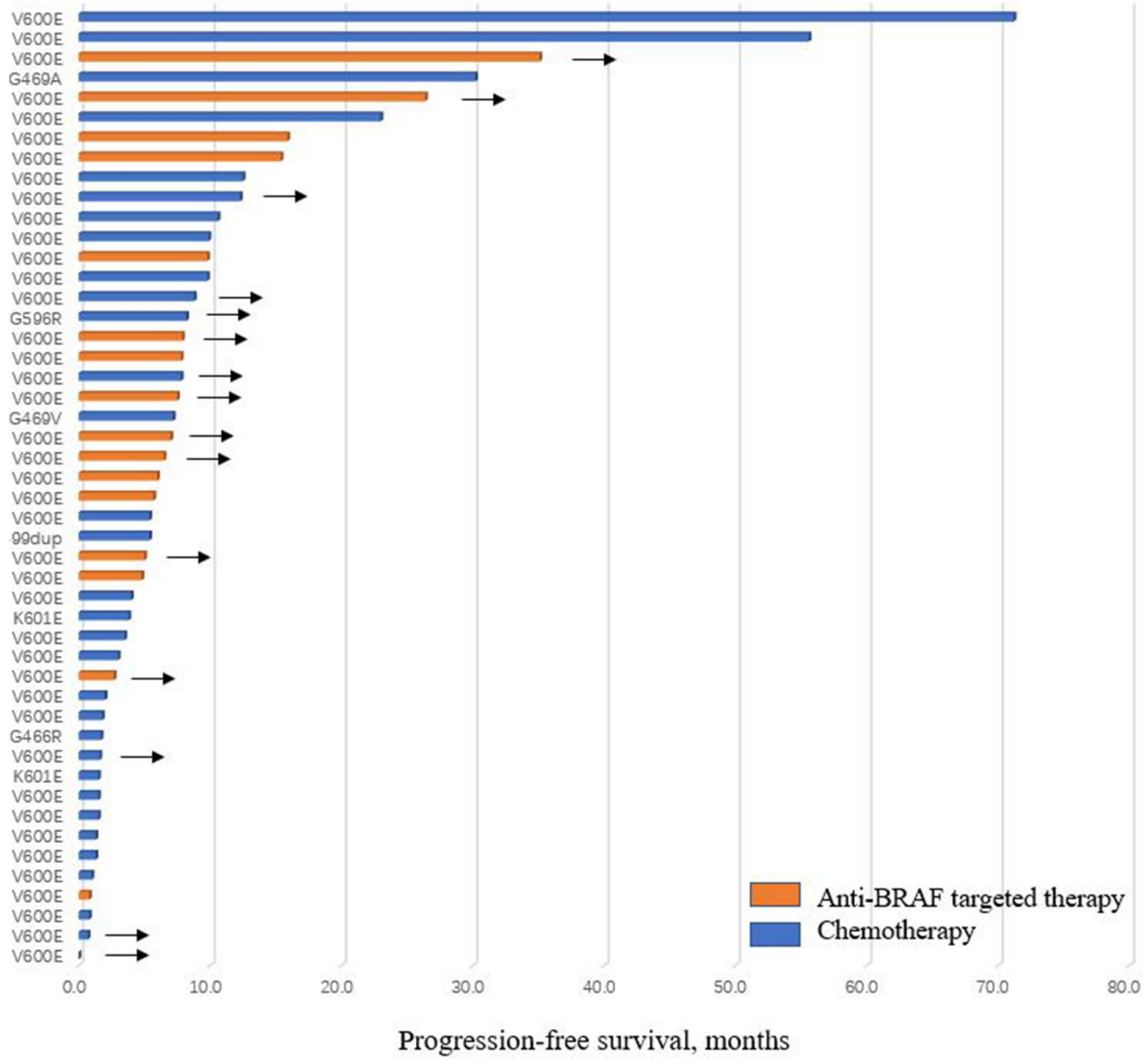

FIGURE 2 | Progression-free survival of patients treated with chemotherapy or anti-BRAF targeted therapy in the first-line. Arrows indicate patients did not progress at last follow-up.

patients progressed on immunotherapy by the time of the analysis. Median PFS was 3.0 months (95%CI 2.9, 3.1). The 2 patients with PR had PFS of 8.9 and 3.0 months, respectively. From the 3 patients with non-V600E, one with K601E had SD with nivolumab plus chemotherapy, while the other two with T599dup and G466R had PD with pembrolizumab and nivolumab plus anlotinib, respectively (**Table 5**). Seventeen of 65 patients tested programmed death ligand 1 (PD-L1) or tumor mutational burden (TMB) during the course of disease, among whom six received checkpoint inhibitors. Considering the various antibody and NGS panel used for PD-L1 and TMB testing of study patients, the relation of these biomarkers, BRAF mutation and treatment efficacy was not analyzed.

DISCUSSION

BRAF mutation was well-reported in papillary thyroid cancer, colorectal cancer, and melanoma, but not NSCLC in Chinese population due to its low prevalence. Some studies have reported the clinical and pathologic characteristics of NSCLC patients harboring BRAF mutations, our study mainly explored the treatment pattern and clinical outcomes of various BRAF genomic subtype among these patients.

BRAF mutation occurred in 0.5–2% of Chinese NSCLC patients (9, 10), which was lower than 2–5% in Caucasian lung cancers (3, 5, 11, 12). Our results showed that BRAF mutations are mostly performed in adenocarcinoma. The prevalence of

TABLE 3 | Efficacy of primary targeted therapy in patients with BRAF V600E mutation.

	Vemurafenib	Dabrafenib	Dabrafenib + Trametinib
First-line	9	2	5
Further-line	4	4	4
Evaluable for response analysis	13	5	9
DCR	12/13, 92.3%	5/5, 100.0%	9/9, 100.0%
PFS, months (95% CI)	7.8 (3.9, 11.7)	5.8 (0.2, 11.4)	6.0 (5.0, 7.0)

DCR, disease control rate; PFS, progression-free survival.

TABLE 4 | Adverse events of targeted therapy.

Type of AE	AE Grade	Vemurafenib *N* = 13	Dabrafenib *N* = 6	Dabrafenib + Trametinib *N* = 11
Pyrexia	1	2	0	4
	2	1	1	0
	3	0	0	1
Arthralgia	1	4	0	1
	2	1	0	1
	3	2	0	1
Rash	1	5	0	1
	2	1	0	0
	3	1	1	0
Hand-foot syndrome	1	1	0	0
	2	2	0	1
	3	1	0	0
Fatigue	1	4	2	1
	2	1	0	1
Pneumonitis	1	0	0	0
	2	0	0	2
Loss of appetite	1	1	0	1
Mucositis oral	1	0	1	0
Nausea	1	2	0	0
Alopecia	1	3	0	0
ALT increased	1	1	0	0
White blood cell decreased	1	1	0	0
Anemia	1	0	1	0
Diarrhea	1	0	0	1

AE, adverse events; ALT, glutamate pyruvic transaminase.

BRAF-V600E mutations is 83.1%, which was consistent with previously reported in Chinese patients (10), while higher than Caucasian population of ∼50% (3, 4, 13, 14). BRAF-V600E and non-V600E are associated with different clinical and pathologic features in our study. BRAF non-V600E mutations were more likely to be smokers and male, while V600E mutation occurred roughly equal both in gender and in smoking status, and micropapillary component was only observed in V600E-mutated population. The clinical features of gender and smoking status among BRAF-mutated NSCLC were different between studies. BRAF mutations in an Australian study occurred all in former smokers (5). Marchetti et al. suggested a significant predominance of female or never-smokers in patients harbored BRAF-V600E mutations and non-V600E mutations in smokers (3), while these studies were mostly focused on white patients. In Chinese studies, Ding et al. showed that BRAF mutations are more likely in never smokers (10), which is similar to patients with EGFR mutations. The discrepancy between studies may due to low sample size of BRAF-mutated NSCLC cases in each study and the difference of distribution of BRAF mutation subtypes between Caucasian and Asian. As for pathologic feature, a majority of BRAF-mutated NSCLC were adenocarcinomas, other histologic type such as squamous cell carcinoma and NSCLC, not otherwise specified (NOS) were also detected (5, 11, 14). The aggressive micropapillary component was a distinctive histologic feature showed partly in BRAF-V600E tumors, and in some studies, was independently associated with poor prognosis (3, 15).

Twelve (18.5%) patients harboring concurrent mutations were observed in our study, including TP53, PIK3CA, NTRK1, and KRAS mutations. The co-occurring rate among patients with BRAF-mutated NSCLC was reported as 14–16% (10, 12). Claire Tissot reported BRAF non-V600E mutations were associated with KRAS mutations in five cases who were all smokers, and suggested the concomitant KRAS mutation may be related to the carcinologic effect of tobacco (14). Whether the cooccurrence of KRAS mutation will impact response to targeted therapies is worthy of further exploration. Villaruz et al. suggested that patients with multiple mutations have inferior OS compared with those harbored single BRAF mutations (12). Additionally, it has been reported that tumors harboring TP53 mutations is associated with aggressive disease profile and worse clinical outcomes (16, 17), we also found that patients with coexisting TP53 mutation had shorter PFS of first-line treatment than those without a TP53 mutation, although the difference did not reach statistical significance. Unfortunately, due to limited cases with coexisting TP53, the clinical implications on treatment selection of such patients was not performed.

In our study, we evaluated the DFS of BRAF-mutated patients with early-stage radically resected NSCLC. Marchetti et al. reported BRAF V600E mutation was associated with a significantly shorter DFS and OS as compared to BRAF wild-type cases, suggesting a negative prognostic factor of BRAF-V600E mutation in early-stage NSCLC patients (3). Cardarella et al. also demonstrated a shorter DFS for BRAF V600-positive resected patients, while no difference between wild-type and mutation positive was observed in advanced-stage patients (11). Litvak et al. further showed that V600 mutant lung cancers performed an improved OS than non-V600 mutant cases in advanced-stage setting (13). The comparisons between studies should be made with caution as the discrepancy of baseline demographic characteristics between studies and the increasing treatment strategies as the development of medical oncology.

TABLE 5 | Characteristics of BRAF-mutated patients treated with immunotherapy.

Patient	BRAF mutation	Regimen	Treatment line	Tumor response	PFS (months)	Status at last follow-up
1	V600E	Pembrolizumab	2	PR	8.9	PR
2	K601E	Nivolumab + Chemotherapy	2	SD	3.5	SD
3	V600E	Nivolumab	1	Not measurable	3.0	PD
4	V600E	Nivolumab + Targeted therapy	3	SD	4.1	PD
5	V600E	Pembrolizumab + Bevacizumab	3	PR	3.0	PD
6	V600E	Nivolumab	2	PD	2.6	PD
7	T599dup	Pembrolizumab	2	PD	2.7	PD
8	V600E	Pembrolizumab + Targeted therapy	3	SD	5.5	PD
9	G466R	Nivolumab + Anlotinib	2	PD	2.0	PD

PR, partial response; SD, stable disease; PD, progressive disease; PFS, progression-free survival.

Chemotherapy and targeted therapy were two basic treatment strategies to BRAF-mutated patients. Cardarella et al. demonstrated a similar results of platinum-based combination chemotherapy between patients with BRAF mutation and those with wild type cancers (11). PFS of first-line pemetrexed-contained chemotherapy was equal in V600E and non-V600E subgroups in our analysis, while several studies (11) observed that response rate and PFS of platinum-based combination chemotherapy appeared a trend of favoring non-V600E population which may be attributed to the micropapillary histology of BRAF V600E-mutated population. We did not explore the association between micropapillary component and clinical outcomes considering the small sample size.

Anti-BRAF targeted therapy is the primary treatment for V600E-mutated cancers. In the NSCLC cohort of a basket study, vemurafenib achieved the ORR of 42% and median PFS of 7.3 months (95% CI, 3.5–10.8) among BRAF V600E–positive pre-treated NSCLC patients (18). The multicenter retrospective EURAF cohort explored the efficacy of known BRAF inhibitors (vemurafenib, dabrafenib, or sorafenib) in BRAF-mutated lung cancer. The median PFS and OS were 5.0 and 10.8 months, respectively, for overall anti-BRAF therapy (8). Dabrafenib was assessed in 78 pre-treated BRAF-V600E NSCLC patients, the ORR and DCR were 33% and 58%, respectively (19). BRAFi combining MEKi has proved to be more effective than single-agents for BRAF V600E-mutated lung cancers. Dabrafenib plus trametinib showed an ORR of 64% and median PFS of 10.9 months in patients with previously untreated BRAF V600E-mutant metastatic NSCLC in a phase 2 trial (7). To our knowledge, because of the low incidence rate of BRAF-mutated NSCLC in China, the efficacy and safety of anti-BRAF targeted therapy among BRAF-mutated Chinese NSCLC population in the real-world clinical practice remains unclear, the results of our study was of clinical importance. The results of our study were similar to that in clinical trials, which was superior to chemotherapy. AEs of targeted therapy were common in our study and performed diverse among patients. Arthralgia and rash were commonly observed in patients receiving vemurafenib, while pyrexia was frequently observed in dabrafenib monotherapy, or dabrafenib plus trametinib. Although it was not uncommon for patients receiving targeted therapy required dose reduction or interruption, most patients continued the doses and no severe AE was observed. Considering the superior efficacy and acceptable toxicity, anti-BRAF therapy was a better choice of first-line treatment for patients with BRAF-V600E mutated NSCLC. However, due to the limited sample size and the lack of head-to-head comparison, the specific choice among targeted agents in Chinese population was still an unmet area and could be guided by patient comorbidity and tolerability. For non-V600E mutation, the efficacy of single anti-BRAF targeted agent remains questionable. Non-V600E mutation was demonstrated lack of activity against BRAFi in clinical practice. The EURAF study (8) included six patients with non-V600E mutations, except for one harboring the G596V achieved PR with vemurafenib, the others (G466V, G469A, G469L, V600K, and K601E mutation) did not respond to BRAF inhibitors. Therefore, none patient with non-V600E mutation in our study received targeted therapy in the first-line. As non-V600E proportion in China was obviously lower than Caucasian, exploring optimal treatment strategy for such patients is even more difficult. Large-scale clinical exploration of diverse treatment strategies in this setting is warranted.

Immunotherapy is another treatment option emerging for patients with NSCLC, whereas the correlation between BRAF mutation and efficacy of immunotherapy is still unclear. Dudnik et al. (20) reported that the expression of PD-L1 was slightly higher in BRAF-mutant NSCLC than unselected population of previously reported, and a higher TMB in BRAF mutated patients was also observed. The median PFS of immunotherapy on BRAF V600E and non-V600E mutated patients was 3.7 and 4.1 months, respectively. The results seemed similar with unselected NSCLC (21, 22). Mazieres et al. (23) demonstrated that median PFS of immune checkpoint inhibitors monotherapy was significantly higher in smokers vs. never smokers (4.1 vs. 1.9 months, $P = 0.03$). In our study, a minority of patients tested PD-L1 or TMB, thus the relation of these biomarkers, BRAF mutation and treatment efficacy was not analyzed. As targeted therapy showed limited efficacy on non-V600E mutations, except for chemotherapy, investigating the efficacy of immunotherapy

is highly in needed. We listed the outcomes of checkpoint inhibitors monotherapy or in combination in our patients, only 1 harboring K601E achieved SD to immunotherapy, the other 2 (T599dup and G466R) performed no response. Due to the limited number of cases, however, the findings need to be careful interpretation. Further researches of larger sample on Chinese population are needed to assess the efficacy of immunotherapy in BRAF-mutated cases.

As a retrospective study, several limitations to our analysis should be acknowledged. Study patients were retrospectively recruited through a patients community, thus a potential of selection bias may be introduced. Additionally, we lack the independent radiological review committee to re-evaluate treatment outcomes from diverse medical centers, and thus we used DCR, not ORR as our primary endpoint. Considering the heterogeneity of the follow-up, the interpretation of the results should be carefully illuminated. Furthermore, the small number of cases with BRAF non-V600E mutations limited the ability to draw conclusions on treatment selection and the power of interpretation to our outcomes. A multicenter, prospective study among Chinese patients harboring BRAF mutation in a larger cohort is needed.

In conclusion, our study confirmed the discrepancy of clinicopathological characteristics in BRAF mutated NSCLC among Chinese population. Anti-BRAF targeted therapy is more effective than chemotherapy, with manageable toxicity among BRAF-V600E mutated Chinese patients in the first-line setting. Chemotherapy was still the dominant treatment strategy for non-V600E population, and the place of immunotherapy for these patients needs further studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by emailing corresponding authors.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

JL and PX designed the study. YM collected, analyzed and interpreted the data, and prepared the manuscript. KY, XH, YW, LW, YL, and LL collected the data. All authors read and approved the final manuscript.

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CASE REPORT
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Cancer Stem-Like Cells in a Case of an Inflammatory Myofibroblastic Tumor of the Lung

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Background: Inflammatory myofibroblast tumor (IMT) is a rare tumor with obscure etiopathogenesis in which different inflammatory cells and myofibroblastic spindle cells are seen histologically. Although the majority of these neoplasms have a benign clinical course, the malignant form has also been reported. The gold standard is surgical treatment for complete removal. Our report describes a 50-year-old woman who underwent surgery for IMT of the lung. The aim is to determine whether cancer stem cells may be present in IMT of the lung.

Methods: In April 2018, the patient underwent surgery for tumor mass asportation through lateral thoracotomy. The histology of the tumor was consistent with IMT of the lung. The ALDEFLUOR assay, after tissue digestion, was used to identify and sort human lung cancer cells expressing high and low aldehyde dehydrogenase (ALDH) activity. SOX2, NANOG, OCT-4, and c-MYC positivity were additionally determined by immunohistochemistry.

Results: The specimen contained 1.10% $ALDH^{high}$ cells among all viable lung cancer cells, which indicates the population of cancer stem cells is not negligible. Immunohistochemically assessed cell positivity for ALDH1A1, SOX2, NANOG, OCT-4, and c-MYC, which are considered as lung cancer stem-like cells markers.

Conclusion: For the first time, we demonstrated the presence of cancer stem cells in a case of IMT of the lung. This finding may provide a base for considering new pathological and molecular aspects of this tumor. This perspective suggests further studies to understand the possibility of developing recurrence depending on the presence of cancer stem cells.

Keywords: inflammatory myofibroblastic tumor of the lung, cancer stem cells, cancer stem-like cells, mitosis, target therapy

INTRODUCTION

The inflammatory myofibroblastic tumor (IMT), first described by Brunn in 1937, is an extremely rare type of inflammatory pseudo-tumor. The prevalence is between 0.04 and 0.7%, independent of gender and race (1–3). It is debated whether an IMT is a benign or malignant lesion and this is often challenging for further clinical decisions. However, the lungs are considered the most common site for the presentation of this tumor (1).

There are cases of recurrence described in the literature, not only in the lungs but also in other organs, however the recurrence rate at 10 year survival is lower than 90% (4, 5). Beside this aspect, the pathological nature of this tumor is still debated.

In 2002 the World Health Organization classified IMT as an intermediate grade malignancy (6). One of the most recent discoveries is related to chromosomal translocation involving the ALK gene, which seems to be present in 50% of cases with malignant characteristics (7). The treatment of choice for IMTs is surgical resection in order to guarantee a favorable prognosis (4).

Our work aims to detect the presence of cancer stem-like cells (CSCs) in a case of IMT of the lung by immunohistochemical testing for the most common CSCs markers, with aldehyde dehydrogenase (ALDH), as well as for the presence of pluripotent transcription factors such as OCT4, SOX-2, NANOG, and C-MYC, which modulate biological CSC activities (8–10). This will provide a base for further studies with a larger cohort of patients considering the presence of CSCs as a new pathological marker and a possible predictive factor of aggressiveness in this type of tumor.

METHODS

Case Presentation

In April 2018, a 47-year-old woman came to our attention for dyspnea and tachycardia under exertion. An x-ray of the chest showed a large mass in the left hemithorax. A CT scan with enhancement presented a 40 × 30 cm mass involving the left upper lobe of the lung. A CT guided biopsy gave a diagnosis of benign lung tumor. An Emission Tomography – Computed Tomography (PET/CT) scan showed a very mild uptake at the level of the nodule (SUV max = 2.3) with no other signs of uptake in other parts of the body (**Figure 1**).

For the symptomatology and the dimension of the mass, the patient underwent a left upper lobectomy through lateral thoracotomy. The final histology showed an IMT of the lung. The cells were positive for actin in smooth muscle, although negative for ALK, MNF116, and estrogenic receptors, as well as for tuberculosis.

Patient underwent a clinical check ten days after surgery by the oncologist who suggested a period of follow up every 6 months for the first and second year from surgery, and every year after the second year, for a total of 5 years of radiological and clinical monitoring.

Abbreviations: ALDH, aldehyde dehydrogenase; FACS, fluorescence-activated cell sorting; CSCs, cancer stem cells; SSC, side scatter; FSC, forward scattered; IMT, inflammatory myofibroblastic tumor.

Cells Extraction and FACS Analysis

Sterile Dulbecco's PBS (L1825-BC—Merck Millipore) was used to wash the IMT tissue, then minced mechanically into millimetric pieces, and further digested using MACS™ C-Tube (Miltenyi) tumor dissociation kit, according to the manufacturer's instructions. The tissue was digested for 60 min at 37°C, filtered through a sterile cell strainer and centrifuged at 300 × g for 5 min, then resuspended in a DMEM and HAM'S F12 media mixture (2:1) (Gibco) containing penicillin-streptomycin and glutamine. The primary single-cell suspension was diluted in an ALDEFLUOR buffer containing BODIPY-aminoacetaldehyde (STEMCELL Technologies, Vancouver, BC). The morphology of the cell population was studied using side scatter (SSC) and forward scatter (FSC). Dead cells were identified and eliminated using 7-AAD (7-amino-actinomycin D) staining. Cell sorting and ALDH analysis were performed using a FACS-ARIA III (Becton Dickinson, Franklin Lakes, NJ). Results were analyzed by fluorescence-activated cell sorting (FACS) Diva software (Becton Dickinson). $ALDH^{high}$ gate was included as gating strategy (11–13).

Immunohistochemistry

The patient's slides were deparaffinized, rehydrated and then washed in PBS. Sodium citrate buffer was used for antigen retrieval. Samples were incubated with anti- ALDH1A1 (1:100) (Abcam, Cambridge, UK), anti-SOX2 (1:200) (MA1-014 Thermo Fisher Scientific, Meridian Road Rockford, IL, USA), anti-NANOG (1:200) (Thermo Fisher Scientific, Meridian Road Rockford, IL, USA), anti-OCT-4 (Cell Marque, Sierra College Blvd. Rocklin, California United States and anti-c-MYC (Ventana Medical Systems, Tucson, Arizona, USA) overnight at 4°C. Images were collected and the positivity was evaluated with Zeiss AxioCam ICc 3 High-Resolution through an Axioskop microscope camera. Section samples were investigated to evaluate the immunoreactivity to the markers used. A semi-quantitative method based on the evaluation of the positivity of the tumor cells was used. Here are shown the score classes: 0 (<5% positive), 1 (5 to 25% positive), 2 (>25 to 50% positive), 3 (>50 to 75% positive), and 4 (>75% positive) (14). Sections were scored by two trained investigators, blinded to patient's outcome and other clinical findings.

RESULTS

Clinical Setting

The patient was discharged after six days from surgery with no complications. A chest x-ray after one week was normal following the operation. The patient underwent follow up and no recurrence was observed within a year after surgery.

$ALDH^{high}$ Stem Cells Were Identified in Primary Cells of an IMT of the Lung

Tumor tissue dissociation efficiently released cancer cells characterized by a heterogeneous morphology, as illustrated in the widespread FSC and SSC values (**Figure 2**) (11–13). The mean viability of the samples was 99.7% based

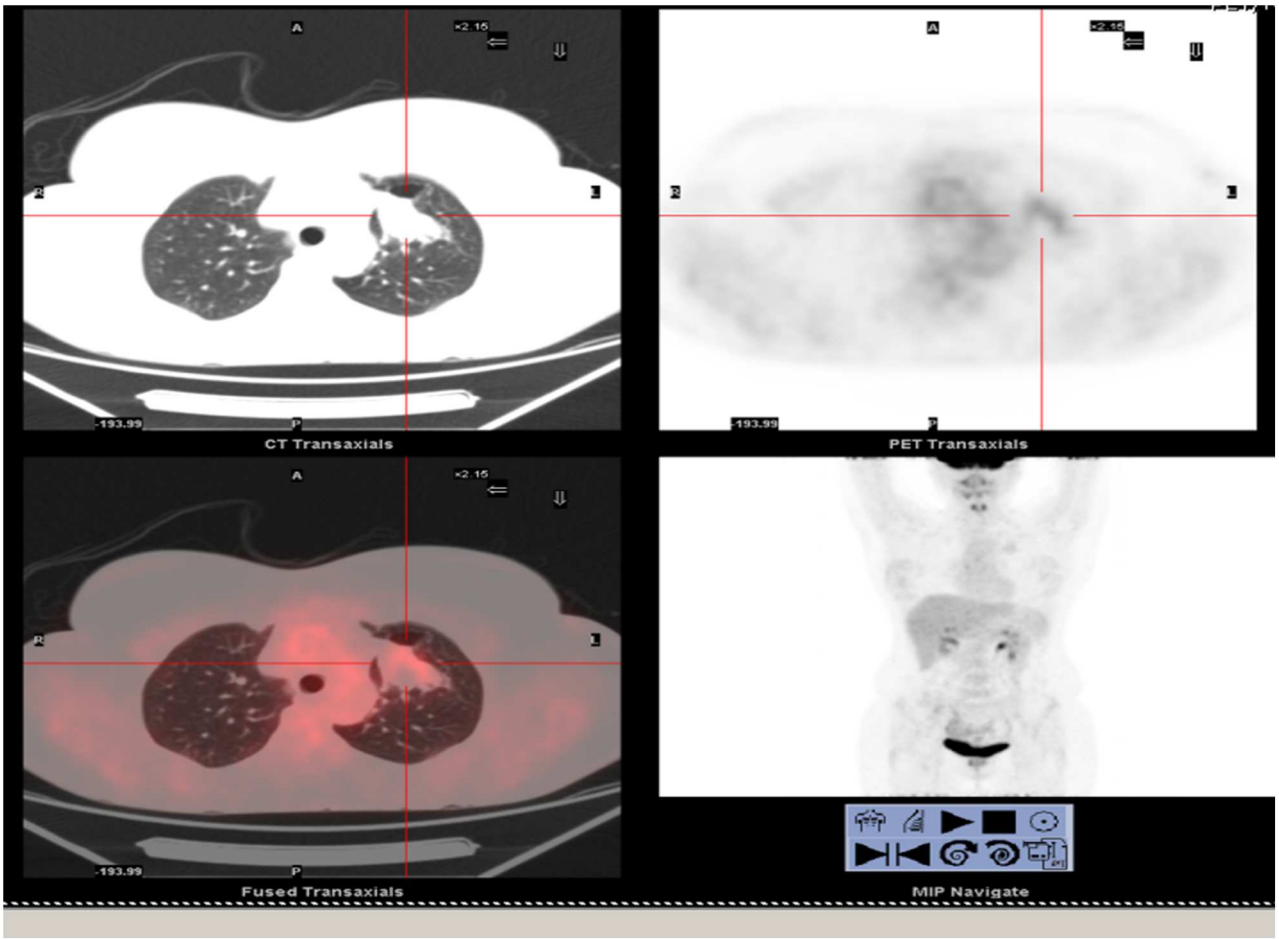

FIGURE 1 | Total body PET/CT scan. Lung nodule at the upper left lobe showed a very low uptake of the lesion.

on 7-AAD staining. These data further confirmed that the developed dissociation procedure was a non-toxic approach to isolating cells from tumor tissues. The CSCs were physically separated from the bulk parental tumor cells and recovered by FACS according to the following gating strategy. Tumor cells were first identified based on their morphological parameters (FSC/SSC), and the ALDH activity was measured in the 7-AAD negative cell population only (**Figure 2**). $ALDH^{low}$ and $ALDH^{high}$ cells were both selected and sorted (**Figure 2**). An $ALDH^{high}$ subpopulation accounted for 1.10% of all viable lung cancer cells, which indicates that a non-negligible population of tumor cells had characteristics of CSCs.

Immunohistochemical Evaluation of the ALDH1A1, SOX2, NANOG, OCT-4, and c-MYC Stem Cell Markers in an IMT of the Lung

To further evaluate the stemness of the cells extracted from the tumor biopsy, SOX2 immunohistochemistry was performed. Positive cells from tissue slides, represented by brown nuclei, were analyzed at 10x and 20x magnification. As expected from the FACS analysis, a non-negligible percentage of the cells were positive for ALDH1A1, SOX2, NANOG, OCT-4, and c-MYC (**Figure 3**).

DISCUSSION

In our report we first described the presence of cancer stem-like cells in a case of IMT of the lung. The connection between benign and malignant lesions has been investigated for a long time (15–17). Besides the different causes of malignancy, infectious disease may be one of the main causes of induced tumorigenesis (18). In fact the inflammatory microenvironment seems to be the key for the development and maintenance of the CSCs' niche (19). These mechanisms can be justified through the enhancement of proliferation, induction and metastatic signaling (15). In this report, our aim is to highlight the presence of cancer stem-like cells in combination with IMT inflammatory components. In particular, the possibility of emerging inflammatory mechanisms inducing stemness of CSCs has been previously described (19). In breast cancer,

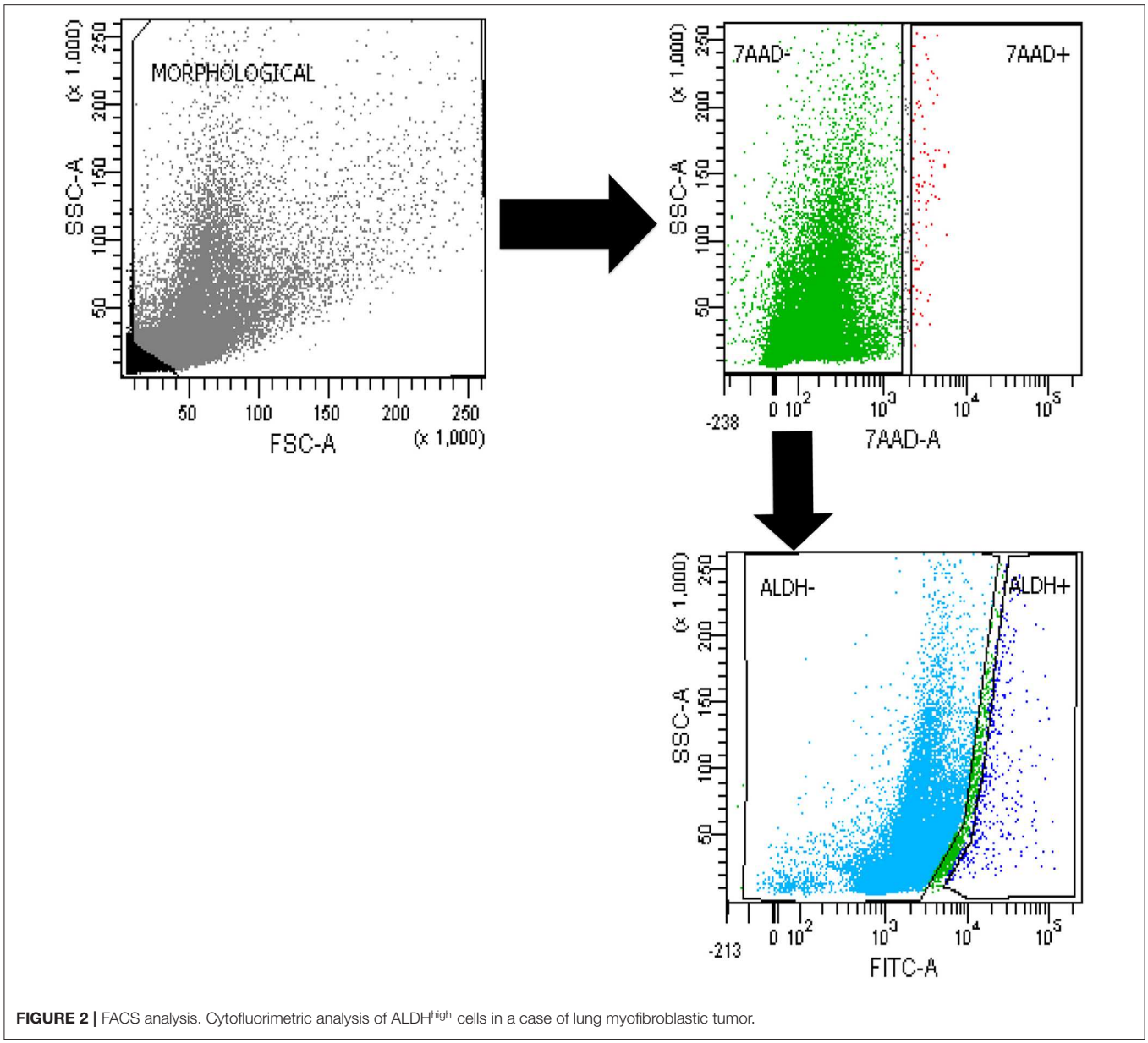

FIGURE 2 | FACS analysis. Cytofluorimetric analysis of ALDH^{high} cells in a case of lung myofibroblastic tumor.

for example, the inflammatory factors are considered as one of the main causes of tumorigenesis (20, 21). However, the histogenesis of IMTs is unclear at the moment; some researchers suggest that IMTs are benign lesions with impacts for surgery, trauma, radiotherapy, steroids, and infectious agents, without convincing explanations for the entire scientific community (22–24). There are other hypothesis suggesting that IMTs are tumors related to genetic modifications, such as the recurrent involvement of chromosomal region 2p23, which seems to induce the aggressive local behavior and metastasis of this tumor (25). In particular, Coffin et al. demonstrated that some IMTs are neoplastic lesions with clonal aberrations (25). Additionally, it is very difficult to define histologically specific characteristics for this disease which can be used to define possible markers, and this is probably due to the impossibility of distinguishing heterogeneity in clinical aspects (26).

As a result, several teams tried to define lesions as falling under the category of IMT.

The World Health Organization has classified IMTs as a distinct entity, being tumors of intermediate biological potential for their capacity to generate local recurrence and, in some cases, distant metastasis (27).

Histologically IMTs are described as cellular or fascicular fibroblastic proliferations associated with chronic inflammatory infiltrate (27). It has been suggested that there are histologic criteria to define the malignant transformation as, for example, round cells associated with necrosis, large nucleoli, several

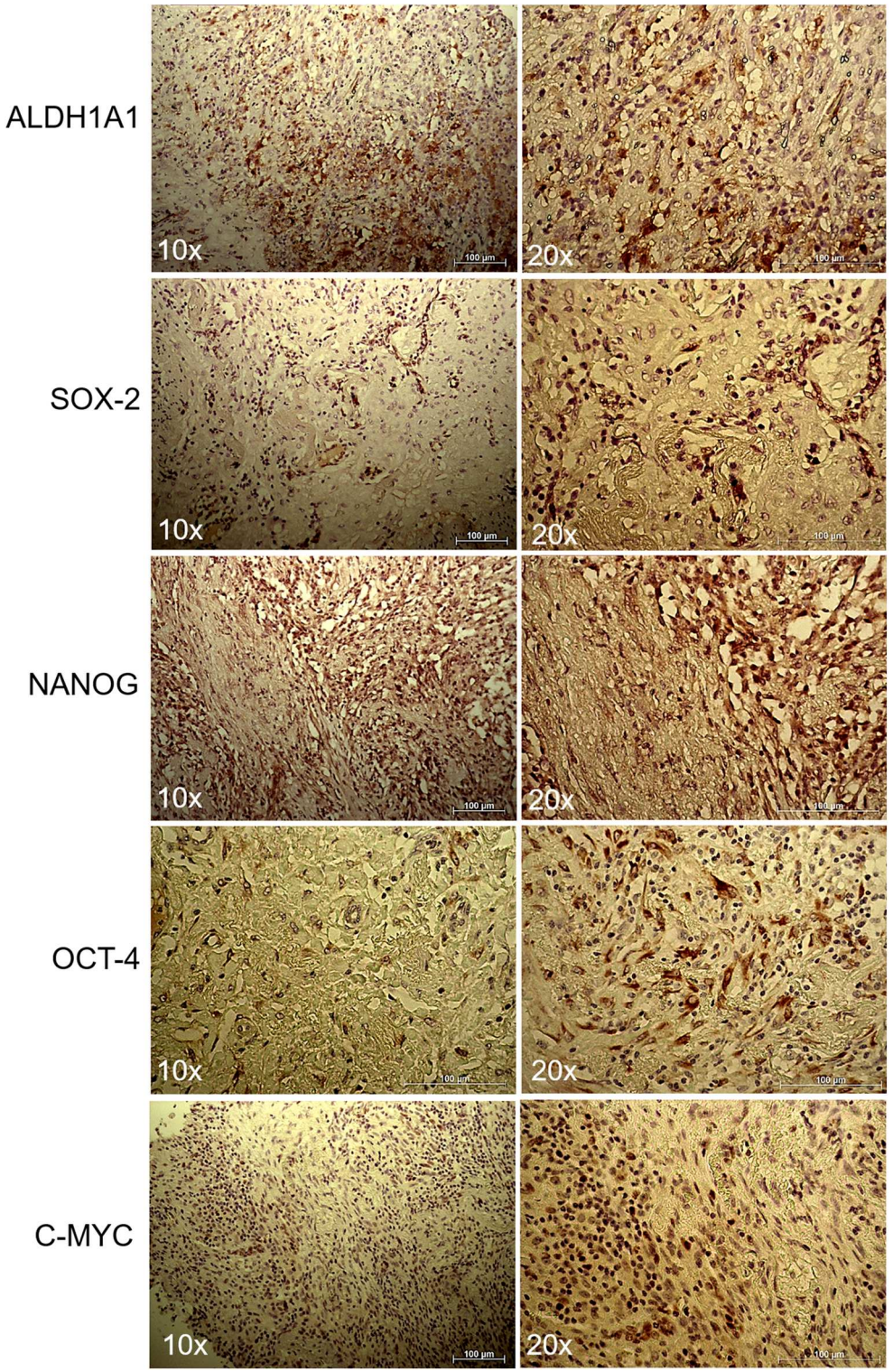

FIGURE 3 | Immunohistochemistry of lung myofibroblastic tumor. Representative immunohistochemical staining of ALDH1A1, SOX2, NANOG, OCT-4, and c-MYC stem cell markers on a myofibroblastic tumor. Images were shown at 10x and 20x magnification.

mitoses, etc. (27). However, the main characteristic of these tumors is the *myofibroblast*, which justified the term of "inflammatory myofibroblastic tumors" (26). However, no attention until now has been given to the possible presence of cancer stem-cells in this tumor (27–30). Our study represents the first attempt at highlighting new cell populations that may be the

key to better characterizing this type of tumor. In conclusion, we aim to determine new pathological markers as well as to aid the development of targeted therapies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee at University Hospital of Modena, MODENA, Italy, on 17 March 2017, Prot. N. 914/C.E and has been performed in accordance with the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The idea for the manuscript was conceived in September 2016 by BA and MD and was further developed by VM, GG, FB, RD'A, AM, LB, AS. AM and PS were involved in histopathological diagnosis. BA, VM, and FB wrote the first draft of the manuscript. BA and UM have been involved in surgery and tissue collection. VM and GG performed laboratory experiments, whereas FB and RD'A performed the statistical analysis. BA, VM, FB, MD, RD'A, AM, and UM reviewed and edited the manuscript before submission. All authors approved the final manuscript before the submission.

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Survival Following Segmentectomy or Lobectomy in Patients With Stage IB Non-small-cell Lung Cancer

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Background: Lobectomy with mediastinal lymph node dissection has always been recognized as the standardized treatment for early-stage non-small-cell lung cancer. However, the feasibility of segmentectomy performed in stage IB non-small-cell lung cancer (NSCLC) patients remains controversial. The present study aims to investigate whether the outcome of stage IB NSCLC patients undergoing segmentectomy was comparable to those who underwent lobectomy.

Method: We retrospectively collected data of 11,010 patients with primary stage IB non-small-cell lung cancer from the Surveillance, Epidemiology, and End Results database. Overall survival (OS) and lung cancer-specific survival (LCSS) were assessed among patients who were performed lobectomy or segmentectomy. To further assess the impact of the surgical procedures on patients with different tumor sizes, subgroups stratified by tumor size were analyzed.

Results: A total of 11,010 patients who were pathologically confirmed to be stage IB were included, of whom 10,453 received lobectomy and 557 received segmentectomy. Both univariate and multivariate Cox regression analyses showed that the patients receiving lobectomy had better OS [hazards ratio (HR) = 1.197, 95% confidence interval (CI) (1.066, 1.343), $P < 0.001$] than those receiving segmentectomy. However, multivariate analysis showed that there was no significant difference in LCSS between lobectomy and segmentectomy [HR = 1.172, 95% CI (0.963, 1.427), $P = 0.114$]. Meanwhile, subgroup analyses showed that lobectomy rather than segmentectomy was associated with better OS [HR = 1.278, 95% CI (1.075, 1.520) $P = 0.006$] and LCSS [HR = 1.118, 95% CI (1.005, 1.280), $P = 0.047$] for patients with a tumor size (TS) of ≤40 and >30 mm, while for patients with a TS of ≤30 mm, lobectomy yielded similar OS [TS ≤ 20 mm: HR = 1.068, 95% CI (0.853, 1.336), $P = 0.566$; TS > 20 mm and ≤30 mm: HR = 1.195, 95% CI (0.961, 1.487), $P = 0.109$] and LCSS [TS ≤ 20 mm: HR = 1.029, 95% CI: (0.682, 1.552), $P = 0.893$; TS > 20 and ≤30 mm: HR = 1.144, 95% CI (0.795, 1.645), $P = 0.469$] to that of segmentectomy.

Conclusion: Segmentectomy achieved equivalent OS and LCSS in stage IB NSCLC patients with TS ≤ 30 mm compared with lobectomy. Lobectomy showed better OS and LCSS than segmentectomy for patients with a TS of >30 and ≤40 mm. Segmentectomy may be acceptable in patients with an older age and a smaller TS.

Keywords: non-small-cell lung cancer, segmentectomy, lobectomy, overall survival, lung cancer-specific survival

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INTRODUCTION

Lung cancer has been the leading cause of cancer mortality worldwide, which makes lung cancer a major public health problem in the world (1). It is estimated that 234,030 cases were newly diagnosed and 154,050 died per year in the United States (2). According to the statistics, non-small-cell lung cancer (NSCLC) accounts for ∼80% of all lung cancer cases (3). Thus, better treatment for NSCLC is urgently needed.

A study based on the Surveillance, Epidemiology, and End Results (SEER) database showed that NSCLC patients with a tumor size (TS) ≤1 cm, who underwent segmentectomy, had equivalent overall survival (OS) compared to those who had lobectomy (4). Later, an observational study using the same database demonstrated that lobectomy yielded better survival than segmentectomy in NSCLC patients with TS ≤ 2 cm who were diagnosed between 2000 and 2012 (5). Moon et al. (6) demonstrated that there were no significant differences in OS or lung cancer-specific survival (LCSS) among patients with TS ≤ 2 cm who underwent lobectomy vs. segmentectomy. Recently, a meta-analysis by Jsseldijk et al. (7) suggested that OS and disease-free survival (DFS) after segmentectomy yielded equal survival compared to lobectomy in NSCLC patients with stage T1aN0M0.

Thus, more and more studies suggest that segmentectomy yielded an equivalent survival rate compared to lobectomy in early-stage NSCLC patients. However, its survival comparison with lobectomy in stage IB non-small-cell lung cancer patients remains unknown. The present study aims to evaluate the impact of lobectomy and segmentectomy on OS and LCSS in stage IB (T2aN0M0) NSCLC patients using the SEER database.

METHODS

Data

Clinical information of all patients was obtained from the SEER database, which was supported by the National Cancer Institute. The database aims to collect and report the cancer incidence and survival data from several registries that involve more than 30% of the U.S. population and has been used for survival analyses in numerous high-quality studies (5, 8–12).

Study Population

The inclusion of the patients involved in the study include (1) NSCLC confirmed by pathology; (2) T2aN0M0 stage tumor based on the eighth edition of NSCLC stage classification (TS > 30 and ≤40 mm or TS ≤ 30 mm but involved with visceral pleural invasion); and (3) surgical history of lobectomy (surgery code: 30 and 33, and extended lobectomy was excluded) or segmentectomy (surgery code: 22, and wedge resection was excluded). And the exclusion criteria include (1) history of chemotherapy; (2) history of radiotherapy; (3) pathologically confirmed small cell lung cancer or all subtypes of sarcoma; (4) age < 18; (5) tumor located in the main bronchus, as a result of which segmentectomy was impossible to be performed.

The baseline characteristics of patients were obtained from the datasets: age, gender, race, year of diagnosis, location of tumor, laterality, pathology classification, the number of resected lymph nodes, TS, survival status, survival time, and cause of death. All eligible patients were divided into the segmentectomy group or lobectomy group according to the surgical strategies. History of malignancy was categorized as No (having no other malignancies before lung cancer diagnosis) and Yes (having one or more malignancies before lung cancer diagnosis). Grade well/moderate group included grade I and II, while poor/undifferentiated included III and IV.

The primary endpoint of this analysis was OS, which is calculated from the day of surgery to the last follow-up or death. The secondary endpoint was LCSS, calculated from the day of surgery to the day of NSCLC-related death or the date of the last follow-up.

Statistical Analysis

Conventional statistics are used to summarize the characteristics of the study. The Wilcoxon tests were used to calculate the distributions of continuous data (age, number of resected regional lymph nodes, and TS), and the Pearson χ^2 test was used in categorical variables (sex, location, laterality, pathology, grade, and history of malignancy). Survival curves of OS and LCSS were calculated by the Kaplan–Meier method, and the significance was assessed by the log-rank test. To evaluate the impact of segmentectomy or lobectomy on the outcome of the patients, univariate and multivariate Cox regression analyses were used to calculate hazards ratios (HR) and 95% confidence intervals (95% CI).

SPSS 22.0 software (IBM Corporation, Armonk, NY, USA) was used for statistical analysis. Kaplan–Meier survival curves were established using R 3.6.1 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant for all statistical tests.

RESULTS

Characteristics of the Study

A total of 11,010 NSCLC patients who were pathologically confirmed to be stage T2aN0M0 were included, of whom 10,453 underwent lobectomy and 557 segmentectomy. The year of diagnosis spanned from 2004 to 2013. The age of the cohort ranged from 22 to 94, and the average was 69. To further explore the impact of age on survival, the cohort was divided into three groups by age: ≤60, 61–75, and >75. The median and mean follow-up times of the entire cohort were 56 and 116.37 months, respectively. Baseline characteristics were depicted in **Table 1**. As shown in **Table 1**, segmentectomy is more likely to be performed in patients with an older age, White race, left lung lesions, advanced tumor grade, and a smaller TS. And a smaller number of lymph nodes were likely to be resected in patients who underwent segmentectomy.

Survival Analysis

The Kaplan–Meier survival analysis showed that lobectomy had better OS ($P < 0.0001$) and LCSS ($P = 0.032$) than segmentectomy (**Figures 1**, **2**). And univariate and multivariate Cox regression analyses were further conducted. As shown in

TABLE 1 | Baseline characteristics of the study population.

	Lobectomy (n = 10,453)	Segmentectomy (n = 557)	P-value
Age			<0.001
≤60	1,839 (17.6%)	63 (11.3%)	
61–75	5,540 (53.0%)	282 (50.6%)	
>75	3,074 (29.4%)	212 (38.1%)	
Sex			0.251
Male	5,084 (48.6%)	257 (46.1%)	
Female	5,369 (51.4%)	300 (53.9%)	
Location			<0.001
Upper	6,251 (58.9%)	336 (59.9%)	
Lower	3,359 (33.3%)	206 (37.4%)	
Others	843 (7.8%)	15 (2.7%)	
Race			0.805
White	8,883 (85.3%)	479 (86.2%)	
Black	831 (8.0%)	41 (7.1%)	
Others	739 (6.7%)	37 (6.8%)	
Pathology			0.400
Adenocarcinoma	6,539 (62.6%)	341 (61.2%)	
Squamous cell carcinoma	2,704 (25.8%)	141 (25.3%)	
Others	1,210 (11.6%)	75 (13.5%)	
Laterality			<0.001
Left	4,272 (40.9%)	313 (56.2%)	
Right	6,181 (59.1%)	244 (43.8%)	
Grade			0.004
Well/moderate	6,310 (60.4%)	297 (53.3%)	
Poor/Undifferentiated	3,560 (34.1%)	223 (40.0%)	
Unknown	583 (5.5%)	37 (6.7%)	
No. of resected lymph nodes			<0.001
0	322 (3.1%)	139 (25.0%)	
1–3	1,716 (16.4%)	172 (30.9%)	
≥4	7,931 (75.9%)	214 (38.4%)	
Unknown	484 (4.6%)	32 (5.7%)	
Tumor size (mm)			<0.001
≤ 20	2,230 (21.3%)	191 (34.3%)	
21–30	2,564 (24.5%)	157 (28.2%)	
31–40	5,659 (54.1%)	209 (37.5%)	
History of malignancy			<0.001
No	5,993 (57.3%)	256 (46.0%)	
Yes	4,460 (42.7%)	301 (54.0%)	

Tables 2, **3**, univariate analyses suggested that an older age, male sex, White race, squamous cell carcinoma, advanced tumor grade, a smaller number of resected lymph nodes, and a larger TS yielded worse OS and LCSS. Patients undergoing segmentectomy had worse OS [HR = 1.442, 95% CI (1.295, 1.606), $P < 0.001$] and LCSS [HR = 1.224, 95% CI: (1.017, 1.473), $P = 0.033$]. In multivariate Cox regression, segmentectomy had poorer OS [HR = 1.197, 95% CI (1.066, 1.343), $P = 0.002$], but not poorer LCSS [HR = 1.172, 95% CI (0.963, 1.427), $P = 0.114$]. An older age, male sex, squamous cell carcinoma, advanced grade, and a smaller number of resected lymph nodes were associated with worse OS and LCSS. Interestingly, patients with a history of malignancy were associated with a better LCSS [HR = 0.311, 95% CI (0.280, 0.345), <0.001] compared with those without a history of malignancy; however, no significant difference was observed in OS [HR = 1.020, 95% CI (0.982, 1.075), $P = 0.465$].

Subgroup Analysis

To further explore the impact of TS on the choice of surgical strategy for stage IB NSCLC patients, subgroup analyses were conducted. As shown in **Table 4**, multivariate Cox regression analysis showed that segmentectomy yielded similar OS to that of lobectomy for NSCLC patients with TS ≤ 30 mm [TS ≤ 20 mm: HR = 1.068, 95% CI (0.853, 1.336), $P = 0.566$; TS > 20 and ≤30 mm: HR = 1.195, 95% CI (0.961, 1.487), $P = 0.109$], while in NSCLC patients with a TS of ≤40 and >30 mm, segmentectomy was associated with poorer OS [HR = 1.278, 95% CI (1.075, 1.520), $P = 0.006$]. We should note that in T2aN0M0 stage patients, if the size of tumor was ≤30 mm, the visceral pleural must have been invaded. As depicted in **Table 5**, segmentectomy achieved similar LCSS to that of lobectomy [TS ≤ 20 mm: HR = 1.029, 95% CI (0.682, 1.552), $P = 0.893$; TS > 20 and ≤30 mm, HR = 1.144, 95% CI (0.795, 1.645), $P = 0.469$]. However, segmentectomy yielded worse LCSS [HR = 1.118, 95% CI (1.005, 1.280), $P = 0.047$] in NSCLC patients with a TS of ≤40 and >30 mm.

DISCUSSION

Although lobectomy is recognized as the standard surgical treatment for patients with stage I NSCLC (13), the optimal surgical management of early-stage lung cancer still remains controversial. In recent years, with the development of surgical technological devices, surgical skills, and perioperative care, minimally invasive surgery has been increasingly accepted. More and more attention has been focused on sublobar resection as an alternative treatment for patients with early-stage NSCLC, especially the elderly ones and those with slight or moderate impairment of lung function. Moreover, patients who had undergone limited resection are more tolerant of reoperation if lung malignancies recurred. In our study, we observed that lobectomy achieved better OS and LCSS than did segmentectomy; however, after being adjusted by other prognostic factors, no significant difference was observed in LCSS between them. To the best of our knowledge, this is the first study to compare the survival rates after lobectomy and segmentectomy for the eighth edition of stage IB NSCLC patients.

In 1995, a study by Ginsberg et al. (13) demonstrated that lobectomy should be recommended as the standard treatment for patients with peripheral T1N0 non-small-cell lung cancer. However, recent studies have tried to assess the difference in outcomes between lobectomy and sublobar resection. Wolf and his team members found that lobectomy was associated with longer OS and recurrence-free survival in NSCLC patients with TS ≤ 2 cm (14). However, other studies (6, 15–18) reported that segmentectomy was comparable to lobectomy in terms of OS for stage IA NSCLC. There was also evidence from several

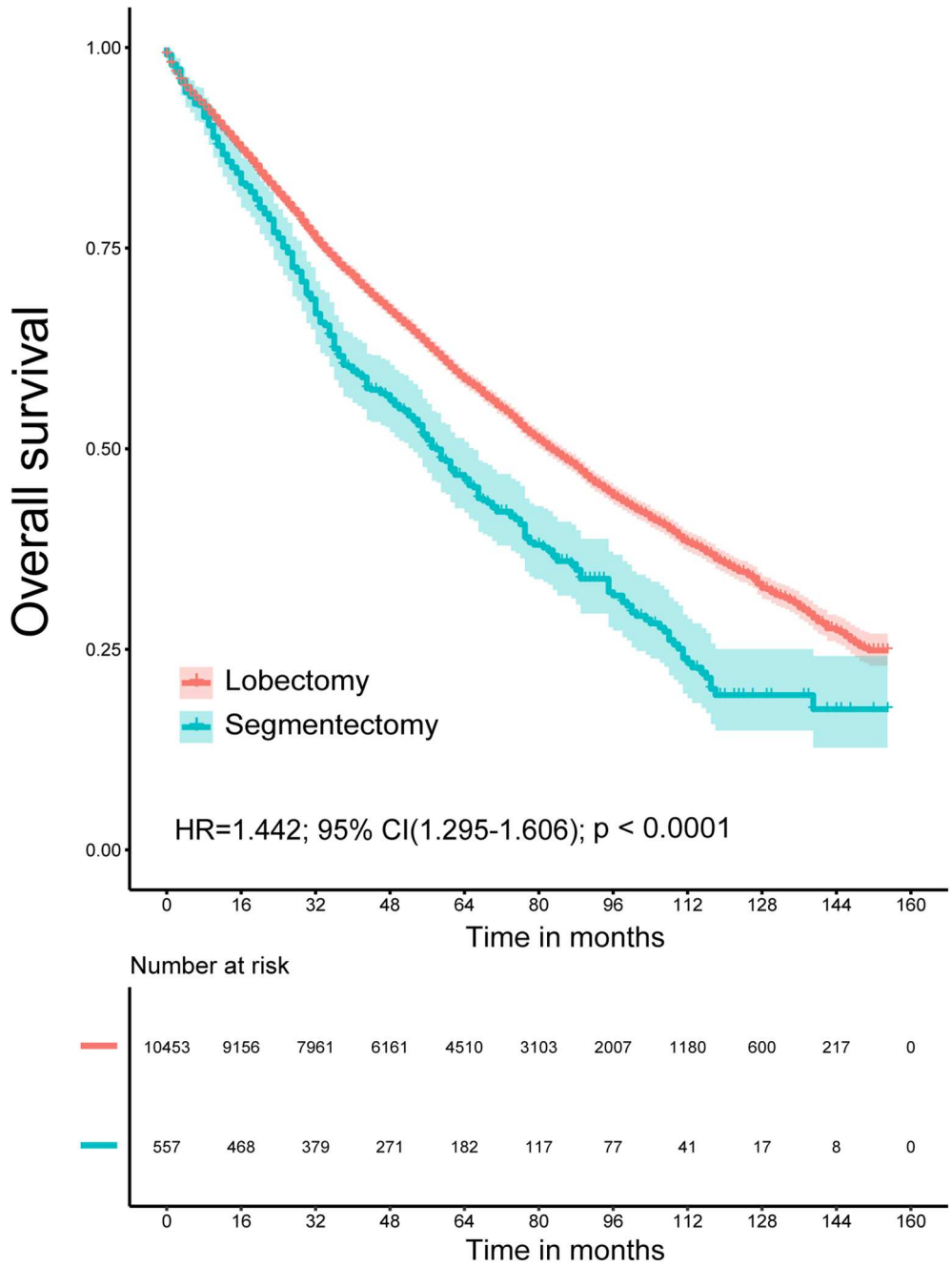

FIGURE 1 | Overall survival for patients with lobectomy and segmentectomy.

studies of meta-analysis suggesting that segmentectomy could achieve comparable OS to lobectomy for stage IA NSCLC (19–21). It seemed that more and more studies suggested that segmentectomy could be the optimal surgical treatment for patients with stage IA NSCLC.

Numerous published studies explored the impact of segmentectomy and lobectomy on the prognosis of patients with stage IA or I NSCLC. It should be noted that stage I contains stages IA and IB. Most of previous studies of lung cancer were based on the seventh or sixth edition. T2N0M0 ($T > 30$ mm) was defined as stage IB in the sixth edition, and T2aN0M0 ($T > 30$ mm and $T \leq 50$ mm) was defined as stage IB in the seventh. In 2012, Schuchert et al. (22) found that patients with stage IB NSCLC who underwent segmentectomy had reduced recurrence-free survival compared to those who underwent lobectomy. However, the TNM stage of the enrolled

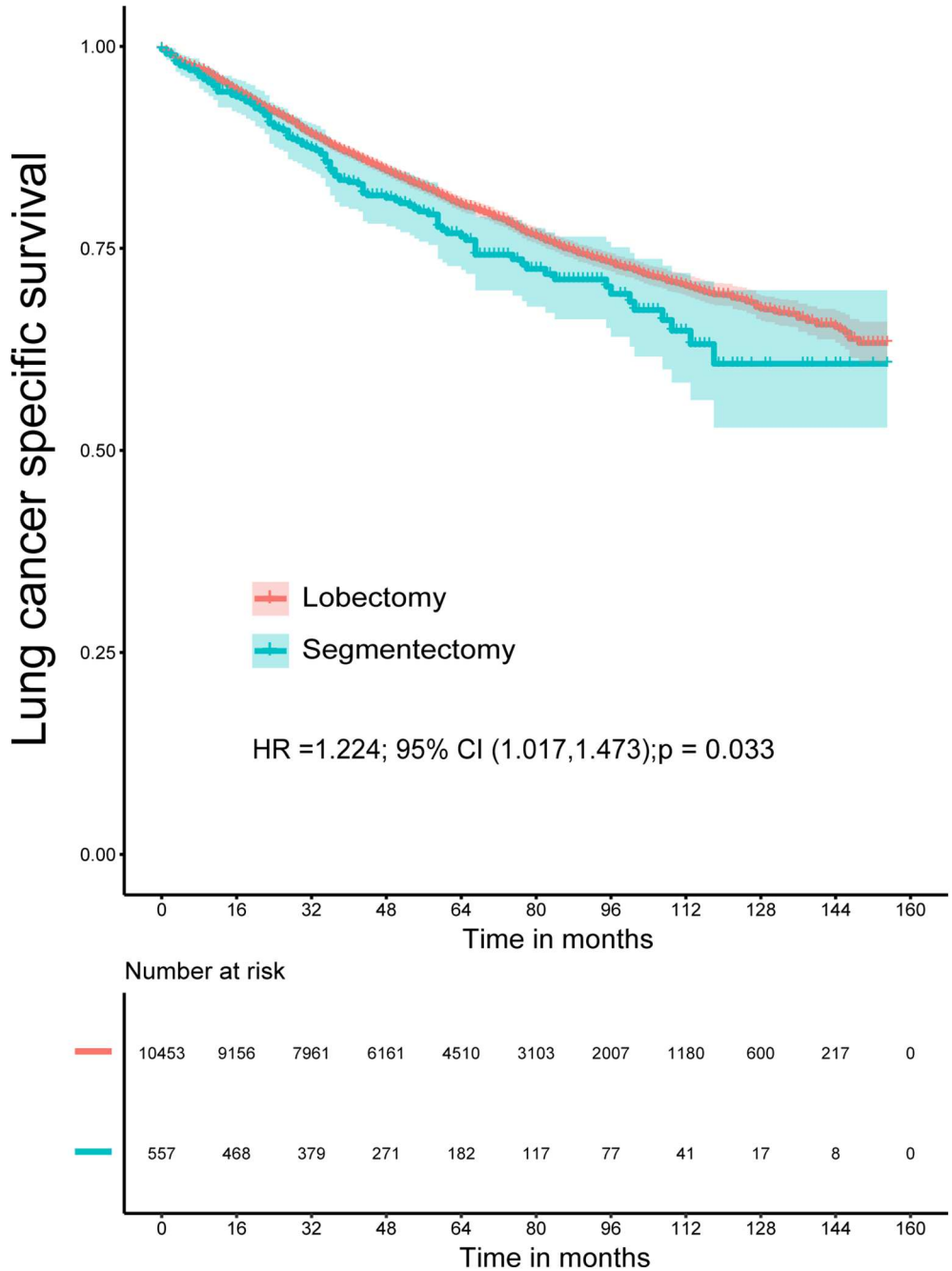

FIGURE 2 | Lung cancer-specific survival for patients with lobectomy and segmentectomy.

patients was classified according to the sixth edition of the American Joint Committee on Cancer (AJCC) Lung Cancer Staging System, and the outcomes of stage IB NSCLC patients would probably be different because of tumor staging based on different editions. Recently, Zhang et al. (23) reported that sublobar resection achieved an equivalent outcome to that of lobectomy, and similar results were also observed in propensity-matched analysis. But we should note that patients who underwent segmentectomy and wedge resection were all included in their study as sublobar resection. Segmentectomy belongs to anatomical resection, while wedge resection does not. Whitson et al. (24) suggested that lobectomy confers a significant survival advantage over segmentectomy in stage I NSCLC patients. However, the tumor staging system was based on the sixth edition lung cancer stage classification of the AJCC. Both the sixth and seventh editions are different from the newest

TABLE 2 | Univariate and multivariate regression analyses for overall survival.

	Univariate analysis			Multivariate analysis		
	HR	**95% CI**	***P***	**HR**	**95%CI**	***P***
Age						
≤60	1			1		
61–75	1.628	1.496–1.773	< 0.001*	1.559	1.430–1.699	< 0.001*
>75	1.777	1.684–1.875	< 0.001*	2.420	2.213–2.647	< 0.001*
Sex						
Male	1			1		
Female	0.703	0.667–0.740	< 0.001*	0.707	0.669–0.746	< 0.001*
Race						
White	1			1		
Black	0.856	0.773–0.947	0.003*	0.929	0.838–1.029	0.159
Others	0.771	0.690–0.861	< 0.001*	0.845	0.756–0.945	0.001*
Laterality						
Left	1			1		
Right	0.991	0.941–1.045	0.748	1.038	0.983–1.096	0.179
Location						
Upper	1			1		
Lower	1.097	1.037–1.160	0.001*	1.067	1.008–1.128	0.025*
Others	0.978	0.884–1.083	0.673	0.956	0.861–1.062	0.402
Pathology						
Adenocarcinoma	1			1		
Squamous cell carcinoma	1.563	1.475–1.656	< 0.001*	1.331	1.252–1.415	< 0.001*
Others	1.273	1.174–1.381	< 0.001*	1.168	1.074–1.270	< 0.001*
Grade						
Well/Moderate	1			1		
Poor/Undifferentiated	1.277	1.210–1.348	< 0.001*	1.177	1.112–1.246	< 0.001*
Unknown	0.846	0.748–0.956	0.007*	0.863	0.763–0.977	0.012*
Resected lymph nodes						
0	1			1		
1–3	0.754	0.666–0.854	< 0.001*	0.775	0.682–0.882	< 0.001*
≥4	0.597	0.532–0.669	< 0.001*	0.625	0.554–0.705	< 0.001*
Unknown	0.645	0.549–0.757	< 0.001*	0.680	0.577–0.802	< 0.001*
Tumor size (mm)						
≤20	1					
21–30	1.272	1.176–1.376	< 0.001*	1.180	1.090–1.276	< 0.001*
31–40	1.314	1.227–1.407	< 0.001*	1.159	1.081–1.243	< 0.001*
History of malignancy						
No	1			1		
Yes	1.126	1.069–1.186	< 0.001*	1.020	0.968–1.075	0.465
Surgical procedure						
Lobectomy	1					
Segmentectomy	1.442	1.295–1.606	< 0.001*	1.197	1.066–1.343	0.002*

**indicates that the difference was statistically significant.*

eighth edition in IB NSCLC tumor staging (25). Obviously, these results are unfit to guide clinical strategies for stage IB NSCLC nowadays. Liu et al. (8) pointed out that NSCLC patients in stage T12N0M0 treated with lobectomy had a better outcome than those treated with sublobar resection. T12N0M0 stage contains stages IA, IB, and IIA. Moreover, sublobar resection contains segmentectomy and wedge resection. Therefore, we considered that the study by Liu et al. has limited directive significance to clinical strategies for patients with stage IB NSCLC. Our study included the patients with stage IB NSCLC according to the eighth edition lung cancer stage classification of the Union for International Cancer Control (UICC), of whom 557 received segmentectomy and 10,453 lobectomy. Due to the nature of retrospective study, multivariate Cox regression analyses were

TABLE 3 | Univariate and multivariate regression analyses for lung cancer specific survival.

	Univariate analysis			Multivariate analysis		
	HR	**95%C**	***P***	**HR**	**95%CI**	***P***
Age						
≤60	1			1		
61–75	1.243	1.103–1.402	< 0.001*	1.394	1.234–1.574	< 0.001*
>75	1.482	1.302–1.687	< 0.001*	1.669	1.462–1.905	< 0.001*
Sex						
Male	1			1		
Female	0.787	0.724–0.856	< 0.001*	0.768	0.703–0.838	< 0.001*
Race						
White	1			1		
Black	0.835	0.707–0.986	0.034*	0.847	0.716–1.003	0.054
Others	1.054	0.901–1.232	0.513	0.989	0.846–1.158	0.895
Laterality						
Left	1			1		
Right	1.060	0.973–1.154	0.181	1.099	1.007–1.199	0.035*
Location						
Upper	1			1		
Lower	1.050	0.959–1.149	0.290	1.064	0.972–1.166	0.180
Others	0.919	0.778–1.085	0.318	0.911	0.768–1.081	0.287
Pathology						
Adenocarcinoma	1			1		
Squamous cell carcinoma	1.407	1.279–1.548	< 0.001*	1.222	1.105–1.351	< 0.001*
Others	1.361	1.200–1.544	< 0.001*	1.213	1.064–1.382	0.004*
Grade						
Well/Moderate	1			1		
Poor/Undifferentiated	1.347	1.235–1.469	< 0.001*	1.255	1.146–1.374	< 0.001*
Unknown	0.807	0.657–0.990	0.040*	0.801	0.651–0.985	0.036*
No. of resected lymph nodes						
0	1			1		
1–3	0.841	0.685–1.034	0.100	0.785	0.635–0.970	0.025*
≥4	0.645	0.533–0.780	< 0.001*	0.587	0.481–0.717	< 0.001*
Unknown	0.706	0.543–0.919	0.009*	0.654	0.500–0.855	0.002*
Tumor size (mm)						
≤20	1			1		
21–30	1.398	1.230–1.589	< 0.001*	1.316	1.157–1.496	< 0.001*
31–40	1.432	1.279–1.603	< 0.001*	1.310	1.167–1.470	< 0.001*
History of malignancy						
No				1		
Yes	0.332	0.299–0.368	< 0.001*	0.311	0.280–0.345	< 0.001*
Surgical procedure						
Lobectomy	1			1		
Segmentectomy	1.224	1.017–1.473	0.033*	1.172	0.963–1.427	0.114

**indicates that the difference was statistically significant.*

performed to remove the bias. The multivariate analyses showed that an older age, male sex, squamous cell carcinoma, advanced grade, a smaller numbers of resected lymph nodes, and a larger TS were independent prognostic factors, and they predicted worse OS and LCSS. Interestingly, patients with a history of malignancy had longer LCSS and seemed to have worse OS. However, the difference in OS was not significant between patients with such a history and those without. We speculated that the reason may be that patients with other malignancies were more likely to die of other recurrent cancers or long-term post-operative complications.

A previous study reported that mediastinal lymph node metastasis was significantly associated with poorer tumor differentiation degree, and a larger number of positive

TABLE 4 | Subgroup analyses stratified by tumor size for overall survival.

	TS ≤ 20 mm			20 mm < TS ≤ 30 mm			30 mm < TS ≤ 40 mm		
	HR	**95% CI**	***P***	**HR**	**95% CI**	**P**	**HR**	**95% CI**	***P***
Surgical procedures									
Lobectomy	1			1			1		
Segmentectomy	1.068	0.853–1.336	0.566	1.195	0.961–1.487	0.109	1.278	1.075–1.520	0.006*
Age									
≤60	1			1			1		
61–75	1.509	1.271–1.792	<0.001*	1.555	1.306–1.851	<0.001*	1.594	1.412–1.800	<0.001*
>75	2.179	1.802–2.635	<0.001*	2.431	2.031–2.910	<0.001*	2.516	2.221–2.849	<0.001*
Grade									
Well/Moderate	1			1			1		
Poor/Undifferentiated	1.118	0.977–1.279	0.103	1.180	1.050–1.325	0.005*	1.198	1.111–1.291	<0.001*
Unknown	0.914	0.694–1.203	0.520	0.880	0.682–1.136	0.327	0.838	0.710–0.990	0.038*

**indicates that the difference was statistically significant.*
TS, tumor size.

TABLE 5 | Subgroup analyses stratified by tumor size for lung cancer specific survival.

	TS ≤ 20 mm			20 mm < TS ≤ 30 mm			30 mm < TS ≤ 40 mm		
	HR	**95% CI**	**P**	**HR**	**95% CI**	**P**	**HR**	**95% CI**	**P**
Surgical procedure									
Lobectomy	1			1			1		
Segmentectomy	1.029	0.682–1.552	0.893	1.144	0.795–1.645	0.469	1.118	1.005–1.280	0.047*
Age									
≤60	1			1			1		
61–75	1.317	1.026–1.691	0.031*	1.283	1.009–1.631	0.042*	1.485	1.251–1.764	<0.001*
>75	1.376	1.010–1.874	0.043*	1.607	1.242–2.81	<0.001*	1.805	1.503–2.167	<0.001*
Grade									
Well/Moderate	1			1			1		
Poor/Undifferentiated	1.266	1.012–1.583	0.039*	1.324	1.103–1.589	0.003*	1.231	1.092–1.387	0.001*
Unknown	0.902	0.562–1.450	0.671	0.970	0.659–1.428	0.878	0.698	0.523–0.933	0.015*

**indicates that the difference was statistically significant.*
TS, tumor size.

lymph nodes were significantly associated with worse OS and progression-free survival (26). Cao et al. (27) found that the more extensive the regional lymph node dissection was, the better the survival of patients who undergo sublobar resection for stage IA NSCLC with TS $\leq$ 2 cm. Our study also strongly demonstrated that a larger number of regional lymph nodes with dissection were associated with better LCSS and OS in stage IB patients. We speculated that the smaller the number of resected lymph nodes, the more likely the potential positive lymph nodes were unremoved. The potential unremoved positive lymph nodes may lead to the recurrence of lung cancer or distant metastasis after operation. Therefore, a thoracic surgeon should attach enough importance to the enlarged lymph nodes on CT/PET-CT before operation and remove the visible regional lymph nodes as much as possible.

In subgroups, multivariate analyses showed that segmentectomy yielded similar OS and LCSS for NSCLC patients with TS $\leq$ 30 mm compared with lobectomy. However, segmentectomy yielded worse OS and LCSS for NSCLC patients with TS $>$ 30 mm and T $\leq$ 40 mm. We acknowledged that segmentectomy was likely to be performed in older patients who had a smaller TS, especially in those with an impaired lung function. In addition, more regional lymph nodes were likely to be resected when lobectomy was performed compared to segmentectomy. As discussed in the previous paragraph, the more the regional lymph nodes were resected, the better the outcome the patients would have. Taking the above factors into consideration, segmentectomy may be acceptable for appropriately selected stage IB NSCLC patients with an older age and a smaller TS, especially for those with comorbidities. However, the conclusion needs to be validated by multicenter randomized controlled trials (RCTs).

Previous studies reported that segmentectomy was associated with less blood loss, shorter operation time, shorter chest

drainage, and shorter hospital stay compared with lobectomy (23, 28). However, other studies suggested that there were no significant differences in post-operative mortality, overall complications, and prolonged air leakage rates between segmentectomy and lobectomy in stage IA NSCLC patients (29, 30). There was also high-quality evidence from the Cancer and Lymphoma Group (CALBG 140503) revealing that no significant differences were observed in post-operative complications in segmentectomy and lobectomy cohorts, except that segmentectomy was associated with a higher rate of air leakage (31). Several reasons may account for these conflicting results, such as differences in surgical technology, perioperative patient care, study population, and study design. Because of lack of information about blood loss, operation time, chest drainage, and hospital stay in SEER database, we could not further evaluate the difference in intraoperative and post-operative complications between segmentectomy and lobectomy. The difference in complications between segmentectomy and lobectomy needs to be further confirmed in large multicenter RCTs.

Additionally, there are some limitations in the study. First, recently, various kinds of targeted therapies and immunotherapies for lung adenocarcinoma have increasingly been applied. The outcome of these patients who received targeted therapy or immunotherapy may greatly differ from those who did not. Due to the lack of detailed data, the impacts of different targeted therapies and immunotherapy on OS and LCSS in patients with segmentectomy and lobectomy could not be further assessed. Nevertheless, early-stage NSCLC patients were less likely to receive such treatment. Therefore, our conclusion may not have been substantially affected. Second, because of the nature of a retrospective study, some bias was inevitable. Our results need to be further validated by a larger randomized study cohort in the future. Finally, no detailed data about the positive rate of resected regional lymph nodes and surgical approach (open vs. VATS) were available, making the investigation further limited.

In conclusion, segmentectomy achieved equivalent OS and LCSS for stage IB NSCLC patients with TS $\leq$ 30 mm compared with lobectomy. Lobectomy yielded longer survival for IB NSCLC patients with TS $>$ 30 mm and TS $\leq$ 40 mm. Therefore, segmentectomy may be acceptable for stage IB patients with an older age and a smaller TS, especially for those with impaired lung function.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

BH, LZ, and TF: study design, manuscript writing, and final approval. BL and WJ: Data collection and analysis. HH and QG: Manuscript revision and final approval.

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Patterns of Extrathoracic Metastases in Different Histological Types of Lung Cancer

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Lung cancer is the leading cause of cancer-related deaths mainly attributable to metastasis, especially extrathoracic metastasis. This large-cohort research is aimed to explore metastatic profiles in different histological types of lung cancer, as well as to assess clinicopathological and survival significance of diverse metastatic lesions. Lung cancer cases were extracted and enrolled from the Surveillance, Epidemiology, and End Results (SEER) database. χ^2-tests were conducted to make comparisons of metastatic distribution among different histological types and odds ratios were calculated to analyze co-occurrence relationships between different metastatic lesions. Kaplan–Meier methods were performed to analyze survival outcomes according to different metastatic sites and Cox regression models were conducted to identify independent prognostic factors. In total, we included 159,241 lung cancer cases with detailed metastatic status and complete follow-up information. In order to understand their metastatic patterns, we elucidated the following points in this research: (1) Comparing the frequencies of different metastatic lesions in different histological types. The frequency of bone metastasis was highest in adenocarcinoma, squamous cell carcinoma, LCLC and NSCLC/NOS, while liver was the most common metastatic site in SCLC. (2) Elaborating the tendency of combined metastases. Bi-site metastases occurred more common than tri-site and tetra-site metastases. And several metastatic sites, such as bone and liver, intended to co-metastasize preferentially. (3) Clarifying the prognostic significance of single-site and bi-site metastases. All single-site metastases were independent prognostic factors and co-metastases ended up with even worse survival outcomes. Thus, our findings would be beneficial for research design and clinical practice.

Keywords: lung cancer, metastasis, histological type, survival, pattern

INTRODUCTION

Lung cancer is one of the most common malignancies and the leading cause of cancer-related deaths (1). Every year, 1.8 million new cases are diagnosed and 1.6 million lung cancer related-deaths occur worldwide (2). This fatal neoplasm represents a typical example for which metastatic patients tend to have extraordinary poorer prognosis than non-metastatic cases (3, 4). In spite of the rapid development of novel therapeutic methods, such as epidermal growth factor receptor

tyrosine kinase inhibitors (EGFR TKI), anaplastic lymphoma kinase (ALK) inhibitors and immune checkpoint inhibitors, 5-year overall survival remains relatively low mainly attributable to high risk of distant metastasis (5–7).

To date, tumor hallmarks, metastatic patterns and prognostic outcomes differ greatly among different histological types of lung cancer (8). Non-small cell lung cancer (NSCLC), including adenocarcinoma, squamous cell carcinoma, large-cell lung cancer (LCLC) and others that are not otherwise specified (NOS), accounts for more than 80% of all lung cancers (9). As for small-cell lung cancer (SCLC), making up <20% of all histological types, it is the most aggressive form of lung cancer and featured by malignant proliferation and early invasive spread (10, 11).

Tumor, regional lymph node and metastasis (TNM) staging system was universally applied for prognostic prediction and therapeutic guidance. According to the 8th TNM staging by American Joint Committee on Cancer (AJCC), M1a was defined as intrathoracic metastases including contralateral lung nodules, pleural metastases and pericardial effusion, and M1b or M1c were defined as single or multiple extrathoracic metastases (12). Previous researches suggested that patients with extrathoracic metastasis had markedly shortened survival than limited intrathoracic metastasis (13–15). Therefore, it is vital to draw a detailed landscape for patients with extrathoracic metastasis.

However, extrathoracic metastatic patterns of lung cancer and their diversity in different histological types are unclear and need further clarification. And prognostic outcomes of diverse extrathoracic sites need to be investigated. Thus, this retrospective, large-cohort study is aimed to explore metastatic profiles in different histological types of lung cancer, as well as to assess clinicopathological and survival significance of diverse metastatic lesions.

METHODS

Cohort Population

We performed a retrospective, population-based research by extracting data from the Surveillance, Epidemiology, and End

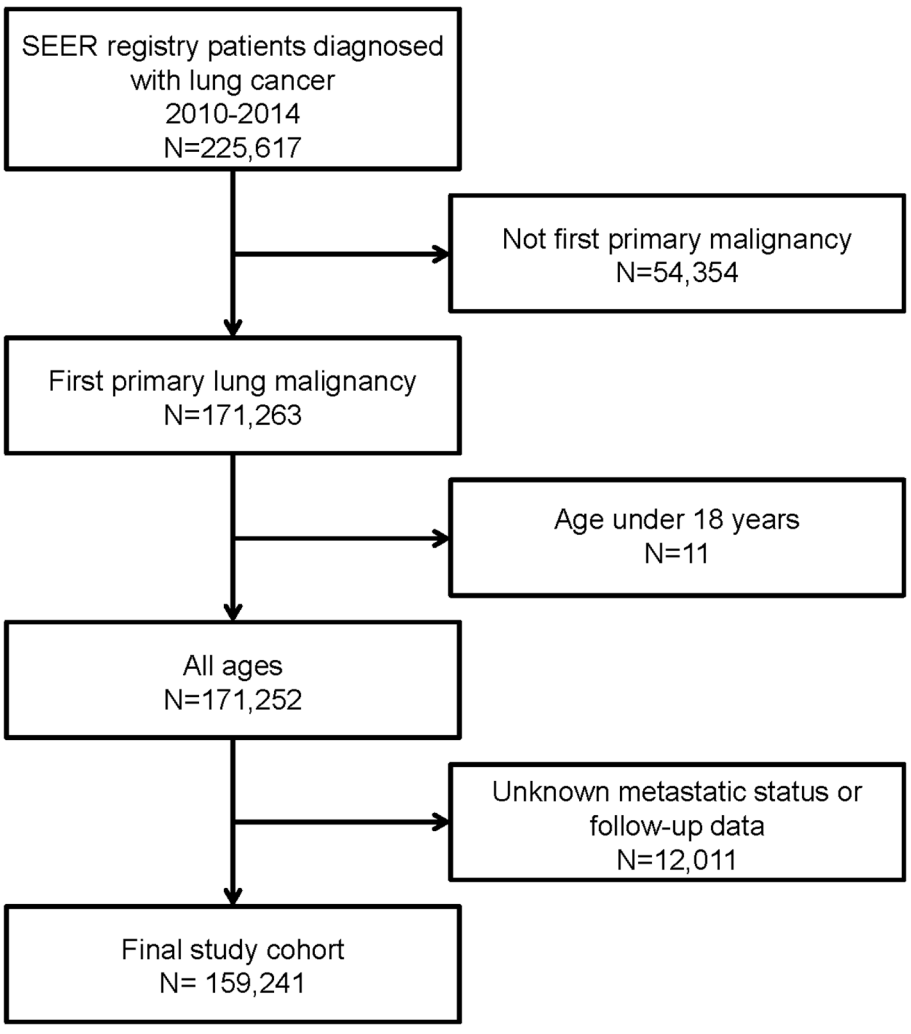

FIGURE 1 | Flowchart of patient selection in this study.

TABLE 1 | Baseline clinical characteristics of lung cancer patients in SEER database.

Characteristics	Bone metastasis			Brain metastasis			Liver metastasis			DL metastasis		
	No (%)	Yes (%)	*P*	No (%)	Yes (%)	*P*	No (%)	Yes (%)	*P*	No (%)	Yes (%)	*P*
Histological type												
Adenocarcinoma	59,090 (78.5)	16,141 (21.5)	<0.001	63,943 (85.0)	11,288 (15.0)	<0.001	68,647 (91.2)	6,584 (8.8)	<0.001	70,399 (93.6)	4,832 (6.4)	<0.001
Squamous cell carcinoma	33,033 (88.8)	4,146 (11.2)		35,061 (94.3)	2,118 (5.7)		34,874 (93.8)	2,305 (6.2)		35,737 (88.0)	1,442 (12.0)	
LCLC	2,322 (82.0)	510 (18.0)		2,342 (82.7)	490 (17.3)		2,441 (86.2)	391 (13.8)		2,601 (91.8)	231 (8.2)	
SCLC	17,427 (76.7)	5,282 (23.3)		18.913 (83.3)	3,796 (16.7)		15,549 (68.5)	7,160 (31.5)		20,308 (89.4)	2,401 (10.6)	
NSCLC/NOS	16,749 (78.7)	4,541 (21.3)		17,736 (83.3)	3,554 (16.7)		18,341 (86.1)	2,949 (13.9)		19,803 (93.0)	1,487 (7.0)	
Gender												
Male	66,188 (79.2)	17,382 (20.8)	<0.001	72,491 (86.7)	11,079 (13.3)	0.295	72,929 (87.3)	10,641 (12.7)	<0.001	77,683 (93.0)	5,887 (7.0)	<0.001
Female	62,433 (82.5)	13,238 (17.5)		65,504 (86.6)	10,167 (13.4)		66,923 (88.4)	8,748 (11.6)		71,165 (94.0)	4,506 (6.0)	
Age												
<50	5,129 (74.9)	1,717 (25.1)	<0.001	5,361 (78.3)	1,485 (21.7)	<0.001	5,936 (86.7)	910 (13.3)	<0.001	6,156 (89.9)	690 (10.1)	<0.001
51–65	39,240 (77.7)	11,276 (22.3)		41,365 (81.9)	9,151 (18.1)		43,568 (86.2)	6,948 (13.8)		46,254 (91.6)	4,262 (8.4)	
≥65	84,252 (82.7)	17,627 (17.3)		91,269 (89.6)	10610 (10.4)		90,348 (88.7)	11,531 (11.3)		96,438 (94.7)	5,441 (5.3)	
Marital status												
Married	62,526 (79.8)	15,860 (20.2)	<0.001	67,634 (86.3)	10,752 (13.7)	<0.001	68,786 (87.8)	9,600 (12.2)	0.625	73,147 (93.3)	5,239 (6.7)	0.035
Unmarried	60,049 (81.7)	13,420 (18.3)		63,833 (86.9)	9,636 (13.1)		64,562 (87.9)	8,907 (12.1)		68,772 (93.6)	4,697 (6.4)	
Unknown	6,046 (81.9)	1,340 (18.1)		6,528 (88.4)	858 (11.6)		6,504 (88.1)	882 (11.9)		6,929 (93.8)	457 (6.2)	
Race												
White	103,885 (80.9)	24,502 (19.1)	<0.001	111,722 (87.0)	16,665 (13.0)	<0.001	112,357 (87.5)	16,030 (12.5)	<0.001	120,108 (93.6)	8,279 (6.4)	0.009
Black	15,612 (81.5)	3,555 (18.5)		16,447 (85.8)	2,720 (14.2)		17,055 (89.0)	2,112 (11.0)		17,889 (93.3)	1,278 (6.7)	
Others	9,124 (78.1)	2,563 (21.9)		9,826 (84.1)	1,861 (15.9)		10,440 (89.3)	1,247 (10.7)		10,851 (92.8)	836 (7.2)	
Grade												
I	7,240 (94.3)	434 (5.7)	<0.001	7,405 (96.5)	269 (3.5)	<0.001	7,521 (98.0)	153 (2.0)	<0.001	7,573 (98.7)	101 (1.3)	<0.001
II	23,464 (90.6)	2,444 (9.4)		24,237 (93.6)	1,671 (6.4)		25,041 (96.7)	867 (3.3)		25,312 (97.7)	596 (2.3)	
III	33,521 (83.9)	6,441 (16.1)		34,842 (87.2)	5,120 (12.8)		36,528 (91.4)	3,434 (8.6)		37,754 (94.5)	2,208 (5.5)	
IV	3,901 (80.4)	951 (19.6)		4,121 (84.9)	731 (15.1)		3,836 (79.1)	1,016 (20.9)		4,434 (91.4)	418 (8.6)	
Unknown	60,495 (74.8)	20,350 (25.2)		67,390 (83.4)	13,455 (16.6)		66,926 (82.8)	13,919 (17.2)		73,775 (91.3)	7,070 (8.7)	
Size (cm)												
<2.0	17,612 (89.1)	2,160 (10.9)	<0.001	18,206 (92.1)	1,566 (7.9)	<0.001	18,536 (93.7)	1,236 (6.3)	<0.001	19,025 (96.2)	747 (3.8)	<0.001
2.0–4.9	51,049 (82.2)	11,029 (17.8)		54,351 (87.6)	7,727 (12.4)		55,967 (90.2)	6,111 (9.8)		58,838 (94.8)	3,240 (5.2)	
5.0–9.9	32,257 (79.1)	8,511 (20.9)		34,305 (84.1)	6,463 (15.9)		35,269 (86.5)	5,499 (13.5)		37,835 (92.8)	2,933 (7.2)	
≥10.0	3,844 (79.8)	974 (20.2)		4,067 (85.4)	751 (15.6)		4,123 (85.6)	695 (14.4)		4,383 (91.0)	435 (9.0)	
Unknown	23,859 (75.0)	7,946 (25.0)		27,066 (85.1)	4,739 (14.9)		25,957 (81.6)	5,848 (18.4)		28,767 (90.4)	3,038 (9.6)	
Regional lymph node invasion												
N0	53,156 (90.4)	5,630 (9.6)	<0.001	54,430 (92.6)	4,356 (7.4)	<0.001	55,846 (95.0)	2,940 (5.0)	<0.001	57,998 (98.7)	788 (1.3)	<0.001
N1	10,999 (82.5)	2,337 (17.5)		11,604 (87.0)	1,732 (13.0)		11,952 (89.6)	1,384 (10.4)		12,824 (96.2)	512 (3.8)	
N2	44,427 (75.6)	14,321 (24.4)		48,970 (83.4)	9,778 (16.6)		48,861 (83.2)	9,887 (16.8)		54,392 (92.6)	4,356 (7.4)	

(Continued)

TABLE 1 | Continued

Characteristics		Bone metastasis			Brain metastasis			Liver metastasis			DL metastasis		
		No (%)	Yes (%)	*P*	No (%)	Yes (%)	*P*	No (%)	Yes (%)	*P*	No (%)	Yes (%)	*P*
	N3	15,408 (70.4)	6,479 (29.6)		17,707 (80.9)	4,180 (19.1)		18,009 (82.3)	3,878 (17.7)		17,249 (79.6)	4,458 (20.4)	
	NX	4,631 (71.4)	1,853 (28.6)		5,284 (81.5)	1,200 (18.5)		5,184 (80.0)	1,300 (20.0)		6,205 (95.7)	279 (4.3)	
Surgery													
	Yes	32,518 (98.8)	400 (1.2)	<0.001	32,346 (98.3)	572 (1.7)	<0.001	32,760 (99.5)	158 (0.5)	<0.001	32,783 (99.6)	135 (0.4)	<0.001
	No	96,103 (76.1)	30,220 (23.9)		105,649 (83.6)	20,674 (16.4)		107,092 (84.8)	19,231 (15.2)		116,065 (91.9)	10,258 (8.1)	
Chemotherapy													
	Yes	58,803 (77.5)	17,117 (22.5)	<0.001	64,268 (84.7)	11,652 (15.3)	<0.001	65,583 (86.4)	10,337 (13.6)	<0.001	69,507 (91.6)	6,413 (8.4)	<0.001
	No	69,818 (83.8)	13,503 (16.2)		73,727 (88.5)	9,594 (11.5)		74,269 (89.1)	9,052 (10.9)		79,341 (95.2)	3,980 (4.8)	
Radiation therapy													
	Yes	50,966 (76.8)	15,387 (23.2)	<0.001	50,744 (76.5)	15,609 (23.5)	<0.001	59,939 (90.3)	6,414 (9.7)	<0.001	62,016 (93.5)	4,337 (6.5)	0.895
	No	77,655 (83.6)	15,233 (16.4)		87,251 (93.9)	5,637 (6.1)		79,913 (86.0)	12,975 (14.0)		86,832 (93.5)	6,056 (6.5)	

ΔOthers include American Indian, AK Native, Asian, and Pacific Islander. LCLC, Large-cell lung cancer; SCLC, Small-cell lung cancer; NSCLC, Non-small cell lung cancer.

Results (SEER) national database. Cases were included in this research on the basis of the following inclusion and exclusion criteria.

Inclusion criteria: (1) Diagnosis of lung cancer was made pathologically between the year 2010–2014; (2) Lung cancer was the first primary malignancy; (3) Detailed information about metastatic status was complete.

Exclusion criteria: (1) Age under 18 years old; (2) Metastatic status was unknown; (3) Follow-up data was missing; (4) Information about histological type was unknown.

Statistical Analysis

Descriptive statistics were used to summarize patients' demographic, clinicopathological, and therapeutic variables in different histological subgroups. We conducted χ^2-tests to make comparisons of metastatic distribution among different histological types. Odds ratios were calculated to analyze co-occurrence relationships between different metastatic lesions. Kaplan–Meier methods were performed to analyze overall survival (OS) and cancer-specific survival (CSS) according to different metastatic sites were conducted to identify independent prognostic factors. Two-sided $P < 0.05$ were defined as statistically significance. We used GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) and SPSS 22.0 (SPSS Inc. Chicago, IL, USA) to perform the statistical analyses.

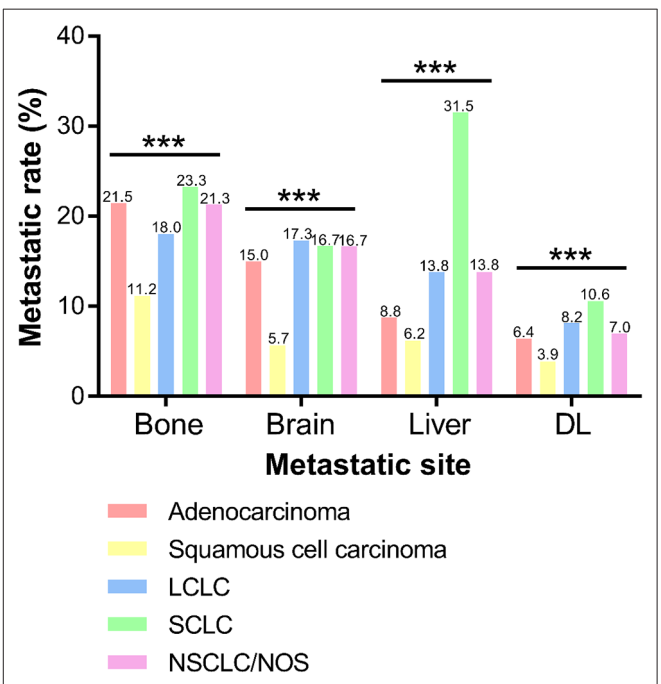

FIGURE 2 | Frequencies of extrathoracic metastatic organs according to different histological types. DL, distant lymph node. (***$P < 0.001$).

RESULTS

Patient Characteristics

According to the inclusion and exclusion criteria, we finally enrolled 159,241 cases diagnosed with lung cancer. Detailed selection flowchart was illustrated in **Figure 1**. Among the final cohort, 75,231 cases (47.2%) were adenocarcinoma, 37,179 cases (23.3%) were squamous cell carcinoma, 2,832 cases (1.8%) were large-cell lung cancer, 22,709 cases (14.3%) were small -cell lung cancer, and 21,290 cases (13.4%) were non-small cell lung cancer. The baseline demographic and clinicopathological parameters according to different metastatic lesions were shown in **Table 1**.

Among the final cohort, 60,580 cases (38.0%) were recorded as extrathoracic metastasis. In total, the four metastatic lesions (bone, brain, liver, and distant lymph node) accounted for 94.0% (56,933/60,580) of all extrathoracic metastatic sites. And the frequencies of bone, brain, liver and distant lymph node (DL) metastasis were 19.2% (30,620/159,241), 13.3% (21,246/159,241), 12.2% (19,380/159,241), and 6.5% (10,393/159,241), respectively.

Metastatic Pattern

As shown in **Figure 2**, incidence rate of bone metastasis was the highest in SCLC (23.3%), followed by adenocarcinoma (21.5%), NSCLC/NOS (21.3%), LCLC (18.0%), and squamous cell carcinoma (11.2%). And frequencies of brain metastasis were 15.0, 5.7, 17.3, 16.7 and 16.7%

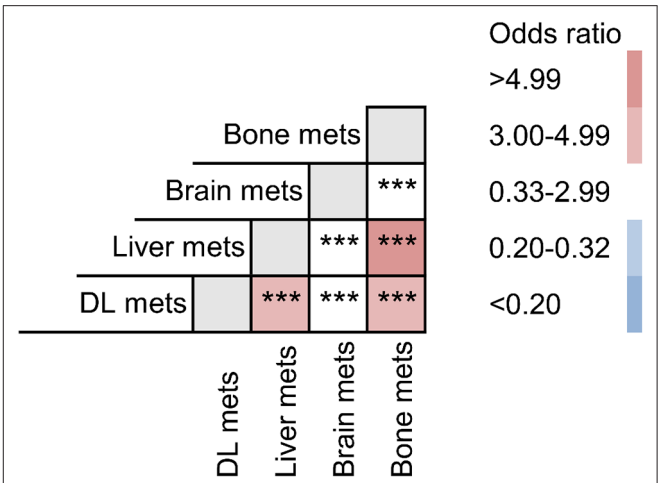

FIGURE 4 | Odds ratio comparison among different metastatic combinations. ***$P < 0.001$.

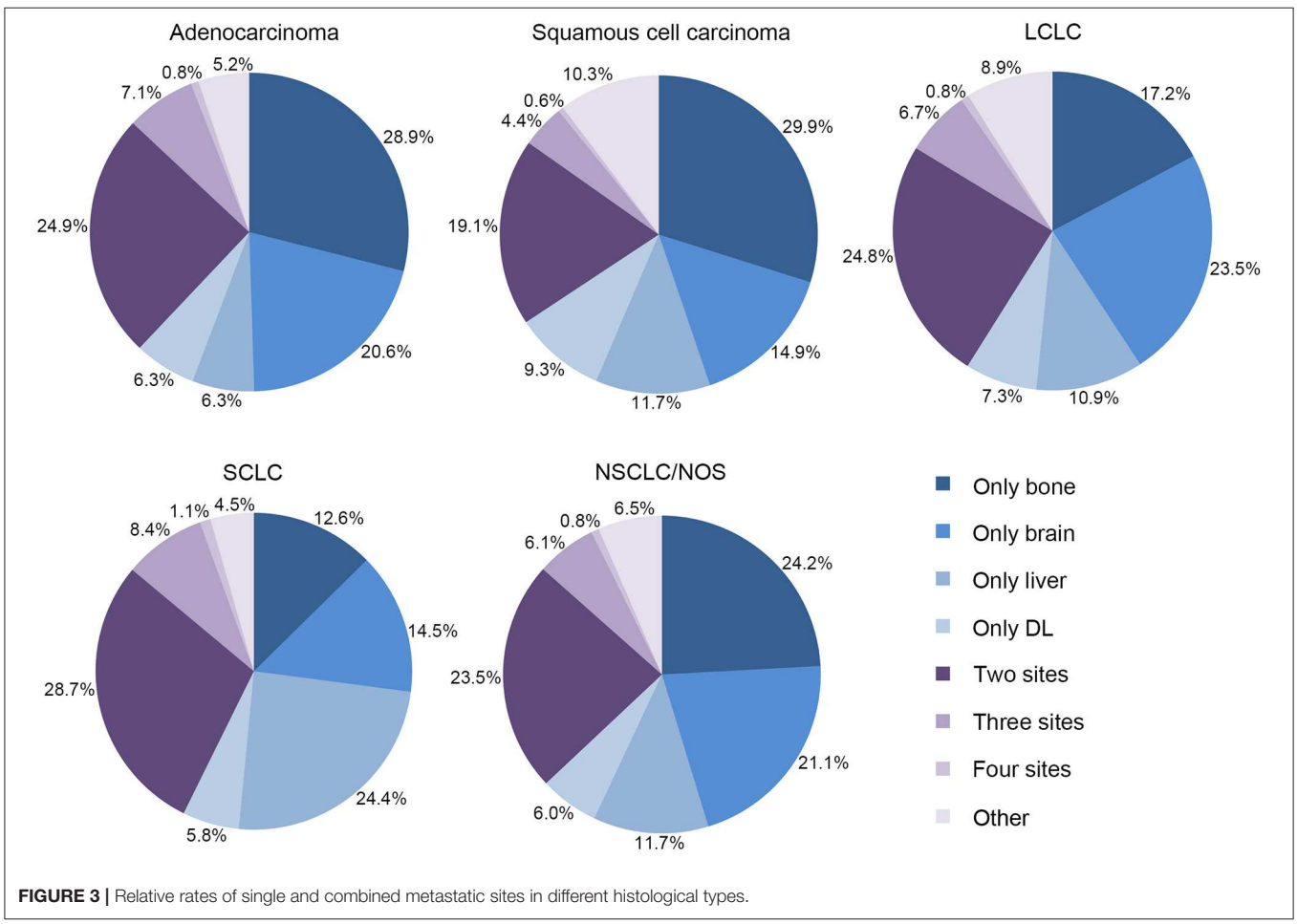

FIGURE 3 | Relative rates of single and combined metastatic sites in different histological types.

in adenocarcinoma, squamous cell carcinoma, LCLC, SCLC, and NSCLC/NOS, respectively. The incidence of brain metasiasis almost the same except squamous cell carcinoma. Also, the metastatic rate of liver was extremely high in SCLC (31.5%) and relatively low in squamous cell carcinoma (6.2%). In addition, the frequency of DL metastasis in SCLC (10.6%) was higher than LCLC (8.2%), NSCLC/NOS (7.0%), adenocarcinoma (6.4%) and squamous cell carcinoma (3.9%).

For clinicopathological features, metastatic group tended to have younger age, poorer tumor differentiation, larger tumor size and higher frequency of regional lymph node invasion (**Table 1**). As for therapies, advanced-stage patients received less surgery and more chemotherapy than non-metastatic patients. And

TABLE 2 | Survival analysis in diverse metastatic organs.

Parameter	1-year OS (%)	Univariate analysis		1-year CSS (%)	Univariate analysis	
		Log rank χ^2 test	*P*		Log rank χ^2 test	*P*
Bone						
No metastasis	50.8	12615.144	<0.001	57.5	13549.160	<0.001
Metastasis	20.0			25.9		
Brain						
No metastasis	48.4	6499.456	<0.001	55.1	7316.606	<0.001
Metastasis	22.1			27.4		
Liver						
No metastasis	49.2	14245.964	<0.001	55.8	14802.929	<0.001
Metastasis	13.6			20.1		
Distant lymph nodes						
No metastasis	46.4	2748.753	<0.001	53.0	2990.572	<0.001
Metastasis	22.9			28.8		

OS, Overall Survival; CSS, Cancer Specific Survival.

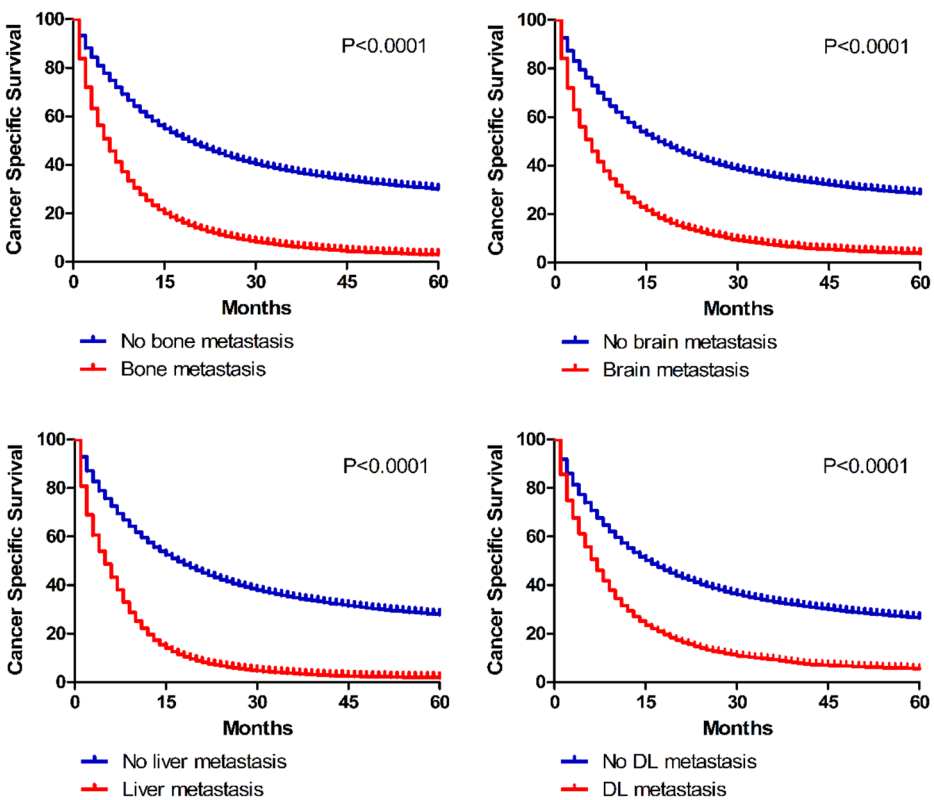

FIGURE 5 | Kaplan–Meier curves of cancer specific survival in patients according to metastatic status.

patients with bone or brain metastasis received more radiation therapy than non-metastatic patients.

Combination of Metastases

For further analyzing combination of metastases, we performed pie charts to investigate single-metastases and co-metastases among different histological types of lung cancer (**Figure 3**). It is shown that bone was the leading lesion as a single metastatic site in adenocarcinoma (28.9%), squamous cell carcinoma (29.9%) and NSCLC/NOS (24.2%). Also, brain was the leading single-metastatic lesion in LCLC (23.5%), and liver was the most frequent site in SCLC (24.4%). As for combination of metastases, bi-site pattern (adenocarcinoma: 24.9%, squamous cell carcinoma: 19.1%, LCLC: 24.8%, SCLC: 28.7%, and NSCLC/NOS: 23.5%) was significantly higher than tri-site (adenocarcinoma: 7.1%, squamous cell carcinoma: 4.4%, LCLC: 6.7%, SCLC: 8.4%, and NSCLC/NOS: 6.1%) and tetra-site pattern (Adenocarcinoma: 0.8%, Squamous cell carcinoma: 0.6%, LCLC: 0.8%, SCLC: 1.1%, and NSCLC/NOS: 0.8%).

Furthermore, we calculated odds ratios to compare each possible combination of different extrathoracic metastatic lesions (**Figure 4**, **Supplementary Figure 1**). Bone preferentially tended to co-metastasize with liver (OR: 5.287) and DL (OR: 3.013). And liver metastasis was significantly correlated with DL metastasis (OR: 3.093).

Survival

In the present study, we analyzed 1-year OS and CSS in cases with diverse extrathoracic metastatic lesions (**Table 2**). Univariate analyses indicated that survival differences existed between non-metastatic and metastatic patients (OS: bone 50.8 vs. 20.0%, brain 48.4 vs. 22.2%, liver 49.2 vs. 13.6%, DL 46.4 vs. 22.9%; CSS: bone 57.5 vs. 25.9%, brain 55.1 vs. 27.4%, liver 55.8 vs. 20.1%, DL 53.0 vs. 28.8%). And Kaplan–Meier curves further illustrated the survival data between non-metastatic and metastatic groups (**Figure 5**).

Furthermore, Cox regression models were conducted to identify independent prognostic factors (**Table 3**). With adjusting for histological type, gender, age, race, marital status, grade, tumor size, regional lymph node invasion and therapies, all extrathoracic metastatic lesions were independent risk factors for OS (bone: HR 1.312, 95%CI 1.302–1.321; brain: HR 1.339, 95%CI 1.328–1.351; liver: HR 1.344, 95%CI 1.333–1.355; DL: HR 1.263, 95%CI 1.235–1.290) and CSS (bone: HR 1.337, 95%CI 1.328–1.348; brain: HR 1.368, 95%CI 1.357–1.381; liver: HR 1.375, 95%CI 1.363–1.388; DL: HR 1.283, 95%CI 1.254–1.313).

Additionally, survival differences between different bi-organ metastases were analyzed (**Figure 6**). It is suggested in the Kaplan–Meier curves that combined metastasis resulted in worse prognostic ending than the separated single-organ metastasis. Once metastasis happens, lung cancer patients might get a worse outcome.

Discussion

Lung cancer related deaths are mainly attributable to extrathoracic metastasis (16, 17). Advanced lung cancer seems to metastasize to lymph nodes and other distant organs, such as brain, bone and liver. Most metastasis could cause corresponding symptoms which is represented by the N and M staging in the TNM system. In order to understand its metastatic patterns, we elucidated the following points in this research: (1) Comparing the frequencies of different metastatic lesions in different histological types; (2) Elaborating the tendency of combined metastases; (3) Clarifying the prognostic significance of single-site and bi-site metastases. As the first comprehensive, population-based research focusing on metastatic patterns in different histological types of lung cancer, the findings may provide sufficient information for clinical decision and cancer research.

According to the reported data, bone and brain were two leading distant targets for metastasis in NSCLC (18, 19). Our results further supported these findings, suggesting that bone was the most common metastatic site, followed by brain, liver and DL in all histological sites of NSCLC (including adenocarcinoma, squamous cell carcinoma, LCLC and NSCLC/NOS). By comparing the frequency of extrathoracic metastasis in diverse histological types of NSCLC, we found that more than 30% of adenocarcinoma and LCLC patients showed distant metastasis, while squamous cell cancer had the lowest rate of distal metastasis. Moreover, among all histological types of lung cancer, SCLC had the highest frequency of extrathoracic metastasis, especially to the liver, which is consistent with the reported data in previous studies (20–22). So, according to these conclusion, adenocarcinoma and LCLC patients could be arranged serious and continual follow-up, more importantly,

TABLE 3 | Multivariate analyses of overall and cancer-specific survival in related to metastatic sites.

Variable	Overall survival		Cancer-specific survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*
No metastasis	Reference		Reference	
Bone metastasis	1.312 (1.302–1.321)	<0.001	1.337 (1.328–1.348)	<0.001
Brain metastasis	1.339 (1.328–1.351)	<0.001	1.368 (1.357–1.381)	<0.001
Liver metastasis	1.344 (1.333–1.355)	<0.001	1.375 (1.363–1.388)	<0.001
DL metastasis	1.263 (1.235–1.290)	<0.001	1.283 (1.254–1.313)	<0.001

Adjusted for histological type, gender, age, race, marital status, grade, tumor size, regional lymph node invasion and therapies. OS, Overall Survival; CSS, Cancer Specific Survival; HR, Hazard Ratio.

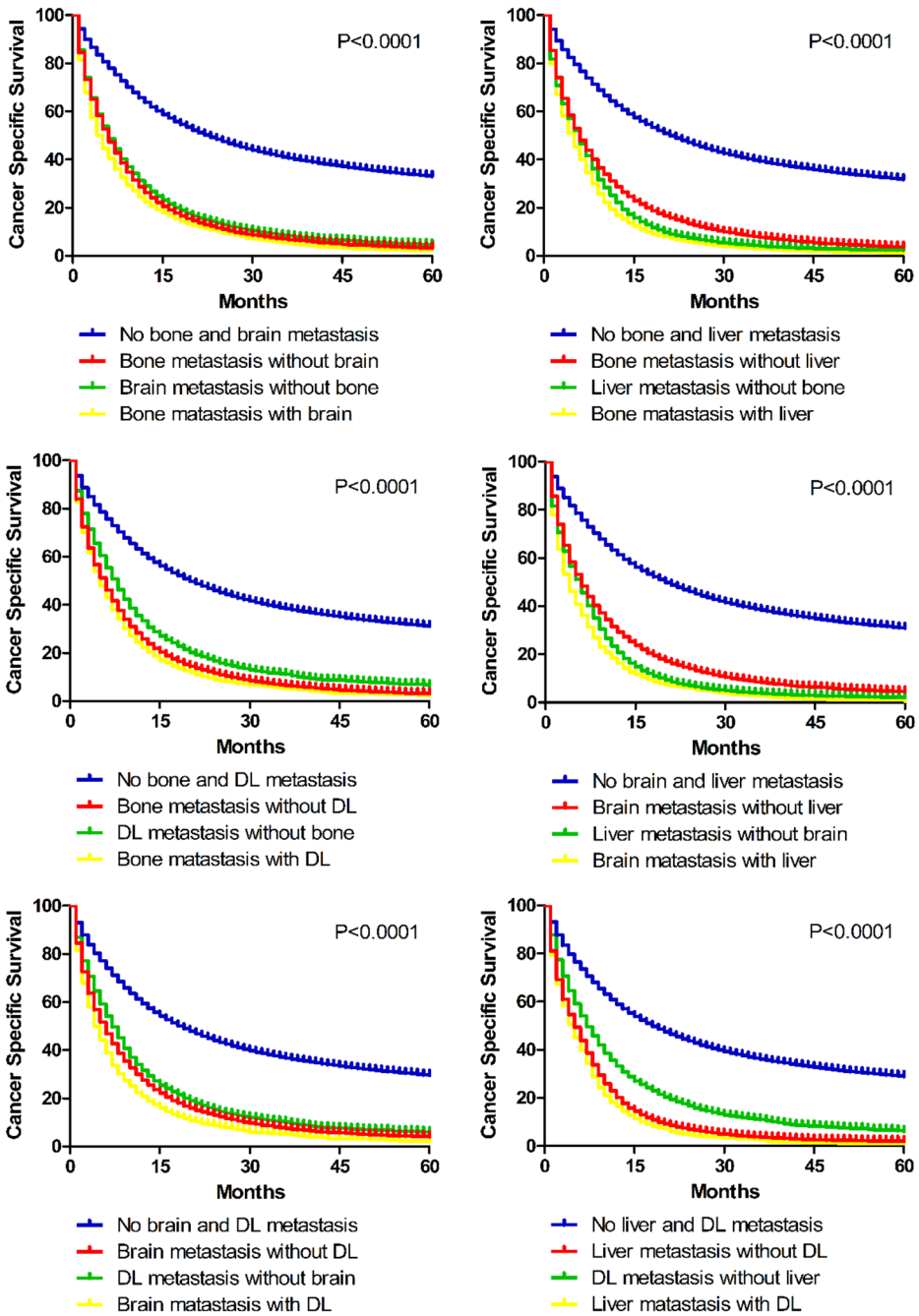

FIGURE 6 | Kaplan–Meier curves of cancer specific survival in patients with different bi-site metastatic patterns.

these patients could take cutting-edge therapies, such as combined immunotherapy, neoadjuvant chemotherapy, and so on. For SCLC patients, liver ultrasound and CT scan need to be focused on.

Notably, according to the clinicopathological features, metastatic group tended to have a poorer tumor differentiation, a larger tumor size and a higher rate of regional lymph node invasion, which indicated a more aggressive and invasive hallmark of tumor biology. Compared to non-metastatic patients, advanced-stage patients received less surgery and more chemotherapy, because they lost the chance of curative resection at the time of diagnosis. And since radiation could control tumor growth of metastatic nodules as well as alleviating symptoms, patients with bone or brain metastasis received more radiation therapy than non-metastatic patients.

But these conclusions have their own historical limitaions. With the development of immunotherapy and neoadjuvant chemotherapy, patients may benefit from these modern and fancy therapies, and they could even get the chance of surgery due to the shrinking tumors. Considering these demographic, clinicopathological and treatment variables that may have impact on survival outcomes, we further conducted multivariate analysis and found that all single-site metastases were independent prognostic factors.

To our knowledge, no previous population-based researches studied the combined metastatic patterns of lung cancer. Our results indicated that bone preferentially tended to co-metastasize with liver and distal lymph nodes. And liver metastasis was significantly correlated with distant lymph node metastasis. To our knowledge, analyzing tendency of co-metastases would be rather useful to assess potential risks and make diagnosis and treatment strategies. Once bone metastasis was found, we need to screen the liver and get an enhanced CT to detect the lymph nodes. Thus, patients may get a comprehensive system treatment. And, if liver metastasis needed to be surgical removed, doctors should note that lymph node dissection is the necessary and best choice. Moreover, we further assessed the prognostic values of bi-site metastases. As shown in Kaplan–Meier curves, combined metastasis resulted in worse prognostic ending than the separated single-organ metastasis. So patients with multi-organ metastasis may need more aggressive therapeutic regimens.

Though we seriously performed this population-based research, there may still be several potential limitations. The first limitation may be the retrospective nature of this study. We only enrolled patients with detailed distal metastasis since SEER database recorded from year 2010. Second, information of extrathoracic metastatic sites was restricted to bone, brain, liver, and DL. However, these four metastatic lesions accounted for the majority of extrathoracic metastatic sites in lung cancer. Third, the metastasis condition from SEER was synchronous when diagnosed, but in the real world, metachronous carcinoma accounts for the majority. These limitations could cause bias in some results.

In a word, we comprehensively analyzed the pattern of extrathoracic metastases in different histological types of lung cancer in this population-based study. We found that the frequency of bone metastasis was the highest in adenocarcinoma, squamous cell carcinoma, LCLC and NSCLC/NOS, while liver was the most common metastatic site in SCLC. Bi-site metastases occurred more common than tri-site and tetra-site metastases. Several metastatic sites, such as bone and liver, intended to co-metastasize preferentially. All single-site metastases were independent prognostic factors and co-metastases ended up with even worse survival outcomes. Thus, our findings would be beneficial for future research design and clinical practice.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

Conception and design: XW and ZW. Development of methodology: XW, ZW, and JP. Acquisition of data, analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z-YL, DX, H-JZ, and S-HW. Writing, review and/or revision of the manuscript: XW, ZW, and JP. Study supervision: D-YH and X-FC. All authors reviewed and approved the final manuscript.

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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.2020.00715/full#supplementary-material

Supplementary Figure 1 | The number of odds ratio comparison among different metastatic combinations.

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Clinical Evaluation of Serum Tumor Markers in Patients With Advanced-Stage Non-Small Cell Lung Cancer Treated With Palliative Chemotherapy in China

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Aim: This study aims to analyze the prognostic value of seven tumor makers and also investigate the response of palliative chemotherapy in advanced NSCLC patients with advanced disease.

Methods: Medical records of 278 advanced NSCLC Chinese patients who received six cycles of palliative chemotherapy were retrospectively reviewed under ethical approval (JSCH2019K-011). Univariate and multivariate Cox regression analyses were performed using SPSS 24 to find the clinical value of these tumor markers and to identify the factors that were associated with progression-free survival (PFS), as well as the response to palliative chemotherapy.

Results: In baseline characteristic, the high levels of CEA, CA-125, CA-199, AFP, NSE, CYFRA21-1, and CA15-3 were detected in 209 (75.18%), 139 (50.0%), 62 (22.30%), 18 (6.47%), 155 (55.75%), 176 (63.30%), and 180 (64.74%) patients, respectively. Univariate analysis revealed that patients with high vs. normal levels of all tumor markers had an increased risk of poor prognosis. In the multivariable Cox regression model, the patient with (high vs. normal) CYFRA21-1 levels (HR = 1.454, $P = 0.009$) demonstrated an increased poor PFS. However, patients with (high vs. normal) CA19-9 levels (HR = 0.524, $P < 0.0001$) and NSE levels (HR = 0.584, $P < 0.0001$) presented a decreased risk of PFS. Also, patients receiving 3-drugs regimen had better PFS compared to those on 2-drugs regimen ($P = 0.043$).

Conclusions: The high levels of CYFRA21-1 was correlated with a poor prognostic factor of PFS for Advanced NSCLC patients. However, the high levels of CA19-9 and NSE were associated with a better prognostic factor of PFS. Additionally, smoking habits and tumor status had a poor prognostic factor of PFS. Moreover, we found that antiangiogenic therapy has high efficacy with first-line chemotherapy and longer PFS of NSCLC patients.

Keywords: non-small cell lung cancer, stage IV, serum tumor markers, prognosis, palliative chemotherapy, six-cycles

INTRODUCTION

Lung cancer is one of the most common and fatal cancers worldwide (1, 2). Lung cancer is a heterogeneous disease comprising mainly non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), which approximately accounted for 85% and 10–15% of all lung cancer cases respectively (2, 3). According to the global cancer statistics of 2018, lung cancer accounted for approximately 2,093,876 (11.6) new cases and 1,761,007 (18.4) of total cancer deaths representing one in five (18.4%) cancer deaths (4).

In China, the incidence and mortality rates of lung cancer have increased markedly over the past decades and accounted for ∼49.94 per 100,000 men and 23.89 per 100,000 women and 40.30 per 100,000 and 17.13 per 100,000 deaths in 2014 (5). A recent study in China reported an annual mortality rate of 0.6 million (majority male) and 0.73 million new cases annually (6). In addition, NSCLC survival rate was estimated at 16.8% for men and 25.1% for women in 2012–2015, which are relatively low compared to other cancers (5). This can be explaining by the fact that about two-thirds of NSCLC patients are usually at an advanced stage (i.e., unresectable stage IIIB and IV) at the time of diagnosis (1, 2). Most of these advanced tumors are not surgically resectable as a result of disseminated (multiple sites) metastatic disease or metastatic sites that are not amenable to surgery. Patients with single metastatic sites may undergo surgical resection of both the primary tumor in the lung and the metastatic site. However, first-line chemotherapy used in most of the advanced NSCLC cases.

The purpose of palliative chemotherapy is to improve patient quality of life and increase the survival rate. Advanced non-small lung cancer patients are treated by either radiotherapy or palliative chemotherapy. Studies have reported that even with radiotherapy survival rates have not been significant (1, 2). Though palliative chemotherapy is not curative, it plays a supportive role to improve patient health state, and limit complications when chances of recovery are slim (7).

Tumor markers are small circulating quantifiable molecules present in blood or tissue which are released by tumor cells or body immune cells in response to tumor growth (8, 9). Tumor markers play a pivotal role in clinical diagnosis, prognosis, and anti-drug surveillance. Tumor markers can also be used to measure the response to chemotherapy (10, 11). Tumor markers have several advantages over conventional diagnostic methods, these are cheap, less time taking, unresting state, and avoid radiation exposure but statistically, it also supports the clinicians to estimate the progression of tumor (12, 13).

Previous studies have reported an association between tumor markers and curative effect in patients with breast cancer, epithelial ovarian cancer, gastric cancer, pancreatic cancer, and colorectal cancer (14–16). There is, however, limited clinical studies on the utilization of tumor markers in advanced-stage NSCLC (17). To the best of our knowledge, this is the first study to evaluate the clinical utility of seven tumor markers CEA, CA19-9, CA125, AFP, NSE, CA15-3, and CYFRA21-1 for prognostic specification as well as for measuring the response of chemotherapy (2-drugs vs. 3-drugs) in terminal stage (IV) NSCLC patients who underwent palliative chemotherapy.

METHODS

Study Site

Jiangsu cancer hospital, also known as Jiangsu Institute of Cancer Research is founded in 1960 and located in Nanjing city, China. The Hospital has 1,161 open beds with 1,635 employees across 25 clinical and medical departments. In 2019, the medical oncology department of Hospital received over 4,874 patients, which present a monthly average of 406 patients.

Study Design

A retrospective study was conducted between January 01, 2013, and March 29, 2019, under the approval of the research ethics committee of Jiangsu Cancer hospital (JSCH2019K-011). In this study, Medical records of 5,445 patients were succinctly reviewed and classified based on defined inclusion and exclusion criteria. The patient demographics, medical history, and physical examination, were verified before study entry. The patient medical record was collected until death, progression of cancer, and last medical fellow-up. The levels of CEA, CA125, CA19-9, AFP, NSE, CYFRA21-1, and CA15-3 were recorded at the baseline and at the start of six chemotherapy cycles. The flowchart and analysis are presented in **Figure 1**.

Inclusion/Exclusion Criteria

The inclusion criteria of this study consisted of: (A) patients with histologically confirmed terminal stage IV NSCLC according to the TNM staging criteria set by the International Union Against Cancer (UICC) in 2009 (2); (B) patients with ECOG performance status of 0–2; (C) patients who received palliative chemotherapy and were followed up at least six chemo-cycles; and (D) Postresection recurrent of NSCLC patients in palliative chemotherapy. The exclusion criteria included: (A) patients who diagnosed previously or had concurrent co-morbid cancers; (B) patients with inadequate medical records or recurrence within six chemo-cycles. Based on the inclusion and exclusion criteria, a total of 278 advanced NSCLC patients were enrolled in this study.

Laboratory Measurement

Assay of tumor markers was performed by electrochemiluminescence (ECL) so as to determine the baseline levels of CEA, CA19-9, CA125, AFP, CYFRA21-1, and NSE and at the beginning of each chemo-cycle until the risk of progression. The levels were compared with the manufacturer cutoff levels of: CEA < 3.5 ng/ml, CA125 < 35 U/ml, CA19-9 $<$ 39 U/ml, AFP < 10 ng/ml, NSE < 16.3 ng/ml, CYFRA21-1 3.3 ng/ml and CA15-3 < 30 U/mL. Serum levels above (high) the cutoff values indicated a positive outcome. Positive detection of all the tumor markers was considered in case of one or more serum marker levels were above the normal cutoff range.

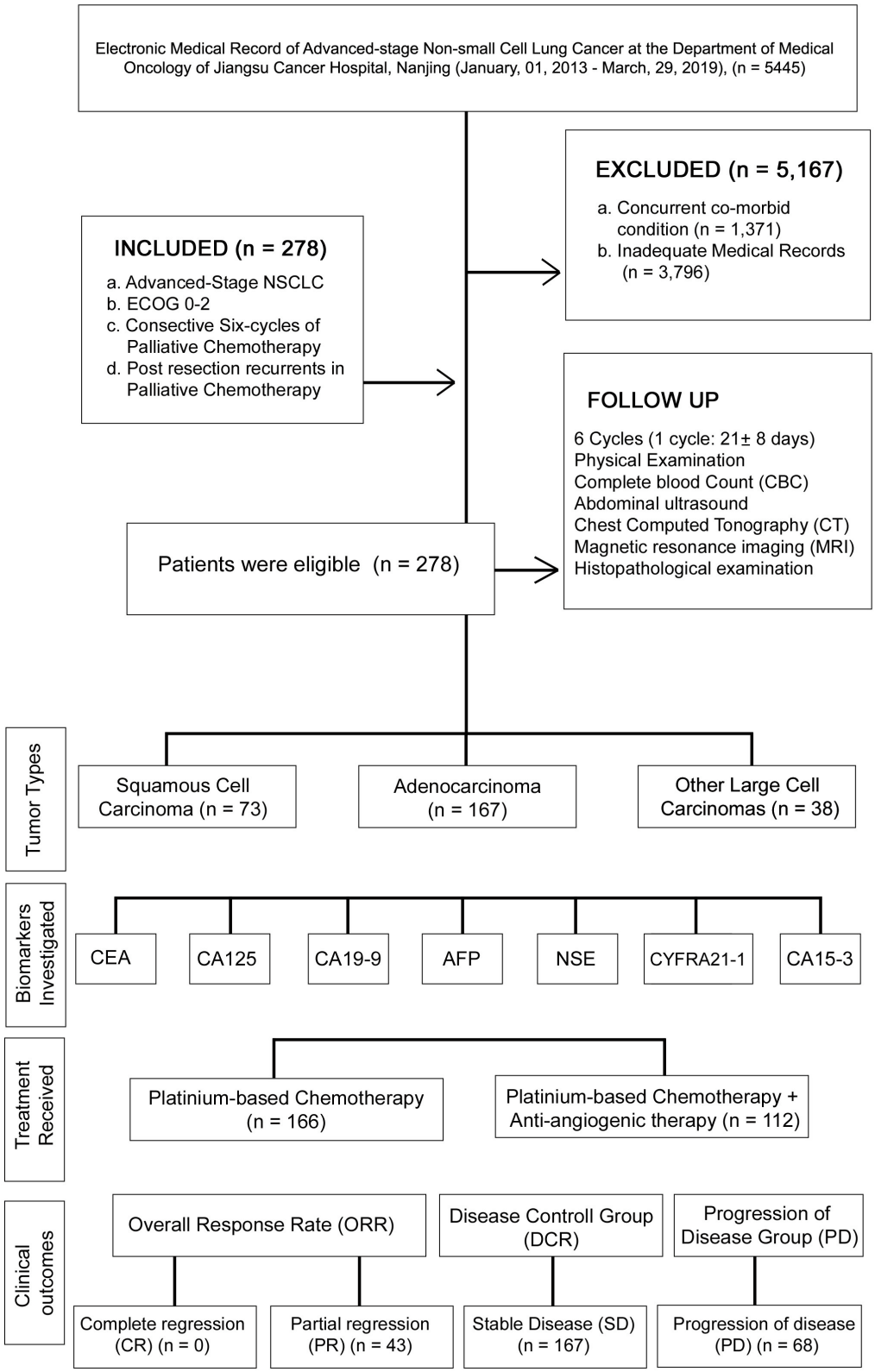

FIGURE 1 | Procedural flowchart of the study.

TABLE 1 | Palliative chemotherapy regimens for advanced-stage NSCLC patients.

Combination of chemotherapy	Dose and cycle
ETOPOSIDE + cisplatin	VP16 100 mg/m^2, d1–3, Cis: 75 mg/m^2, d1, q3w
PEMETREXED DISODIUM + carboplatin	Pem 500 mg/m^2, d1, Carbo AUC 5, d1, q3w
PEMETREXED DISODIUM + irinotecan	Pem 500 mg/m^2, d1, Iri 200 mg/m^2, d1, q3w
DOCETAXEL + cisplatin	Doc 60–75 mg/m^2, d1, Cis 60–75 mg/m^2, d1
GEMCITABINE + vinorelbine	Gem 1,000 mg/m^2 d1, d8, Vin 25 mg/m^2 d1, d8, q3w
PACLITAXEL ALBUMIN + nedaplatin	Nab-Pac 125 mg/m^2 d1, d8, Neda 80 mg/m^2 d1, q3w
DOCETAXEL + epirubicin	Doc 60–75 mg/m^2, d1, Epi 60 mg/m^2, d1
BLEOMYCIN HCL + CARBOPLATIN	Bleo 15 mg, d1–5,Carbo AUC 5 d1, q3w
DOCETAXEL + oxaliplatin	Doc 60–75 mg/m^2, d1, Oxol 120 mg/m^2 d1, q3w
ETOPOSIDE + lobaplatin	VP16 100 mg/m^{2*3} (d1–3), Lobaplatin 30 mg/m^2 d1, q3w
DISODIUM CANTHARIDINATE; PYRIDOXINE + pemetrexed	VP16 100 mg/m^2, d1–3, Lobo 30 mg/m^2, d1
PACLITAXEL ALBUMIN + cisplatin	Nab-Pac 125 mg/m^2 d1, d8, Cis 60–75 mg/m^2, q3w
PEMETREXED + tegafur; gimeracil; oteracil	Pem 500 mg/m^2 d1, Tegafur 50 mg Bid*14, q3w
VINORELBINE TARTRATE + epirubicin	Vin 25 mg/m^2 d1–3, Epi 60 mg/m^2 d1, q3w

TABLE 2 | Combination of chemotherapy plus anti-angiogenic agents' palliative chemotherapy-based regimens for advanced-stage NSCLC patients.

Combination of chemotherapy plus anti-angiogenic agents	Dose and cycle
PEMETREXED DISODIUM + carboplatin + bevacizumab	Pem 500 mg/m^2, Carbo AUC 5 *1, Bev 7.5 mg/kg d1, q3w
DOCETAXEL + cisplatin + bevacizumab	Doc 60–75 mg/m^2, d1, Cis 60–75 mg/m^2 d1, Bev 7.5 mg/kg, d1, q3w
PEMETREXED DISODIUM + carboplatin + gefitinib	Pem 500 mg/m^2, d1, Carbo AUC 5, d1, Gefi 250 mg/day, unti PD, q3w
PEMETREXED DISODIUM + carboplatin + osimertinib	Pem 500 mg/m^2, d1, Carbo AUC 5, d1, Gefi 250 mg/day, unti PD, q3w
DOCETAXEL + oxaliplatin + icotinib	Doc 60–75 mg/m^2, d1, Oxol 120 mg/m^2 d1, Icotinib 125 mg tid until PD, q3w
Paclitaxel + carboplatin + bevacizumab	Pac 175 mg/m^2, d1, Carbo AUC 5, d1, bev 7.5 mg/m^2, d1, q3w
Gemcitabine + cisplatin + bevacizumab	Gem 1,000 mg/m^2 d1, d8, Carbo AUC 5, d1, bev 7.5 mg/m^2, d1, q3w

Clinical Outcomes

The clinical outcomes were evaluated by progression free survival (PFS). PFS was an initial time of taking therapy to the tumor progression or death. The Curative response was measured by tomography accordingly to the Response Evaluation Criteria in Solid Tumor (RECIST) (1, 2). These were divided into complete regression (CR), stable disease (SD), partial response (PR), and progression disease (PD). Objective response rate (ORR) measured as CR and PR while SD considered as disease control rate (DCR).

Treatment Received

All patients received palliative chemotherapy and were divided into two groups to assess the effectiveness of the chemotherapy: (1) patients receiving 2-drugs (Combination of chemotherapy) as indicated in **Table 1** and (2) those receiving 3-drugs (Combination of chemotherapy + antiangiogenic therapy) as shown in **Table 2**.

Follow-Up

A standardized follow-up was received by all patients, for 2 years at an interval of 3 months, and 6 months, then 3 years and thereafter. On each cycle of follow-up, patients' physical examination, complete blood count (CBC), abdominal ultrasound, chest computed tomography (CT), and brain magnetic resonance imaging (MRI) were performed. Whenever possible local recurrence and distant metastases were also confirmed histologically.

Statistical Analysis

All patients' medical record was analyzed using SPSS 24.0. The association between tumor markers and clinicopathological features were determined by Chi-square analysis. PFS distribution was estimated through Kaplan–Meier curves. The independent prognostic value of each tumor marker and clinicopathological features that highly affect the PFS was evaluated by Cox regression multivariate analysis. Change in the tumor marker levels and effectiveness of pre- and post-palliative chemotherapy were determined using Wilcoxon signed ranks test. And $P < 0.05$ was considered statistically significant.

RESULTS

Patients' Characteristics

The Baseline characteristics of the 278 advanced NSCLC patients are summarized in **Table 3**. The Mean Age of the patients was (59.11 ± 10.39) years, and the majority of patients were males (65.8%) with no statistical differences ($P = 0.357$). In addition, 56.6% of patients had non-smoking habits with significant differences ($P < 0.0001$). Patients were classified according to the standard classification system of World Health Organization/International Association for the study of Lung Cancer (WHO/IASLC) (1, 2). With respect to the clinicopathological features, the majority of patients had metastasis (69.8%) with significant differences ($P = 0.015$). The histological diagnosis revealed 26.3, 60.1, and 13.7% of patients had squamous cell carcinoma, adenocarcinoma, and large cell carcinomas, respectively, with no significant differences ($P = 0.152$). Among these patients, there were 52.5% poorly differentiated, 15.1% moderate, and 32.4% well-differentiated. Most of the patients (59.7%) were on a 2-drugs regimen (Combination of chemotherapy), while the remaining (40.3%)

TABLE 3 | Baseline characteristics of tumor markers parameters of advanced-stage NSCLC patients.

Variables	Patients *N* = 278 (%)	*P*-value	CEA level			CEA125			CA19-9			AFP			NSE			CYFRA21-1			CA15-3			Combined detection	
			Normal	High	*P*-value	Normal	High	*P*-value	Normal	High	*P*-value	Normal	High	*P*-value	Normal	High	*P*-value	Normal	High	*P*-value	Normal	High	*P*-value	Combined +	*P*-value
Age (Mean ± SD) years	(59.11 ± 10.39)	0.994			0.950			0.810			0.632			0.989			0.316			0.381			0.942		0.993
<60	124 (44.6)		31 (25.0)	93 (75.0)		61 (49.2)	63 (50.8)		98 (79.0)	26 (21.0)		116 (93.5)	8 (6.5)		59 (47.6)	65 (52.4)		49 (39.5)	75 (60.5)		44 (35.5)	80 (64.5)		124 (100)	
≥60	154 (55.4)		38 (24.7)	116 (75.3)		78 (50.6)	76 (49.4)		118 (76.6)	36 (23.4)		144 (93.5)	10 (6.5)		64 (41.6)	90 (58.4)		53 (34.4)	101 (65.6)		54 (35.1)	100 (64.9)		153 (99.3)	
Gender		0.357			0.678			0.528			0.955			0.555			0.451			0.017			0.893		0.352
Male	183 (65.8)		44 (24.0)	139 (76.0)		89 (48.6)	94 (51.4)		142 (77.6)	41 (22.4)		170 (92.9)	13 (7.1)		78 (42.6)	105 (57.4)		58 (31.7)	125 (68.3)		64 (35.0)	119 (65.0)		183 (100)	
Female	95 (34.2)		24 (26.3)	70 (73.7)		50 (52.6)	45 (47.4)		74 (77.9)	21 (22.1)		90 (94.7)	5 (5.3)		45 (47.4)	50 (52.6)		44 (46.3)	51 (53.7)		34 (35.8)	61 (64.2)		94 (99)	
Smoking status		<0.0001			0.784			0.503			0.149			0.033			0.391			0.112			0.520		0.0001
Non-smoker	157 (56.5)		42 (26.8)	115 (73.2)		82 (52.2)	75 (47.8)		127 (80.9)	30 (19.1)		151 (96.2)	6 (3.8)		68 (43.3)	89 (56.7)		640.84	93 (59.2)		59 (37.6)	98 (62.4)		156 (99.3)	
Smoker	88 (31.7)		15 (17.0)	73 (83.0)		40 (45.5)	48 (54.5)		65 (73.9)	23 (26.1)		80 (90.9)	8 (9.1)		35 (39.8)	53 (60.2)		28 (31.8)	60 (68.2)		26 (29.5)	62 (70.5)		88 (100)	
Unknown	33 (11.9)		12 (36.4)	21 (63.6)		17 (51.5)	16 (48.5)		24 (72.7)	9 (27.3)		29 (87.9)	4 (12.1)		20 (60.6)	13 (39.4)		10 (30.3)	23 (69.7)		13 (39.4)	20 (60.6)		33 (100)	
Metastasis		0.015			0.018			0.118			0.934			0.766			0.965			0.095			0.081		0.015
Yes	194 (69.8)		56 (28.9)	138 (71.1)		103 (53.1)	91 (46.9)		151 (77.8)	43 (22.2)		182 (93.8)	12 (6.2)		86 (44.3)	108 (55.7)		65 (33.5)	129 (66.5)		62 (32.0)	132 (68.0)		193 (99.48)	
No	84 (30.2)		13 (15.5)	71 (84.5)		36 (42.9)	48 (57.1)		65 (77.4)	19 (22.6)		78 (92.9)	6 (7.1)		37 (44.0)	47 (56.0)		37 (44.0)	47 (56.0)		36 (42.9)	48 (57.1)		84 (95.45)	
Differentiation		0.001			0.801			0.090			0.865			0.210			0.222			0.556			0.377		0.0001
Poor	146 (52.5)		36 (24.7)	110 (75.3)		79 (54.1)	67 (45.9)		113 (77.4)	33 (22.6)		139 (95.2)	7 (4.8)		59 (40.4)	87 (59.6)		52 (35.6)	94 (64.4)		55 (37.7)	91 (62.3)		145 (99.31)	
Moderate	42 (15.1)		9 (21.4)	33 (78.6)		22 (52.4)	20 (47.6)		35 (83.3)	7 (16.7)		39 (92.9)	3 (7.1)		21 (50.0)	21 (50.0)		14 (33.3)	28 (66.7)		14 (33.3)	28 (66.7)		42 (100)	
Unknown	90 (32.4)		24 (26.7)	66 (73.3)		38 (42.2)	52 (57.8)		68 (75.6)	22 (24.4)		82 (91.1)	8 (8.9)		43 (47.8)	47 (52.2)		36 (40.0)	54 (60.0)		29 (32.2)	61 (67.8)		90 (100)	
Tumor		0.152			0.213			0.063			0.670			0.890			0.786			0.088			0.413		0.155
Squamous cell	73 (26.3)		23 (31.5)	50 (68.5)		30 (41.1)	43 (58.9)		58 (79.5)	15 (20.5)		69 (94.5)	4 (5.5)		32 (43.8)	41 (56.2)		34 (46.6)	39 (53.4)		25 (34.2)	48 (65.5)		73 (100)	
Adenocarcinoma	167 (60.1)		36 (21.6)	130 (77.8)		87 (52.1)	80 (47.9)		129 (77.2)	38 (22.8)		155 (92.8)	12 (7.2)		73 (43.7)	94 (56.3)		55 (32.9)	112 (67.1)		56 (33.5)	111 (66.5)		166 (99.4)	
Other	38 (13.7)		9 (23.7)	29 (76.3)		22 (57.9)	16 (42.1)		29 (76.3)	9 (23.7)		36 (94.7)	2 (5.3)		18 (47.4)	20 (52.6)		13 (34.2)	25 (65.8)		17 (44.7)	21 (55.3)		38 (100)	
Drug		0.644			0.080			1.00			0.243			0.901			0.038			0.818			0.159		0.646
2-Drugs	166 (59.7)		35 (21.1)	131 (78.9)		83 (50.0)	83 (50.0)		125 (75.3)	41 (24.7)		155 (93.4)	11 (6.6)		65 (39.2)	101 (60.8)		60 (36.1)	106 (63.9)		53 (31.9)	113 (68.1)		166 (100)	
3-Drugs	112 (40.3)		34 (30.4)	78 (69.6)		56 (50)	56 (50)		91 (81.3)	21 (18.7)		105 (93.8)	7 (6.3)		58 (51.8)	54 (48.2)		42 (37.5)	70 (62.5)		45 (40.2)	67 (59.8)		111 (99.1)	
Response of therapy		0.012			0.969			0.164			0.343			0.366			0.592			0.045			0.082		0.012
CR (complete response)	0																								
PR + SD (stable disease)	210 (75.5)		52 (24.8)	158 (75.2)		110 (52.4)	100 (47.6)		166 (79)	44 (20)		198 (94.3)	12 (5.7)		91 (43.3)	119 (56.7)		84 (40.0)	126 (60.0)		80 (38.1)	130 (61.9)		209 (99.5)	
PD (progressive disease)	68 (24.5)		17 (25.0)	51 (75.0)		29 (42.6)	39 (57.4)		50 (73.5)	18 (26.5)		62 (91.2)	6 (8.8)		32 (47.1)	36 (52.9)		18 (26.5)	50 (73.5)		18 (26.5)	50 (73.5)		68 (100)	

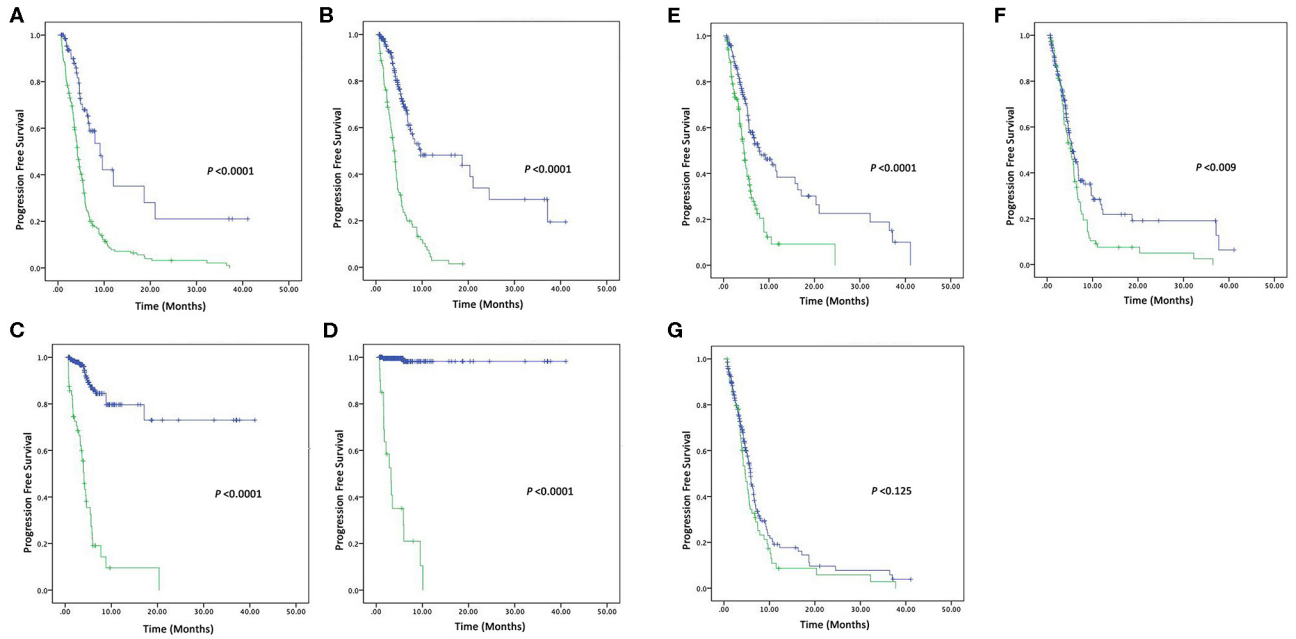

FIGURE 2 | Kaplan–Meier survival curve of four Tumor markers **(A)** PFS of CEA (high vs. normal) levels, **(B)** PFS of CA125 (high vs. normal) levels, **(C)** PFS of CA19-9 (high vs. normal) levels, and **(D)** PFS of AFP (high vs. normal) levels. Kaplan–Meier survival curve of three Tumor markers **(E)** PFS of NSE (high vs. normal) levels, **(F)** PFS of CYFRA21-1 (high vs. normal) levels, and **(G)** PFS of CA15-3 (high vs. normal) levels.

received a 3-drugs regimen (Combination of chemotherapy plus antiangiogenic therapy). Of all patients, 75.9% presented a stable disease, while 24.5% had progression disease, with significant differences ($P = 0.012$).

Association of Tumor Markers With Patients' Characteristics

In the pre-treatment, patients with high levels of CEA, CA-125, CA-199 AFP, NSE, CYFRA21-1, and CA15-3 were as follows: 209 (75.18%), 139 (50%), 62 (22.30%), 18 (6.47 %), 155 (55.75%), 176 (63.30%), and 180 (64.74%), respectively. In **Table 3**, CEA was found to significantly correlate with metastasis ($P = 0.018$). Similarly, CYFRA21-1 has strong correlation with gender ($P = 0.017$) and clinical response ($P = 0.045$). AFP correlated with smoking ($P = 0.033$) while NSE correlated only with therapy ($P = 0.038$). However, the combined positive detection of tumor markers was highly correlated with smoking ($P = 0.0001$), metastasis ($P = 0.015$) and cancer cell differentiation ($P = 0.0001$). There were no significant correlations in pre-treatment levels of CA125, CA-199, and CA15-3 levels with patients' characteristics (all $P > 0.05$), as shown in the **Table 3**.

In this present study, the tumor was progressed in 68 out of 278 patients, 166 patients used 2-drugs, while 112 patients used 3-drugs, and their overall median of PFS was 5.9 (4.1–8.7) months. Patients with CEA (high vs. normal) levels had a median PFS of 4.7 (4.15–5.31; $P < 0.0001$). Similarly, CA-125 (high vs. normal) levels median PFS was 6.26 (5.33–7.20; $P < 0.0001$) months. CA19-9 (high vs. normal) levels median PFS was 24.63 (20.41–28.85; $P < 0.0001$) months. AFP (high vs. normal) levels median PFS was 35.58 (32.40–38.76; $P < 0.0001$) months. NSE (high vs. normal) levels had median PFS was 5.6 (5.01–6.18; $P < 0.0001$) months. Similarly, patients with CYFRA21-1 (high vs. normal) levels median PFS was 5.4 (4.86–6.04; $P = 0.009$) months. However, patients with CA-153 (high vs. normal) levels were found poorly correlated with overall median PFS 5.53 (5.04–6.02; $P = 0.125$). Patients with elevated pre-treatment levels of CEA, CA125, CA19-9, AFP, NSE, CYFRA21-1, and CA15-3 noted shorter PFS compared to normal levels, as shown in the **Figures 2A,G**.

Furthermore, to find the pivotal role of these tumor markers as independent prognostic factors of PFS for NSCLC, univariate, and multivariate analyses were carried out, as shown in **Tables 4**, **5**. Univariate Cox regression analysis was performed to assess the factors which correlated with PFS. CEA/CA125, and CA19-9 levels were found as highly associated with PFS. In addition, AFP and NSE levels were also statistical associated with PFS except in the following variables, i.e., Age > 60, Smoking status, Differentiation status, Tumor status, Therapy (3-drugs), and Curative response (disease progression).

In multivariable Cox regression model, smoking status (Ever vs. Never, $P = 0.037$), Tumor (Others vs. Adenocarcinoma, $P = 0.001$), CA19-9 (high vs. normal, $P = <0.0001$) levels, NSE (high vs. normal, $P = <0.0001$) levels, CYFRA21-1 (high vs. normal, $P = 0.009$) levels, CA15-3 (high vs. normal, $P = 0.073$) levels and Sex* Tumor ($P = 0.022$) were found to be independent prognostic factors of PFS for NSCLC.

Prognostic values of all these tumor markers in advanced-stage NSCLC patients were evaluated in eight groups, i.e., (1)

TABLE 4 | Univariate analysis of tumor markers for progression free survival using Cox regression model in advanced-stage NSCLC patients.

Variables	CEA High vs. normal		CA125		CA19-9		AFP		NSE		CYFRA21-1		CA15-3	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Age														
<60	2.662 (1.464–4.842)	0.001	3.321 (1.995–5.530)	<0.0001	11.013 (4.768–25.439)	<0.0001	97.792 (12.014–796.013)	<0.0001	2.887 (1.695–4.918)	<0.0001	1.547 (0.977–2.450)	0.063	1.034 (0.639–1.675)	0.891
>60	3.469 (1.904–6.318)	<0.0001	4.593 (2.814–7.499)	<0.0001	12.019 (5.879–24.572)	<0.0001	1.000 (0.075–13.399)	1.000	1.915 (1.247–2.940)	0.003	1.360 (0.917–2.017)	0.126	1.552 (1.006–2.393)	0.047
Sex														
Male	3.399 (1.917–6.027)	<0.0001	3.737 (2.427–5.756)	<0.0001	12.183 (6.146–24.153)	<0.0001	180.730 (23.400-−1395.894)	<0.0001	2.104 (1.413–3.133)	<0.0001	1.527 (1.073–2.175)	0.019	1.360 (0.923–2.004)	0.119
Female	2.594 (1.391–4.839)	0.003	4.579 (2.491–8.419)	<0.0001	10.286 (4.134–25.593)	<0.0001	65.309 (7.237–589.403)	<0.0001	2.600 (1.414–4.780)	0.002	1.294 (0.726–2.309)	0.382	1.077 (0.610–1.900)	0.799
Smoking status														
Non-smoker	2.567 (1.507–4.374)	0.001	4.939 (3.061–7.969)	<0.0001	11.319 (5.355–23.925)	<0.0001	46.447 (8.351–258.316)	<0.0001	2.941 (1.862–4.644)	<0.0001	1.289 (0.847–1.963)	0.236	1.419 (0.925–2.178)	0.109
Smoker	3.977 (1.889–8.36)	<0.0001	2.861 (1.527–5.360)	0.001	6.007 (2.606–13.845)	<0.0001	170554.306 (0.0001–1.509E+45)	0.797	1.622 (0.944–2.788)	0.080	1.684 (1.005–2.821)	0.048	1.275 (0.732–2.223)	0.391
Unknown	5.231 (0.692–39.598)	0.109	3.394 (1.231–9.361)	0.018	1129.174 (0.028–45709448.1)	0.194	11248.858 (0.0001–3.731E+21)	0.650	2.108 (0.593–7.497)	0.249	1.198 (0.495–2.900)	0.689	0.794 (0.299–2.111)	0.644
Metastasis														
Yes	3.368 (2.068–5.486)	<0.0001	6.252 (3.859–10.130)	<0.0001	13.335 (6.805–26.133)	<0.0001	1.000 (0.072–13.794)	1.000	2.304 (1.545–3.435)	<0.0001	1.378 (0.973–1.951)	0.071	1.107 (0.764–1.602)	0.592
No	1.956 (0.845–4.526)	0.117	2.007 (1.113–3.622)	0.021	8.953 (3.517–22.791)	<0.0001	35.267 (4.042–307.693)	0.001	2.150 (1.189–3.887)	0.011	1.887 (1.041–3.421)	0.037	1.905 (1.027–3.534)	0.041
Differentiation														
Poor	2.310 (1.375–3.883)	0.002	4.363 (2.546–7.479)	<0.0001	11.727 (5.406–25.441)	<0.0001	1.000 (0.020–50.669)	1.000	2.103 (1.344–3.291)	0.001	1.211 (0.800–1.832)	0.366	1.698 (1.058–2.726)	0.028
Moderate	4.243 (1.284–14.020)	0.018	3.920 (1.586–9.690)	0.003	31.435 (2.743–360.219)	0.006	161926.676 (0.0001–6.586E+68)	0.872	2.165 (0.829–5.658)	0.115	0.993 (0.457–2.158)	0.986	1.105 (0.477–2.557)	0.816
Unknown	4.202 (1.672–10.558)	0.002	3.575 (2.038–6.271)	<0.0001	12.361 (4.949–30.872)	<0.0001	63.787 (7.817–520.508)	<0.0001	2.493 (1.351–4.601)	0.003	2.376 (1.372–4.116)	0.002	1.183 (0.704–1.989)	0.526
Tumor														
Squamous	3.279 (1.288–8.347)	0.013	5.359 (2.798–10.265)	<0.0001	15.383 (4.874–48.554)	<0.0001	46.186 (4.718–452.122)	0.001	2.368 (1.246–4.500)	0.008	2.831 (1.480–5.414)	0.002	1.461 (0.811–2.634)	0.207
Adenocarcinoma	3.107 (1.810–5.332)	<0.0001	4.104 (2.548–6.610)	<0.0001	10.431 (5.245–20.748)	<0.0001	163.477 (20.983–1273.627)	<0.0001	2.478 (1.598–3.841)	<0.0001	1.069 (0.730–1.567)	0.731	1.461 (0.973–2.194)	0.067
Others	2.678 (1.016–7.060)	0.046	2.550 (0.914–7.116)	0.074	9.735 (2.566–36.943)	0.001	262777.899 (0.0001–3.364E+105)	0.915	1.547 (0.620–3.860)	0.350	1.118 (0.443–2.822)	0.814	0.861 (0.278–2.673)	0.796
Drug														
2–Drugs	2.483 (1.466–4.206)	0.001	3.843 (2.450–6.027)	<0.0001	9.267 (4.840–17.743)	<0.0001	57.450 (12.335–267.582)	<0.0001	2.004 (1.340–2.999)	0.001	1.666 (1.135–2.444)	0.009	1.230 (0.834–1.815)	0.297
3–Drugs	3.836 (1.912–7.697)	<0.0001	4.273 (2.426–7.527)	<0.0001	13.035 (5.122–33.172)	<0.0001	1.000 (0.034–29.436)	1.0000	2.667 (1.484–4.791)	0.001	1.262 (0.779–2.046)	0.344	1.256 (0.703–2.244)	0.441
Curative response														
CR	0													
PR + SD	2.773 (1.714–4.485)	<0.0001	3.459 (2.302–5.199)	<0.0001	13.619 (7.235–25.637)	<0.0001	69.883 (15.263–319.972)	<0.0001	2.114 (1.444–3.095)	<0.0001	1.616 (1.133–2.305)	0.008	1.545 (1.066–2.239)	0.022
PD	3.848 (1.632–9.074)	0.002	6.023 (2.831–12.817)	<0.0001	7.073 (2.539–19.704)	<0.0001	1.000 (0.032–31.230)	1.000	2.854 (1.434–5.681)	0.003	1.198 (0.682–2.104)	0.530	0.776 (0.400–1.507)	0.455

patients with one elevated tumor marker level, (2) patients with two elevated tumor markers levels, (3) patients with three elevated tumor markers levels, (4) patients with four elevated tumor markers levels, (5) patients with five elevated tumor markers levels, (6) patients with six elevated tumor marker levels, (7) patients with seven elevated tumor markers levels. However, only one patient found normal pre-treatment levels of all the seven tumor markers. On comparison of all the seven tumor markers, patients with six and seven were recorded shorter PFS compared to patients with normal pre-treatment levels ($P = 0.025$) as shown in the **Figure 3A**.

TABLE 5 | Multivariate analysis of tumor markers for progression free survival using Cox regression model in advanced-stage NSCLC patients.

Variables	HR	95%CI	*P*-value
Age: <60 vs. >60	0.846	(0.655–1.092)	0.199
Sex: male vs. female	0.863	(0.612–1.218)	0.401
Smoking: ever vs. never	1.379	(1.020–1.864)	0.037
Unknown vs. never	1.1651	(0.786–1.727)	0.447
Treatment: 2- vs. 3-Drugs	1.183	(0.898–1.557)	0.231
Distant metastases: yes vs. no	0.954	(0.706–1.289)	0.758
Tumor: squamous vs. adenocarcinoma	0.650	(0.380–1.110)	0.114
Others vs. adenocarcinoma	4.030	(1.795–9.232)	0.001
Differentiation: moderate vs. poor	1.028	(0.709–1.492)	0.882
Unknown vs. poor	1.043	(0.730–1.492)	0.816
CEA: ≤3.5 vs. >3.5 ng/ml	0.851	(0.632–1.145)	0.286
CA125: ≤35 vs. >35 Uml	0.955	(0.724–1.261)	0.747
CA19-9: ≤39 vs. >39 U/ml	0.524	(0.375–0.731)	<0.0001
AFP: <10 vs. >10	0.672	(0.407–1.110)	0.121
NSE: ≤15.2 vs. >15.2 ng/ml	0.584	(0.446–0.763)	<0.0001
CYFRA21-1: <3.3 vs. >3.3	1.454	(1.098–1.926)	0.009
CA15-3: <30 vs. >30	1.310	(0.975–1.758)	0.073
Curative response: PR + SD vs. PD	0.886	(0.644–1.217)	0.454
Sex* Tumor (Squamous cells)	1.227	(0.671–2.244)	0.507
Sex* Tumor (Others)	0.336	(0.132–0.853)	0.022

Association of Treatment With Progression-Free Survival

In this study166 (59.7%) patients were on a 2-drug regimen, while 112 (40.3%) received a 3-drug treatment regimen. These therapies (2-drugs and 3-drugs) were compared for progression free survival, and those on the 3-drugs regimen found to have better PFS compared to the ones receiving the 2-drugs treatment regimen ($P = 0.043$), as shown in **Figure 3B**.

Association of Tumor Markers With Response to Palliative Chemotherapy

In this study, 278 patients received palliative chemotherapy, and their clinical responses were recorded. None of the patients had fully recovered, while 43 patients achieved partial response (PR), 167 patients had stable disease (SD), and 68 patients had disease state progress (PD). Some patients had also experienced following side effects while receiving chemotherapy, i.e., alopecia, anorexia, nausea, vomiting. No patient death due to treatment was recorded.

Mean of the initial and final levels of the tumor markers were analyzed using Wilcoxon signed ranks test. The results revealed significant statistical Mean differences levels of CEA, CA-125,

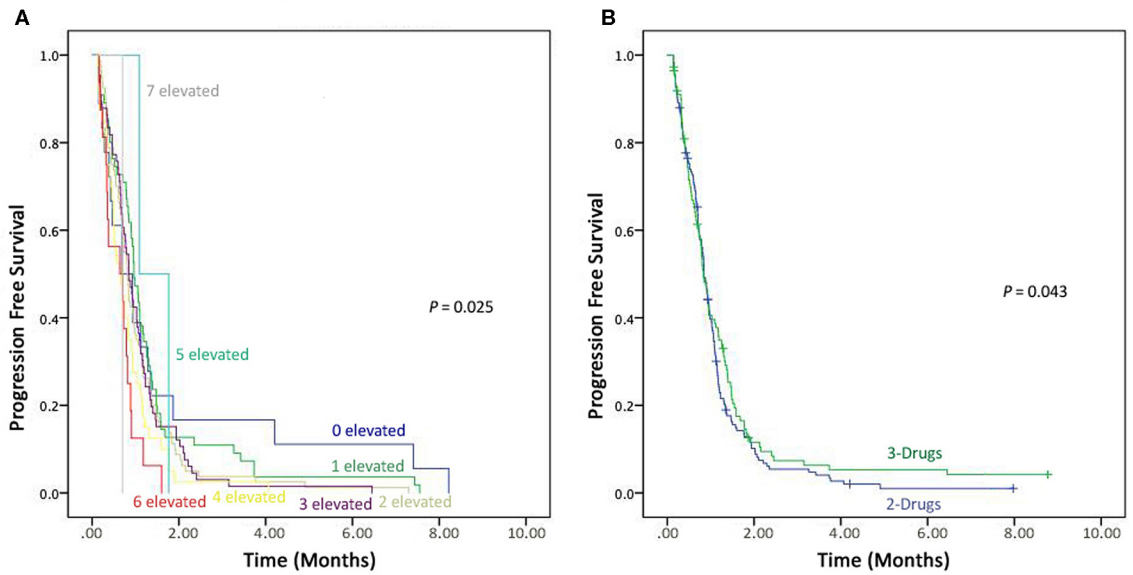

FIGURE 3 | Kaplan–Meier progression-free survival. **(A)** Combined detection of elevated seven Tumor markers. **(B)** Comparison the effectiveness of 3-drugs regimen vs. 2-drugs regimen.

TABLE 6 | Mean levels of seven tumor markers in pre- and post-palliative chemotherapy in advanced-stage NSCLC patients.

	CEA Initial–CEA final	CA125 Initial–CA125 final	CA19-9 Initial–CA19-9 final	AFP Initial–AFP final	NSE Initial–NSE final	CYFRA21-1 Initial–CYFRA21-1 final	CA15-3 Initial–CA15-3 final
z (Wilcoxon signed ranks test)[a]	−2.352[b]	−3.419[b]	−2.272[c]	−4.748[c]	−2.513[b]	−0.997[b]	−0.490[c]
P-value	0.019	0.001	0.023	<0.0001	0.012	0.319	0.624

[a]*Wilcoxon signed ranks test.*
[b]*Based on positive ranks.*
[c]*Based on negative ranks.*

CA-199, AFP, and NSE ($P = 0.019$, 0.001, 0.023, $P < 0.0001$, and $P = 0.012$, respectively) between the pre-and post-treatment. Meanwhile, the Mean levels of CYFRA21-1 and CA15-3 were not statistically significant ($P = 0.319$ and 0.624, respectively), as shown in **Table 6**.

All the seven tumor marker levels were measured at baseline and after 6th cycle of palliative chemotherapy. When stratified the Mean levels of all tumor markers by the disease control group and the progression disease group, there were statistical significant decreasing of CEA ($P < 0.0001$), CA-125 ($P < 0.0001$), AFP ($P < 0.0001$), NSE ($P = 0.050$), and CYFRA21-1 ($P = 0.050$) levels after the 6th cycle of palliative chemotherapy in the disease control group. However, no significant differences were observed in the Mean levels of pre- and post-treatment for CA19-9 ($P = 0.151$) and CA15-3 ($P = 0.436$) in the same group, as shown in **Table 7**.

In addition, when stratified by the progression disease group, there was statistical significant decrease of CA19-9 ($P = 0.047$) levels between the pre-and post-treatment. However, no significant differences were observed for CEA, CA125, AFP, NSE, CYFRA21-1, and CA15-3 levels in the progression disease group (all $P > 0.05$), as shown in **Table 7**.

Furthermore, we also evaluated the response to therapy in patients receiving the two forms of palliative chemotherapy (i.e., 2-drugs or 3-drugs regiment). As evinced from **Table 7,** patients receiving a 3-drugs treatment regimen achieved better therapeutic outcomes compare to those on a 2-drugs regimen. Also, the pre- and post-treatment levels of the tumor markers were compared. When stratified by 3-drugs regimen, the results showed significant differences in CA125 ($P = 0.009$), AFP($P < 0.0001$), NSE ($P = 0.014$) and CYFRA21-1 ($P = 0.43$) levels. However, no significant differences were observed for CEA ($P = 0.122$), CA19-9 ($P = 0.071$), and CA15-3 ($P = 0.983$) levels. Meanwhile, when stratified by the 2-drugs regimen, no statistical significant differences were observed in all tumor markers (all $P > 0.05$), as shown in **Table 8**.

DISCUSSION

This retrospective study is one of the few studies that assess the clinical utility of tumor markers CEA, CA19-9, CA125, AFP, NSE, CA15-3, and CYFRA21-1 for prognostic specification as well as for measuring the response to chemotherapy. CEA is non-specific with an abnormal countenance in solid tumors including, non-small lung cancer. Moro et al. (18) reported CEA as a negative prognostic factor. One study reported that CEA has a poor prognostic specification in NSCLC for survival (19). In our present study, patients having elevated CEA pre-treatment levels were correlated with shorter PFS and poor prognosis compared to those with normal levels, as similarly found in previous studies (19, 20). Moreover, in univariate Cox regression analysis, CEA was a correlated factor with PFS, but the multivariate analysis demonstrated that CEA is not an independent prognostic factors of PFS ($P = 0.286$).

Previously, the role of CA125 as a prognostic marker was not well defined (21). A limited number of studies had explored its prognostic value in an advanced-stage of cancer (22, 23). Herein, patients with increased pre-treatment levels of CA125 had not shown any significance, but in univariate Cox regression, CA125 was found statistical associated with risk of progression. But the multivariate analysis found no statistical significant ($P = 0.747$). Similarly, the role of CA19-9 was not previously well-elucidated with PFS in NSCLC patients (19, 24). However, in our study patients with increased pre-treatment levels of CA19-9 had not shown any significant differences ($P > 0.05$), but in univariate Cox regression and multivariate variable models, CA19-9 was found as an independent prognostic factor associated with risk of progression.

The prognostic value of AFP is already reported in several types of cancers (e.g., gastric cancer and ovarian cancer) (25), but there is no study available that explored its diagnostic and prognostic value in lung cancer (26). Our study is the first to our best knowledge to identify the potential role of AFP in NSCLC. Our results showed that AFP levels have a significance difference in high pre-treatment levels. Moreover, AFP was found associated with PFS in univariate Cox regression, but not in multivariate analysis ($P = 0.121$). Further studies are, however needed to validate our results.

The role of NSE as a tumor marker is widely accepted in small cell lung cancer (SCLC). However, its prognostic value is controversial in NSCLC (27). Numerous studies explored the prognostic role of NSE in local advanced and metastatic NSCLC and found it as a vital prognostic factor for PFS. (28, 29) Our findings are also consistent with them and found NSE levels were associated with worse prognosis and shorter PFS. On the contrary, one study on 67 operable early stage NSCLC patients reported a non-correlation of NSE with prognosis (30). In addition, studies explored the prognostic reliability of CYFRA21-1 and its levels were highly expressed the in blood of NSCLC (31). In alignment with our study, we found a significant correlation of CYFRA21-1 with gender and curative response. Furthermore, univariate cox regression and multivariate variable

TABLE 7 | Mean levels of serum tumor markers in pre-and post-palliative chemotherapy in DC group (CR + PR + SD) and PD group respectively, in advanced-stage NSCLC patients.

Efficacy comb	CEA initial–CEA final	CA125 Initial–CA125 final	CA19-9 Initial–CA19-9 final	AFP Initial–AFP final	NSE Initial–NSE final	CYFRA21-1 Initial–CYFRA21-1 final	CA15-3 Initial–CA15-3 final
CR + SD + PR Z Asymp. Sig. (2-tailed)[a]	−3.517[b]	−4.559[b]	−1.435[c]	−4.476[c]	−1.897[b]	−1.958[b]	−0.779[c]
	0.000	0.000	0.151	0.000	0.050	0.050	0.436
PD Z Asymp. Sig. (2-tailed)[a]	−1.265[c]	−0.956[c]	−1.983[c]	−1.725[c]	−1.752[b]	−0.947[c]	−0.633[b]
	0.206	0.339	0.047	0.084	0.080	0.344	0.527

[a] *Wilcoxon signed ranks test.*
[b] *Based on positive ranks.*
[c] *Based on negative ranks.*

TABLE 8 | Comparing the clinical response of palliative chemotherapy (3-Drugs and 2-Drugs) in advanced-stage NSCLC patients.

Efficacy	CEA Initial–CEA final	CA125 Initial–CA125 final	CA19-9 Initial–CA19-9 final	AFP Initial–AFP final	NSE Initial–NSE final	CYFRA21-1 Initial–CYFRA21-1 final	CA15-3 Initial–CA15-3 final
3-Drugs Z Asymp. Sig. (2-tailed)[a]	−1.546[b]	−2.622[b]	−1.805[c]	−4.807[c]	−2.468[b]	−2.021[b]	−0.021[b]
	0.122	0.009	0.071	0.000	0.014	0.043	0.983
2-Drugs Z Asymp. Sig. (2-tailed)[a]	−1.839[b]	−2.170[b]	−1.424[c]	−1.365[c]	−0.887[b]	−0.866[c]	−0.731[c]
	0.066	0.30	0.154	0.172	0.375	0.386	0.465

[a] *Wilcoxon signed ranks test.*
[b] *Based on positive ranks.*
[c] *Based on negative ranks.*

model results showed that CYFRA21-1 is a reliable tumor marker of NSCLC. Our findings are also in line with previous studies that found CYFRA21-1 as an independent predictor of gender and metastasis (32).

CA15-3 is a mucin-1 soluble form that is associated with non-squamous carcinoma (33). We did not find any significant difference in pre-treatment levels of CA15-3. However, univariate Cox regression revealed that CA15-3 was associated with Age, poor differentiation, and disease control group, but no significant differences were observed in multivariate analysis. In accordance with our findings, Liu et al. (34) reported that CA15-3 is not a reliable tumor marker. Furthermore, CEA, CA125, CA19-9, AFP, NSE, CYFRA21-1, and CA15-3 may not have significant prognostic values individually, but their combined detection can help in diagnosis, prognosis, and further, it can also evaluate the response of therapy. One study reported that changes in tumor marker levels in patients taking pre- and post-gefitinib-based chemotherapy were associated with tumor response and PFS (35). Therefore, the clinical utilization of these tumor markers could play a promising role in predicting the outcomes of therapy in NSCLC. The combined positive detection was highly correlated with smoking status, metastasis, differentiation, and curative response.

In the Kaplan–Meier survival curve, patients with 5-, 6-, or 7-elevated pre-treatment tumor markers have short PFS compared to those with 0, 1-, 2-, 3-, or 4-elevated pre-treatment tumor markers. Therefore, clinicians/oncologists should consider the detection of the combined tumor markers before prescribing the chemotherapy (36–38). The role of chemotherapy in advanced-stage NSCLC in the past two decades has been well-established. However, an antiangiogenic drug also gained attention in recent years, antiangiogenic drugs, e.g., bevacizumab has proved its efficacy in numerous solid tumors, and also show high efficacy with first-line chemotherapy in NSCLC patients (39, 40). Numerous studies reported the safety profile and synergistic effects of bevacizumab in combination with chemotherapy (40, 41). Herein, patients who received 3-drugs regimen had longer PFS compared to those on 2-drugs. Those findings were consistent with previous studies (42).

The association between tumor markers and curative effect has already been studied in breast cancer, colorectal cancer, gastric cancer, pancreatic cancer, and ovarian cancer, but limited clinical studies are available to identify the role of tumor markers and response to chemotherapy in advanced stage of NSCLC (14, 16, 43–45). In this study, we sought to determine the clinical potential of tumor markers in monitoring the response of patients to palliative chemotherapy. Our results showed a significant reduction of tumor marker levels after palliative chemotherapy, especially in the disease control group (CR + PR + SD), as compared to the progression disease group, as aforementioned in **Table 7**.

In the present study, we also compared the effectiveness of a 2- and 3-drugs combination therapy. Our results showed significant differences in the tumor marker levels of patients using 3-drugs than those on a 2-drugs therapy, as shown in **Table 8**. Previously published studies supported the hypothesis that antiangiogenic therapy, e.g., bevacizumab, can penetrate inside the tumor with or without first line

chemotherapy (1, 2). It can therefore be inferred that combination of antiangiogenic therapy with chemotherapy could improve patient survival and improve their quality of life.

The limitation of this retrospective study is that the socio-demographic data may be subject to bias, especially for the classification of being smoker, considering the fact it was a self-report. Nonetheless, our findings require confirmation in additional large prospective studies.

CONCLUSION

The high levels of CYFRA21-1 were correlated with poor a prognostic factor of PFS for Advanced NSCLC patients. However, the high levels of CA19-9 and NSE were associated with a better prognostic factor of PFS. Additionally, smoking habits and tumor status had a poor prognostic factor of PFS. Moreover, we found that antiangiogenic therapy has high efficacy with combination of chemotherapy and longer PFS of NSCLC patients.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics research committee of Jiangsu Cancer hospital, Nanjing, China. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MA and MS: conceptualization. MA: methodology and writing—original draft preparation. MA and SK: formal analysis. MA and XL: data curation. MA, SK, and MH: writing—review and editing. Z-CW, MS, YH, and H-LZ: supervision and project administration.

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CASE REPORT
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A Novel Oncogenic Driver in a Lung Adenocarcinoma Patient Harboring an *EGFR*-KDD and Response to Afatinib

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Introduction: Oncogenic mutations in the epidermal growth factor receptor (*EGFR*) occur frequently in patients with lung cancer. These mutations may serve as critical predictive biomarkers in patients with non-small cell lung cancer (NSCLC). Among them, *EGFR* exon 18–25 kinase domain duplication (*EGFR*-KDD) mutations have been identified as a novel *EGFR* gene subtype in NSCLC.

Case Presentation: We reported a rare case of a 59-year-old male diagnosed with adenocarcinoma. A biopsy revealed an *EGFR*-KDD identified by the next generation sequencing (NGS). Effective treatment outcome has been observed after administration with afatinib.

Conclusion: This case highlights that comprehensive NGS technique is valuable in detecting novel genetic mutations in tumors.

Keywords: non-small cell lung cancer, epidermal growth factor receptor, kinase domain, next-generation sequencing, afatinib

INTRODUCTION

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related death, with adenocarcinoma being one of the most common forms (1). Epidermal growth factor receptor (*EGFR*) mutations could be detected in 30–60% of Asian patients and 10–20% of Caucasian patients with lung cancer (2). Being as a driver oncogene, double-blinded randomized clinical trials have indicated that application of EGFR tyrosine kinase inhibitors (TKIs) are effective against NSCLC cases harboring *EGFR* mutations (3).

EGFR mutations most commonly occur in exon 19 or exon 21 within the EGFR tyrosine kinase domain. The rare *EGFR* mutations are usually not detected by the first-generation testing techniques. However, advanced precise detection techniques (e.g., next generation sequencing, NGS) enable the discovery of more rare *EGFR* mutations, including exons 18–25 kinase domain duplications (KDDs) (4). Herein, we reported a first case of an oncogenic EGFR-KDD in lung adenocarcinoma who were responsive to treatment with afatinib in Chinese populations.

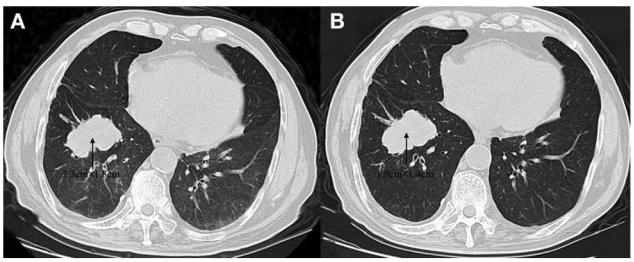

FIGURE 1 | Computed tomography (CT) scans before **(A)** and after **(B)** afatinib therapy.

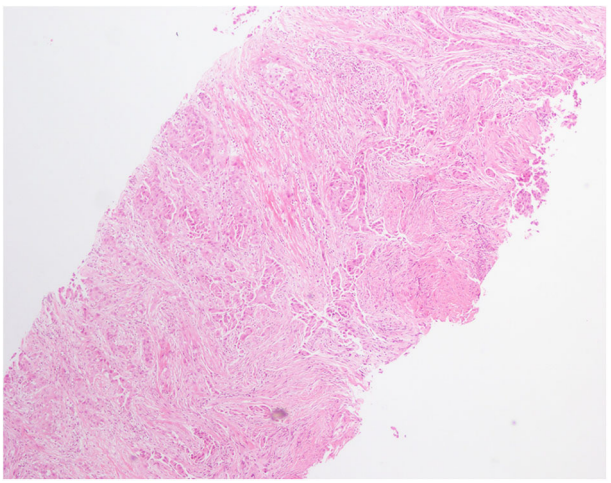

FIGURE 2 | Hematoxylin and eosin (H&E) staining of lung biopsy showed a typical morphology for adenocarcinoma cells (H&E × 100).

CASE PRESENTATION

A 59-year-old male was referred to our hospital for detection of a right lung mass on physical examination. He had no history of pulmonary disease or smoking. A mass (2.3 × 1.8 cm) in the right lower lung was observed by computed tomography (CT) scan (**Figure 1A**). F-18 fluorodeoxyglucose hypermetabolic speckles in fourth vertebral body; no hypermetabolic lesions were demonstrated in other sites, and a MRI of the brain or CT of the head with IV contrast was not performed. We detected a typical morphology for adenocarcinoma cells by hematoxylin and eosin (H&E) staining (**Figure 2**). Immunohistochemical staining showed positive for the expression of NapsinA, TTF-1, and CK7. The patient was classified as stage IV ($T_1N_0M_1$), in accordance to the 7th edition of TNM staging. The ARMS assay, the first-generation sequencing technique, revealed wild-type for sensitive *EGFR* mutations, including *EGFR* 18-21, and negative for *ALK* rearrangement or *ROS1* rearrangement. Then, a NGS analysis of the tumor biopsy identified a *EGFR*-KDD mutation (copy number 2.0) accompanied *TP53* p.R282W (frequency 13.0%) and *CTNNB1* p.S37Y (frequency 5.1%) in the tumor (Geneplus, Beijing, China) (**Figure 3**), and PD-L1 staining was not done. Therefore, he underwent oral afatinib treatment (30 mg qd). Afterwards, the patient showed a stable tumor response to afatinib (1.9 × 1.4 cm) (**Figure 1B**). Besides, there were no adverse events, including gastrointestinal reactions, hepatic and renal function, and cardiac damage. Currently, the disease is stable and treatment with afatinib continues for 10 months.

DISCUSSION

Oncogenic *EGFR* mutations are detected in 30–60% of Asian patients and 10–20% of Caucasian patients. Such mutations are most detected as small in-frame deletions in exon 19 or point mutations in exon 21. Uncommon *EGFR* alterations, including rare point mutations and gene rearrangements, have also been reported previously (5).

EGFR-KDDs could activate EGFR signaling by forming an intra-molecule dimer (6). *EGFR*-KDD of exons 18–25, firstly discovered in a glioblastoma (7), has been recognized as a driver gene in lung cancer. In 2015, Baik et al. (8) first reported an *EGFR*-KDD in a female patient with a bronchoalveolar carcinoma and responsive to the first-generation EGFR-TKIs, including erlotinib and gefitinib. Another case reported a male patient with lung adenocarcinoma and an *EGFR*-KDD mutation detected with NGS, who had a preferable anti-tumor response to afatinib, a second-generation EGFR-TKI (6). Zhu et al. (9) reported the first case involving the presence of oncogenic *EGFR*-KDD in China, who had stable disease to treatment with an EGFR-TKI icotinib. Another case report of a prolonged multi-year response to gefitinib and then erlotinib has been described for advanced *EGFR*-KDD mutated lung adenocarcinoma (8). Therefore, it seems these *EGFR* variants are sensitive to first- and second- generation EGFR-TKIs. Consistently, *in vitro* study showed that *EGFR*-KDD is constitutively active, and computational modeling provides potential mechanistic support for its auto-activation (9). Herein, we for the first time detected EGFR-KDD in a Chinese patient who achieved sustained anti-tumor responses from treatment with afatinib.

Polymerase chain reaction (PCR) is frequently applied for detection of common *EGFR* variants in NSCLC patients, which is unable to identify some rare types of *EGFR* alterations (10). By contrast, NGS allows for multiplex testing and enables the detection of known as well as uncommon genomic events, as reported in this case (11). Thus, clinical treatment should improve with clinical diagnostics for multiple gene testing to provide personalized cancer therapy.

In summary, the present case increases the evidence supporting afatinib treatment of NSCLC patients harboring *EGFR*-KDD variants. The NGS assay provides a useful way to identify rare and uncommon *EGFR* gene mutations in NSCLC patients.

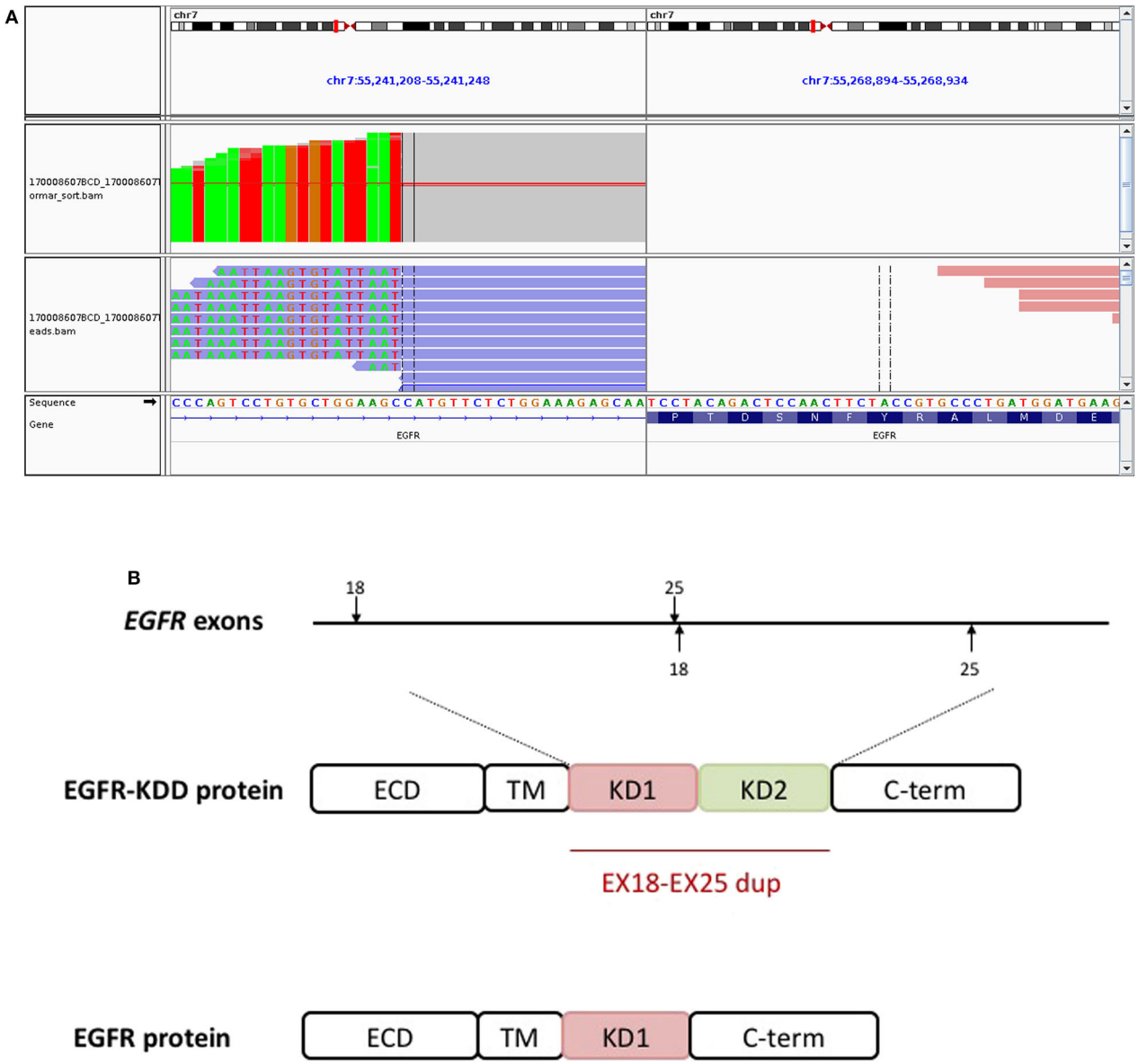

FIGURE 3 | The *EGFR*-KDD is an oncogenic *EGFR* alteration. **(A)** The Integrative Genomics Viewer snapshot of paired NGS reads of tumor and matched blood. **(B)** Schematic representation of *EGFR*-KDD depicting the genetic and protein domain structures. ECD, extracellular domain; TM, transmembrane domain; KD1, first kinase domain; KD2, second kinase domain; C-term, carboxyl terminus. Blue, *EGFR* exons 18–25 #1; orange, *EGFR* exons 18–25 #2.

ETHICS STATEMENT

This study was approved by the Ethic Committee of Zhejiang Rongjun Hospital. The patient provided written informed consent for the publication of this case report.

AUTHOR CONTRIBUTIONS

DC, XL, and BW conceptualized and designed the entire study. XZ, HC, and MF carried out patient clinical management and sample collection. WW analyzed the data. WW, YD, and CX wrote the manuscript. WW and CX revised the manuscript. All authors read and approved the final version of manuscript for submission.

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ORIGINAL RESEARCH
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Mutational Portrait of Lung Adenocarcinoma in Brazilian Patients: Past, Present, and Future of Molecular Profiling in the Clinic

*Helano C. Freitas[1†], Giovana Tardin Torrezan[2,3†], Isabela Werneck da Cunha[4,5†], Mariana Petaccia Macedo[4], Vanessa Karen de Sá[1], Marcelo Corassa[1], Elisa Napolitano e Ferreira[2,6], Augusto Obuti Saito[1], Graziela Zibetti Dal Molin[1,7], Vladmir C. Cordeiro de Lima[1,8] and Dirce Maria Carraro[2,3]**

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Objectives: Approximately 60% of lung adenocarcinomas (LAs) carry mutations that can guide treatment with tyrosine-kinase inhibitors (TKI) and other targeted therapies. Data on activating mutations in *EGFR* and other tyrosine-kinase receptor (TKR) genes in highly admixed populations, such as that of Brazil, are scarce. In this study, we comprehensively analyzed the actionable alteration profile of LA in Brazilian patients.

Materials and Methods: *EGFR* driver mutation data were collected from a large Brazilian LA cohort covering an 8-year period of molecular testing in a single institution. Tests were performed using three distinct methods, and demographic and histopathological data were analyzed. For a subset of patients, driver mutations in *KRAS*, *NRAS*, and *BRAF* and gene fusions involving TKR genes (before TKI treatment) and *EGFR* T790M (after TKI treatment) were assessed.

Results: *EGFR* mutations were detected in 25% of 1,316 LAs evaluated, with exon 19 deletions and exon 21 L858R TKI sensitizing mutations representing 72.5% of all mutations. Mutation rates were higher in women and non-smokers ($p < 0.001$). Next-generation sequencing was very sensitive, with a lower rate of inconclusive results compared with Sanger sequencing and pyrosequencing. *EGFR/RAS/BRAF* hotspot gene panels were applied in 495 LA cases and detected oncogenic mutations in 51.3% of samples, most frequently in *EGFR* (22.4%) and *KRAS* (26.9%). In subgroups of 36 and 35 patients, gene fusions were detected in 11.1% of tumors and *EGFR* T790M resistance mutations were detected in 59% of plasma samples from patients previously treated with TKI, respectively.

Conclusion: This report provides the first comprehensive actionable alteration portrait of LA in Brazil. The high rate of actionable alterations in *EGFR* and other driver genes in LA reinforces the need to incorporate TKI guided by molecular diagnostics into clinical routines for patients in both public and private healthcare systems.

Keywords: EGFR, lung adenocarcinoma, driver mutations, targeted therapies, molecular testing

INTRODUCTION

Non-small cell lung cancer (NSCLC) accounts for 85% of primary lung malignancies. Adenocarcinoma is the most common histological subtype of lung cancer, accounting for half of cases (1). Recent years have been marked by changes in the treatment paradigm for lung adenocarcinoma (LA) according to genomic portrait, which in turn, has contributed to the identification of molecular drivers implicated in the clinical behavior of the disease (prognostic value) and in treatment response (predictive value). In consequence, it is currently established that more than 60% of LA cases carry driver mutations that could guide treatment tailoring (2).

LA presents a variety of structural genomic alterations that lead to the activation of oncogenes, especially those involving the tyrosine-kinase receptors *ALK*, *ROS1*, and *RET*; and point mutations, especially in genes of EGFR-pathway, such as *EGFR* and *KRAS* genes (2). Mutations in *EGFR* were first described in 2004, and several clinical trials have since demonstrated the efficacy of EGFR-targeted tyrosine-kinase inhibitors (TKIs) in this scenario (3–5). EGFR TKIs have been incorporated into clinical practice and are now a part of standard treatment worldwide.

The incidence of *EGFR*-mutant LA is greater in eastern Asia than in other regions, with more than 40% of tumors carrying a somatic mutation in this gene (6, 7). In Europe and the US, the incidence ranges from 10 to 15% (6, 8). In Latin America and Brazil, small series have suggested that the frequency of *EGFR*-mutant LA is higher than observed in Europe and the US (6, 9).

In this study, we present a historical perspective on the application of molecular testing of patients with LA at a Brazilian reference center for cancer treatment. First, we compared the detection rates of *EGFR*-activating mutations in 1,316 consecutive LA cases using three approaches—Sanger sequencing, pyrosequencing, and next-generation sequencing (NGS)—and investigated the association of *EGFR* mutations with demographic and histopathological data for different subsets of cases. We also assessed the frequency of *EGFR*-, *KRAS*-, and *BRAF*-activating mutations and other gene fusions in a subset of tumors using focused NGS gene panels. Finally, we described the rate of *EGFR*-T790M resistance mutations detected in circulating tumor DNA (ctDNA) after treatment with TKI in a group of patients. Altogether, we have generated a comprehensive portrait of *EGFR*-activating alterations in Brazilian patients with LA, considering methodological and pathological variables.

MATERIALS AND METHODS

Patient Cohort

This retrospective analysis included 1,316 lung cancer samples tested for *EGFR* mutation between August 2010 and October 2018 at the Laboratory of Genomic Diagnostics of the A.C. Camargo Cancer Center. The samples were collected from 1,316 patients for whom we had access to test results and demographic data (age at diagnosis and gender). For subsets of cases, 579, 470, and 436, we also we had access to tumor histology, smoking behavior, and presence of metastases, respectively. Patients were tested according to different methodologies, which were current at the corresponding timepoints during the study period. Thirty-five patients were also tested using a liquid biopsy approach to search for resistance mutations in ctDNA after being exposed to TKI treatment.

Sample Preparation and DNA/RNA Extraction

Tumor samples were derived from routine formalin-fixed paraffin-embedded (FFPE) blocks obtained from biopsies and resected lung specimens. Two medical pathologists (IW, MP) reviewed the histological diagnoses and classified the LA samples according to the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma (10). Samples were subjected to histological analysis to assess the percentage of tumor cells and to select adequate tumor areas. Manual dissection of selected tumor regions was performed on unstained slides after paraffin removal with xylene and ethanol. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) with QIAcube equipment. Tumor RNA was extracted using the AllPrep DNA/RNA FFPE Kit (Qiagen).

For liquid biopsy analysis, blood samples were collected and processed within 2 h of collection to avoid plasma contamination with leucocyte DNA. Briefly, peripheral blood (4 ml) was collected in BD Vacutainer®/Hemogard™ EDTA K2 Plus tubes or BD Vacutainer® PPT™ tubes (BD Biosciences, NJ, USA) and submitted to centrifugation at 1,600 g for 10 min. The plasma was transferred to new tubes and centrifuged again at 1,600 g for 10 min. DNA was extracted from the plasma using the MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific, MA, USA), according to the manufacturer's instructions. DNA quantity and quality were assessed with a Nanodrop 1000 and/or Qubit dsDNA HS kit (Thermo Fisher Scientific).

Tumor Mutation Analysis

EGFR exons 18, 19, 20, and 21 were investigated by Sanger sequencing, pyrosequencing, or three distinct NGS strategies, as follows.

Sanger Sequencing

PCR amplification of *EGFR* exons 18, 19, 20, and 21 was performed with 80–150 ng genomic DNA using primers developed in house and the Platinum Taq DNA Polymerase High Fidelity Kit (Invitrogen). PCR products were verified in 1% agarose gels using SYBR safe DNA gel stain (Invitrogen) and purified with ExoSap (USB, OH, USA). Sequencing reactions were performed using BigDye v3.1 reagents (Thermo Fisher Scientific), according to the manufacturer's instructions. The sequencing products were purified using an ethanol precipitation protocol. Automated sequencing was performed by capillary

Abbreviations: LA, Lung adenocarcinomas; TKI, tyrosine kinase inhibitor; TKR, tyrosine-kinase receptor; NSCLC, non-small cell lung cancer; NGS, next generation sequencing; ctDNA, circulating tumor DNA.

TABLE 1 | Methodologies used for tumor molecular testing in patients with LA.

Method	Genes	Regions	Test type	Years utilized	N of tested patients
Sanger	*EGFR*	Full exons (18, 19, 20, 21)	In house protocol	2010–2014	352
Pyrosequencing	*EGFR*	Hotspot regions in exons (18, 19, 20, 21)	Therascreen EGFR Pyro Kit (Qiagen)	2014	101
NGS - panel1	*EGFR*	Full exons (18, 19, 20, 21)	In house protocol	2014–2018	374
NGS - panel 2	*EGFR, KRAS, NRAS, BRAF*	Full exons (*EGFR* 18, 19, 20, 21), Hotspot regions in other genes	In house protocol	2016–2018	459
NGS - panel 3	14 genes for point mutations and 3 genes for fusions	Full exons (*EGFR* 18, 19, 20, 21), Hotspot regions in other genes, frequent fusions in *ALK, RET, ROS1*	In house + Lung Fusion panel (ThermoScientific)	2017–2018	36
				Total of NGS tests	869
				Total of tested patients	1,322
				Total of unique tested patients*	1,316

Six patients were tested using more than one methodology.

electrophoresis on an ABI3130xl or ABI3500 device (Applied Biosystems). The sequences were aligned and electropherograms were analyzed using CLC Main Workbench software (Qiagen).

Pyrosequencing

Pyrosequencing of *EGFR* exons 18, 19, 20, and 21 was performed using the commercial EGFR Pyro Kit (Qiagen). PCR amplification was performed with 80–120 ng of genomic DNA, according to the manufacturer's instructions. PCR products were verified in 1% agarose gels using SYBR safe DNA gel stain (Invitrogen). Template preparation and sequencing were performed with PyroMark Gold Q24 reagents in a PyroMark Q24 device, following the manufacturer's instructions. Mutations were detected using PyroMark Q24 software and the default analysis parameters recommended by the manufacturer (Qiagen). A somatic mutation was considered to be present when the variant allele was detected at a frequency >5%.

NGS

Tumor somatic mutations were investigated by target sequencing using a custom Ion AmpliSeq™ Panel (Thermo Fisher Scientific) containing hotspot regions of 14 genes frequently mutated in solid tumors, including the complete exons 18, 19, 20, and 21 of *EGFR* and hotspot regions of *KRAS*, *NRAS*, and *BRAF*. Depending on the requested test (NGS types 1–3; **Table 1**), only regions of the gene of interest were analyzed and reported. Gene fusions were analyzed using the commercial Ion AmpliSeq RNA Lung Cancer Research Fusion Panel (Thermo Fisher Scientific). Multiplex amplification was performed with 10 ng of DNA or RNA using the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific), and high-throughput sequencing was performed using the Ion PGM or Ion Proton platform (Thermo Fisher Scientific), according to the manufacturer's instructions. For DNA point mutation analyses, mapping of sequencing reads, and variant calling were performed using the Torrent Suite Browser/TVC (Thermo Fisher Scientific) and CLC Genomics Workbench (Qiagen). A somatic mutation was considered to be present when the variant allele was detected in >2% of the reads, considering a minimum coverage depth of 100X. Gene fusion analyses were performed with Ion Report software (Thermo Fisher Scientific) using commercial pipelines.

Liquid Biopsy Mutation Analysis

For liquid biopsy analyses, tumor mutations in ctDNA were investigated using a custom Ion Ampliseq™ Panel containing hotspot regions of seven genes or with a specific amplicon designed for the evaluation of only the T790M mutation. For the gene panel, amplification was performed as described for the NGS tumor analyses. For the T790M amplicon, libraries were prepared using the Ion Plus Fragment Library Kit (Thermo Fisher Scientific). Sequencing and mutation analyses were performed as described for the tumors, with appropriated differences in the variant frequency cut-off (>0.5% of reads) and coverage (minimum coverage depth of 20,000X for negative results).

Statistical Analysis

Frequencies were used to describe categorical variables and medians were used for continuous variables. The chi-square test (or Fisher's exact test, when applicable) was used to compare frequencies of categorical variables. The Mann–Whitney *U*-test was used to compare median values of continuous variables (age and smoking load). Significance was established at $p \leq 0.05$. Analyses were performed using SPSS® Statistics version 20 (IBM).

RESULTS

EGFR Mutation Results

In this study we compiled the results of *EGFR* mutation testing of 1,316 consecutive LA patients from a single institution. Molecular testing was performed during an 8-year period (2010–2018) using three sequencing platforms, resulting in an overall *EGFR* mutation rate of 25.4%. Basic demographic and histological characteristics were collected (**Table 2**). The male/female rate was almost 1:1 and only 36% of patients were

non-smokers. *EGFR* mutation was more frequent among women and non-smoking patients ($p < 0.001$). Less than 10% (56/579) of the patients had non-adenocarcinomas (mostly squamous cell carcinomas), of whom only 5 had *EGFR* mutations (3.1% of all *EGFR* mutated patients) (**Table 2**).

Regarding mutation rate of three platforms used in this study (Sanger sequencing, pyrosequencing, and NGS), pyrosequencing and NGS had higher mutation rates (26.7 and 25.8%, respectively) than Sanger sequencing (23.3%; **Table 3**). NGS had the lowest rate of inconclusive test results (1.8%, compared with 4.0% for pyrosequencing and 17% for Sanger sequencing; $p < 0.001$; **Table 3**). Variants of unknown clinical significance were detected only with Sanger sequencing and NGS, as both are open-source sequencing technologies that are able to detect all types of genetic variation in the four evaluated exons.

Concerning the clinical relevance of identified *EGFR* mutations, the frequency of TKI-sensitizing, or likely-sensitizing mutations among *EGFR*-positive patients was 82.6% (74.2, 100, and 82.6% according to Sanger sequencing, pyrosequencing, and NGS, respectively; **Table 3**). Most mutations identified occurred in exons 19 and 21 (43.7 and 38.9%, respectively), and the test employed did not impact the distribution of mutations within exons (**Table 3**). The rates of exon 18 and exon 20 variants were 8.1 and 9.2%, respectively. Exon 19 deletions (39.2%) and exon 21 L858R (33.3%) sensitivity mutations were the most common alterations, representing 72.5% of all mutations (**Figure 1A**).

Most exon 20 insertions have been associated with TKI resistance, as have other SNVs in exons 18 (E709X), 19 (L747R), and 20 (Q787R and T790M). These resistance mutations were found in only 7.8% of *EGFR*-mutated tumors in our cohort (10.8, 0, and 7.6% according to Sanger sequencing, pyrosequencing, and NGS, respectively; **Table 3**). We found more than one *EGFR* mutation (complex or compound mutations) in only 19 patients (6.7% of *EGFR*-mutated tumors). Two patients presented the T790M mutation at diagnosis (**Figure 1A**).

TABLE 2 | Demographic and histopathological data and EGFR status.

	All patients	WT EGFR	MUT EGFR	*P*-value
Sex				
Male	503 (49.5%)	418/762 (54.9%)	85/254 (33.5%)	<0.001
Female	513 (50.5%)	344/762 (45.1%)	169/254 (66.5%)	
Median age at diagnosis	64	64	64.8	0.72
Histology				
Adenocarcinoma	523 (90.3%)	367/418 (87.8%)	156/161 (96.9%)	0.01
Non-adenocarcinoma	56 (9.7%)	51/418 (12.2%)	5/161 (3.1%)	
Smoking status				
Non-smoker	157 (36%)	81/308 (26.3%)	52/128 (40.6%)	<0.001
Smoker/Former smoker	279 (64%)	227/308 (73.7%)	76/128 (59.4%)	
Median smoking load (pack-years)	40	40	17.5	<0.001
Metastases at diagnosis				
Yes	343 (73%)	238/333 (71.5%)	105/137 (76.6%)	0.25
No	127 (27%)	95/333 (28.5%)	32/137 (23.4%)	

WT, wild type; MUT, mutated.

NGS Panel Results

For a subset of the 1,316 patients tested for *EGFR* mutations, NGS tests including other cancer mutation hotspots were performed.

TABLE 3 | Numbers and types of mutations detected according to test methodology.

	Sanger	Pyroseq	NGS	*P*-value	Aggregate
EGFR mutated patients	82/352 (23.3%)	27/101 (26.7%)	225/863 (26.1%)	0.57	334/1316 (25.4%)
Inconclusive test	60/352 (17%)	4/101 (4%)	16/863 (1.8%)	<0.00001	80/1316 (6.1%)
Patients with compound EGFR variants*	8/82 (9.8%)	1/27 (3.7%)	10/225 (4.4%)	0.31	19/334 (4.7%)
Total number of EGFR variants detected	93	28	236		357
Variant type					
SNV	51/93 (54.8%)	11/28 (39.3%)	124/236 (52.5%)		186/357 (52.1%)
Indel	42/93 (45.2%)	17/28 (60.7%)	112/236 (47.5%)		171/357 (47.9%)
Variant significance					
Sensitizing/Likely sensitizing	69/93 (74.2%)	28/28 (100%)	198/236 (83.9%)	0.11[#]	295/357 (82.6%)
Resistance	10/93 (10.8%)	0/28 (0%)	18/236 (7.6%)		28/357 (7.8%)
Uncertain significance	14/93 (15.1%)	0/28 (0%)	20/236 (8.5%)		34/357 (9.5%)
Variant location					
Exon 18	10/93 (10.8%)	1/28 (3.6%)	18/236 (7.6%)	0.58	29/357 (8.1%)
Exon 19	39/93 (41.9%)	17/28 (60.7%)	100/236 (42.4%)		156/357 (43.7%)
Exon 20	9/93 (9.7%)	2/28 (7.1%)	22/236 (9.3%)		33/357 (9.2%)
Exon 21	35/93 (37.6%)	8/28 (28.6%)	139/236 (40.7%)		139/357 (38.9%)

**Nineteen patients presented two or more EGFR variants. SNV, single nucleotide variant. indel, insertion/deletion. Sensitivity variants: G719X, exon 19 deletions, S768I, L858R, L861Q, and L861R. Resistance variants: E709X, exon 20 insertions, T790M, Q787R, and T854A. All other variants were considered to be of uncertain significance. Inconclusive refers to tests in which one or more exons could not be analyzed. [#]Calculated only between Sanger sequencing and NGS, as pyrosequencing is directed at hotspots of clinically significant variants.*

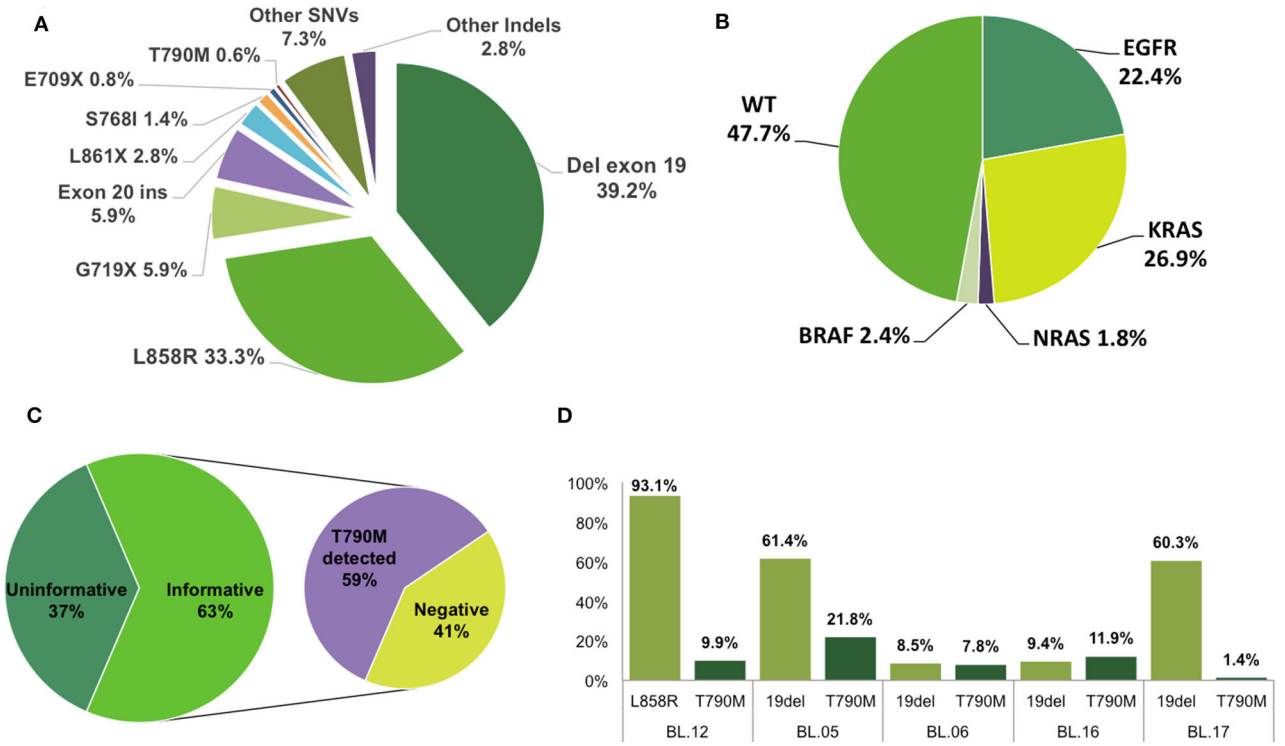

FIGURE 1 | Mutation patterns in Brazilian patients with LA. **(A)** Frequencies of *EGFR* mutation types detected in all 334 mutated patients (357 mutations). **(B)** Oncogenic mutations detected in 510 patients tested with NGS gene panels. Together, *EGFR* and *KRAS* mutations were detected in 49.3% of patients. **(C)** *EGFR* mutation detection in ctDNA from plasma. Twenty-two of thirty-fifth results were informative, and 59% of these patients were positive for the T790M mutation. **(D)** Variant allelic fractions of sensitizing and resistance mutations in five cases with both mutations detected in NGS of ctDNA.

Of 495 patients tested with gene panels, 459 patients were tested for hotspots in *EGFR*, *KRAS/NRAS*, and *BRAF*, and 36 patients were tested with a larger panel containing hotspots for 14 genes (including the four genes mentioned above) and lung cancer gene fusions. The patterns of mutations in these two groups are detailed in **Table 4**.

Briefly, we detected oncogenic point mutations in 51.3% (254/495) of these patients. The most frequently mutated genes were *EGFR* and *KRAS*, with 111/495 (22.4%) patients harboring *EGFR* mutations and 133/495 (26.9%) patients presenting *KRAS* mutations. *NRAS* mutations were detected in 9 (1.8%) patients, and *BRAF* mutations were found in 12 (2.4%) patients (**Figure 1B**). Most driver mutations were mutually exclusive, with 95.7% of patients presenting only one driver and co-occurrence of hotspot mutations in at least two genes detected in 11 patients (**Table 4**). Among the 36 patients evaluated for gene fusion, EML4-ALK, and KIF5B-RET fusions were detected in two (5.6%) patients each.

Plasma Screening for the T790M Mutation

We used NGS to analyze ctDNA mutations in the plasma of 35 patients harboring sensitizing *EGFR* mutations who were undergoing TKI treatment, using a gene panel covering the four exons of *EGFR* or a single amplicon for the T790M mutation. For 13 patients, the ctDNA analysis was considered to be uninformative, as neither the T790M resistance mutation nor the original sensitizing *EGFR* mutation (L858R or exon 19 deletion) could be detected with panel testing. Among 22 patients with informative results, 13 (59%) were positive for the T790M mutation (**Figure 1C**), with the mutant allele detected at a mean frequency of 5.2% (range, 0.88–21.8%). Of the 13 patients positive for T790M, 11 underwent ctDNA testing with the gene panel capable of detecting resistance and sensitizing mutations; we detected both mutations in plasma in five cases (mutation frequency, 1.36–93.1%; **Figure 1D**), and only the T790M mutation in six cases (mutation frequency, 0.88–1.95%).

For 10 patients, multiple samples were collected for ctDNA analyses at different time points (two samples from seven patients, three samples from two patients, four samples from one patient) because analyses of the first samples were considered to be uninformative. Subsequent results were informative for six of these patients (five T790M positive, one T790M negative), and uninformative for four patients.

DISCUSSION

Since 2009, abundant evidence for the benefit of TKIs in the treatment of *EGFR*-mutant NSCLC has accumulated. Nevertheless, health insurance companies in Brazil did not reimburse for molecular tests until sometime later, and such

TABLE 4 | Mutation detection in tumors evaluated with NGS panels.

	EGFR/RAS/BRAF panel		14 gene panel + fusions		Total	
	Count	%	Count	%	Count	%
Completely inconclusive tests	5	1.1	0	0.0	5	1.0
Wild-type	216	47.1	16	44.4	232	46.9
Point mutation detected	238	51.9	16	44.4	254	51.3
Fusion detected	NE	NE	4	11.1	4	NE
Total	459	100	36	100	495	100
Mutated Genes						
EGFR	94	20.5	8	22.2	102	20.6
EGFR/KRAS	3	0.7	1	2.8	4	0.8
EGFR/BRAF	2	0.4	0	0.0	2	0.4
EGFR/NRAS	2	0.4	0	0.0	2	0.4
EGFR/KRAS/NRAS	1	0.2	0	0.0	1	0.2
KRAS	120	26.1	6	16.7	126	25.5
KRAS/MET	NE	NE	1	2.8	1	0.2
KRAS/NRAS	1	0.2	0	0.0	1	0.2
NRAS	5	1.1	0	0.0	5	1.0
BRAF	10	2.2	0	0.0	10	2.0
EML4-ALK	NE	NE	2	5.6	NE	NE
KIF5B-RET	NE	NE	2	5.6	NE	NE

NE, not evaluated.

testing is still not widely available to patients in the private or public health system, making available data of *EGFR* mutation rates scarce for this population. Here, we compiled the results of *EGFR* mutation testing of 1,316 consecutive LA patients from a single institution, achieving an *EGFR* mutation rate of 25.4%. To our knowledge, this is the largest published cohort of LA cases tested for *EGFR* mutation using DNA sequencing–based platforms in Brazil and the largest comprehensive analysis of driver mutations in lung cancer in our population.

In previously published series of Brazilian patients, *EGFR* mutation rates were 21.6, 30.4, 22.7, and 19.2% in 125, 207, 444 and 619 LA cases, respectively (11–14). Studies conducted in other Latin American countries suggest that *EGFR* mutation rates are higher on this continent than in European countries and the USA, which are around 10–15% (6, 8, 9), especially in countries with greater contributions of *mestizo*/indigenous ancestries (11). *EGFR* mutation rates of 51.1% in Peru, 34.3% in Mexico, 24.7% in Colombia, and only 14.4% in Argentina have been reported (12).

These higher-than-expected mutation rates in this study and others from Brazil, compared with those in LA diagnosed in other Western populations, could be explained by demographic characteristics, such as gender and smoking behavior, and by genetic backgrounds. However, demographic characteristics do not seem to have introduced bias in our cohort, as the male/female rate was almost 1:1 and only 36% of patients were non-smokers. By the other side, the genetic background of the population could have contributed to the high mutation frequency. In this sense, a greater proportion of Asian ancestry (7.3%) was recently reported to be associated with *EGFR* mutation in a Brazilian cohort from São Paulo state (13). In addition, a high prevalence of *EGFR* activating-mutations was recently detected in LA diagnosed in Brazilian patients with Li-Fraumeni syndrome harboring the Brazilian *TP53* R337H founder mutation; however these patients comprised only 2.7% of our cohort (14). Interestingly, in the previously reported series of Brazil a considerably variation in terms of mutation rate was observed −19% in South of Brazil and 21.9–30% in Southeast (more specifically in São Paulo city) that has a higher proportion of Amerindian and Asian ancestries.

Regarding the clinical relevance of *EGFR* mutations, exon 19 deletions, and exon 21 L858R sensitivity mutations represented 72.5% of all mutations. The frequency of L858R mutation was 33.3%, similar to those reported for other series (29–45%) (15–19). In contrast, the rate of exon 19 deletions (39.2%) was slightly lower than described in the literature (44–57%) (15–19), and the rates of exon 18 and exon 20 variants (8.1 and 9.2%, respectively) were 2-fold higher than in other published series (4 and 2–5%, respectively) (8, 17, 18, 20, 21). Currently, evidence supports the sensitivity of mutations other than L858R and exon 19 deletions to available TKIs. For instance, single nucleotide variants (SNVs) such as exon 21 L861Q and L861R, and exon 18 G719X, are well-recognized as being sensitive to *EGFR* TKI treatment (18, 19, 22). Thus, considering these rare variants, the overall frequency of TKI-sensitizing or likely-sensitizing mutations among *EGFR*-positive patients was 82.6%.

Mutations related to primary or secondary resistance to EGFR TKIs are also of clinical relevance, and they were identified in only 7.8% of *EGFR*-mutated tumors in our cohort. Of note, only two patients presented the T790M mutation at diagnosis, representing <1% of untreated *EGFR*-mutated tumors, similar to rates reported in other studies (**Figure 1A**) (8, 23).

The three test platforms used in this study reflect the evolution of laboratory expertise in the detection of *EGFR*-activating mutations through the 8-year study period. Although similar results were obtained for most data with these different molecular testing methodologies, a smaller mutation detection rate and the highest rate of inconclusive tests were observed for Sanger sequencing, reflecting the improvement of sensitivity and robustness of more recent methods. Also, is noteworthy the NGS detection of a non-LREA exon 19 deletion in one patient with a previous negative test result from the Cobas® platform. This patient was treated with erlotinib for 18 months and is currently receiving second-line therapy with osimertinib. This case emphasizes that even high-quality standard platforms do not cover all clinically relevant variants.

A subset of patients tested for *EGFR* mutations were evaluated with NGS tests that include other cancer mutation hotspots, enabling assessment of other oncogenes from the EGFR pathway that are frequently mutated in lung cancer. In this group of 495 patients, oncogenic point mutations were detected in 51.3%, most frequently in *KRAS* (26.9%) and *EGFR* (22.4%). Mutation rates for other oncogenes have been described for patients with LA from other populations. *KRAS* is usually the first or second most frequently mutated gene in LA, with mutation frequencies similar to those for *EGFR*, which are strongly associated with a positive smoking status; these mutations are more frequent in white than

in Asian populations and show no sex predilection (24, 25). In Western countries, *KRAS* mutations are identified in 20–25% of patients with LA (25). In a recent update for Latin American countries, the overall *KRAS* mutation rate was 14.0% (range, 9.1–18.9%) (11). This lower frequency of *KRAS* mutations could be a result of a lower frequency of smokers in that cohort and a high *EGFR* mutation frequency, as the two mutation types are usually mutually exclusive. In the Brazilian population, *KRAS* mutation frequencies of 14.6–30.2% have been reported (12, 13, 26, 27). These differences could be partially explained by differences in detection methods and population characteristics.

From a clinical perspective, *KRAS* mutations are negative predictors of TKI response. Additionally, *KRAS*-mutant LA has been associated with poorer overall survival in several studies (28, 29), including a study conducted with a Brazilian population (13). However, new discoveries about *KRAS* biology and its impact in the tumor microenvironment, together with the advent of immunotherapies and targeted therapies, may result in the development of effective treatment strategies and optimal therapeutic stratification of *KRAS*-mutant LA (25). Indeed, the recent promising results of a phase I study with AMG 510 targeting specifically the G12C *KRAS* mutations reinforce this perspective, and in our cohort this mutation was detected in 35.3% (47/133) of *KRAS* mutated patients or 9.5% of all LA patients (47/495).

The recent advances in liquid biopsy methods and the development of third-generation TKIs, such as osimertinib, targeting the T790M mutation, have resulted in the rapid implementation of ctDNA analysis in clinical practice. In this study, we evaluated *EGFR* mutations in ctDNA from plasma of 35 patients who were undergoing TKI treatment, most of them receiving first generation agents (erlotinib or gefitinib). Among patients with informative results, 59% were positive for the T790M mutation, with the mutant allele detected at a mean frequency ranging from 0.88–21.8%. The ctDNA analysis was considered to be uninformative for 37% (13/35) of the patients, since neither the T790M resistance mutation nor the original sensitizing *EGFR* mutation could be detected, and most likely in these cases the tumor is not shedding adequate levels of DNA for detection (30). For 10 of these uninformative patients, we performed multiple plasma collections at different time points and in 6 of them an informative result was obtained in at least one ctDNA analysis (five T790M positive, one T790M negative). Our results highlight the ability of serial plasma collection to overcome the low sensitivity for mutation detection in cell-free DNA from patients with tumors shedding small amounts of ctDNA, especially when tissue biopsy is not possible. Additionally, our results emphasize the importance of using a method, such as NGS, that enables the detection of sensitizing, and resistance mutations to differentiate true-negative from uninformative results.

Our study has several limitations. Since this was a diagnostic laboratory cohort, demographic, and clinical data from these patients were limited and outcome data were not evaluated. Additionally, for some analysis, such as the expanded NGS panel covering gene fusions and the liquid biopsy analysis the number of evaluated patients were limited.

In summary, we report a higher-than-expected *EGFR* mutation rate in a cohort of Brazilian patients, with most mutations being associated with EGFR TKI sensitivity. This high *EGFR* mutation rate highlights the negative impact of not performing *EGFR* mutation testing and underscores the urgent need for broader discussion regarding the incorporation of molecular testing and targeted therapy for lung cancer in the Brazilian public and private healthcare systems. Finally, our preliminary results from expanded gene panels and liquid biopsy analysis underscore the rapid evolution of genomic tests and the importance of prompt incorporation of these advances into clinical practice.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by A.C. Camargo Institutional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HF, IC, and DC conceived the study. HF analyzed and interpreted the EGFR and patient data. VC, MC, AS, and GD contributed with patient data. IC and MM performed the histological examination. VK, GT, EF, and DC analyzed and interpreted the genetic data. HF, GT, and DC were the major contributors to manuscript writing. VC and VK contributed to manuscript writing. DC contributed funding. All authors have read and approved the final manuscript.

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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.2020.01068/full#supplementary-material

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CASE REPORT
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Resistance to Both Chemotherapy and EGFR-TKI in Small Cell Lung Cancer With EGFR 19-Del Mutation: A Case Report

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Epidermal growth factor receptor (EGFR) mutations are common in non-small cell lung cancers, but rare in small cell lung cancers (SCLCs). In previous reports, some SCLC patients with EGFR mutations could benefit from EGFR tyrosine kinase inhibitors (TKIs). In this study, we reported a case in which an SCLC patient with EGFR exon 19 deletion (19-Del) mutation did not benefit from EGFR-TKIs. Interestingly, the standard treatment strategies for SCLC also failed to control tumor progression. Moreover, we screened 43 SCLC patients in China and found that the frequency of EGFR mutations in Chinese SCLC patients was about 4.65% by next-generation sequencing (NGS). Collectively, this case illustrated a rare subtype of SCLCs which harbored EGFR mutations and was intrinsically resistant to standard treatments and EGFR-TKIs. We also tried to explore the mechanisms underlying drug resistance. The literature concerning SCLCs with EGFR mutations is reviewed.

Keywords: SCLC, EGFR 19-Del, EGFR-TKI, drug resistance, PTEN mutation

INTRODUCTION

Epidermal growth factor receptor (EGFR) mutations are found in 25–45.6% of Asian non-small cell lung cancer (NSCLC) patients and about 24% of white patients (1–3). It is a biomarker for the use of EGFR tyrosine kinase inhibitors (TKIs). However, EGFR mutations were rarely detected in small cell lung cancers (SCLCs) (4, 5). According to previous studies, 1.8% of 113 Italian SCLC patients and 4% of 122 Japanese SCLC patients had EGFR mutations (4, 6). In China, the patients with EGFR mutations accounted for 2.6–7.1% of SCLC patients (7–10).

SCLCs with EGFR mutations were reported to be sensitive to EGFR-TKIs (4, 11–13). EGFR-mutated SCLC patients tended to be female, non-smoker, and had limited disease (6, 14, 15). EGFR mutations may indicate a possible positive prognostic effect (6, 14, 15). Here, the patient we reported was also female and non-smoker, but her cancer was aggressive. The tumor metastasized rapidly to distant organs including brain, liver, adrenal gland, spinal cord, and vertebrae. Our case may suggest that EGFR mutations are not the only significant predictors for a positive outcome in SCLCs. More importantly, we also explored the possible mechanisms underlying tumor resistance to both chemotherapy and EGFR-TKIs in EGFR-mutated SCLCs.

GENOMIC ANALYSIS OF PURE SCLC TUMORS

We excluded SCLC cases with adenocarcinoma components and collected 43 pure SCLC cases from hospitals in Zhejiang Province, China. The cases were identified as SCLC by pathological diagnosis according to the standard criteria of WHO classification. The median age of the study group was 62 years (range, 36–85). Seventy-seven percent of the cohort were male, and 63% of them had extended disease (**Supplemental Table 1**). The samples in these cases were subjected to next-generation sequencing (NGS) utilizing the Illumina HiSeq 4000 platform. The sequencing time ranged from July 2016 to September 2019. We found that the frequency of EGFR mutations in Chinese SCLC patients was 4.65% (**Figure 1**). Also, we demonstrated the genomic profiling of the 43 Chinese SCLC patients (**Figure 1**).

CASE PRESENTATION

A 59-year-old woman without smoking history presented with intermittent cough and dizziness. Computed tomography (CT) of the chest and brain revealed a right middle lobe lung mass and a left occipital lobe brain metastasis. Magnetic resonance imaging (MRI) of the brain demonstrated an enhancing mass in the left occipital lobe (1.7 × 1.4 cm). Bronchoscopic examination revealed a lesion occluding the right lateral middle lobe bronchus. Transbronchial biopsy of the right middle lobe mass was positive for small cell carcinoma. The histological H&E examination of biopsy specimens showed small and poorly differentiated cells with round or oval nuclei. The biopsies were devoid of any evidence of non-small-cell components. Histopathology reported a TTF-1-positive, Syn-positive, Ki-67 (60–70%), small cell carcinoma of the lung. Whole-body positron emission

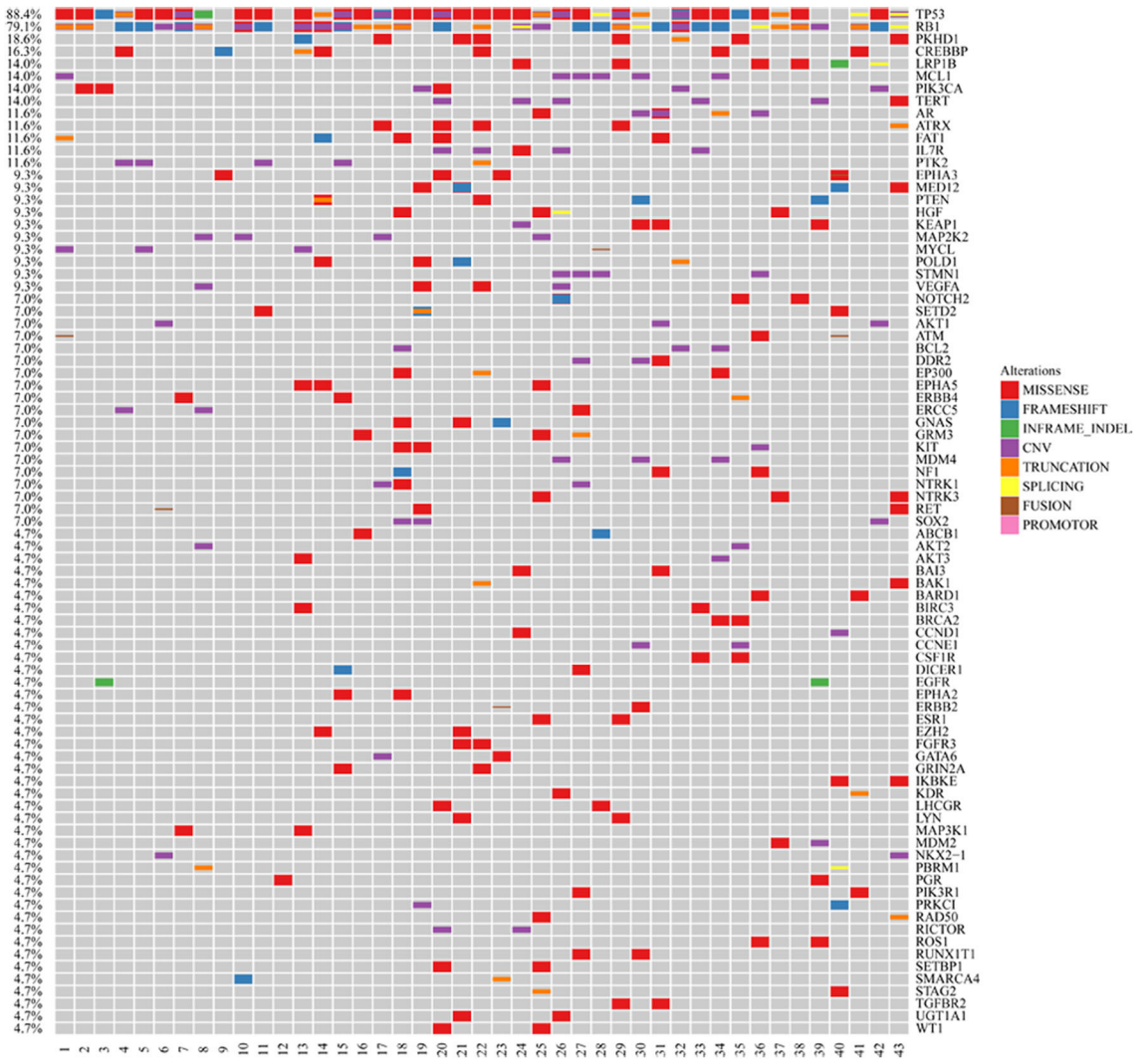

FIGURE 1 | Genomic profiling of the 43 Chinese SCLC patients. The abscissa was tumor specimens, and the ordinate was gene names. Genetic alterations annotated according to the color panel on the right side of the image.

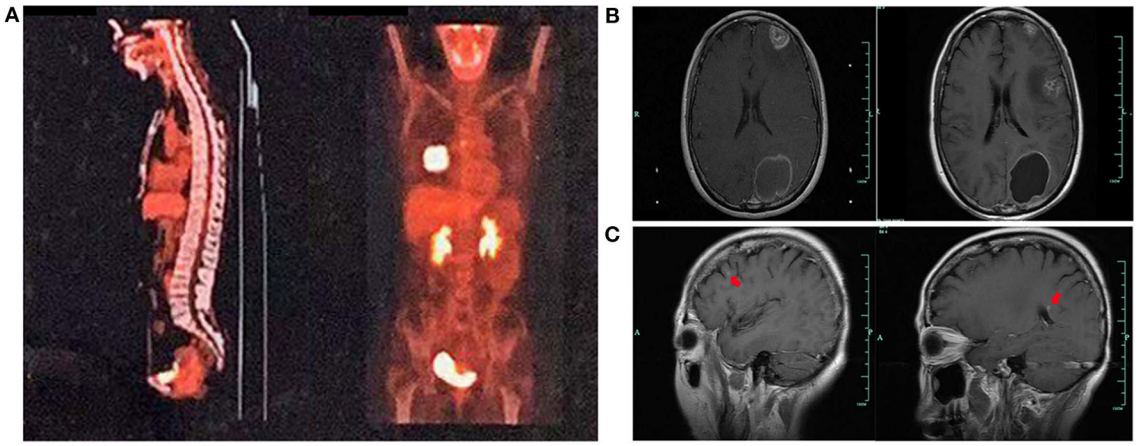

FIGURE 2 | Tumor lesions detected at various times. PET-CT showing a mass in the middle lobe of the right lung **(A)**. Tumor lesions in the brain before and after EP therapy **(B)**. New masses in the brain after EP therapy **(C)**.

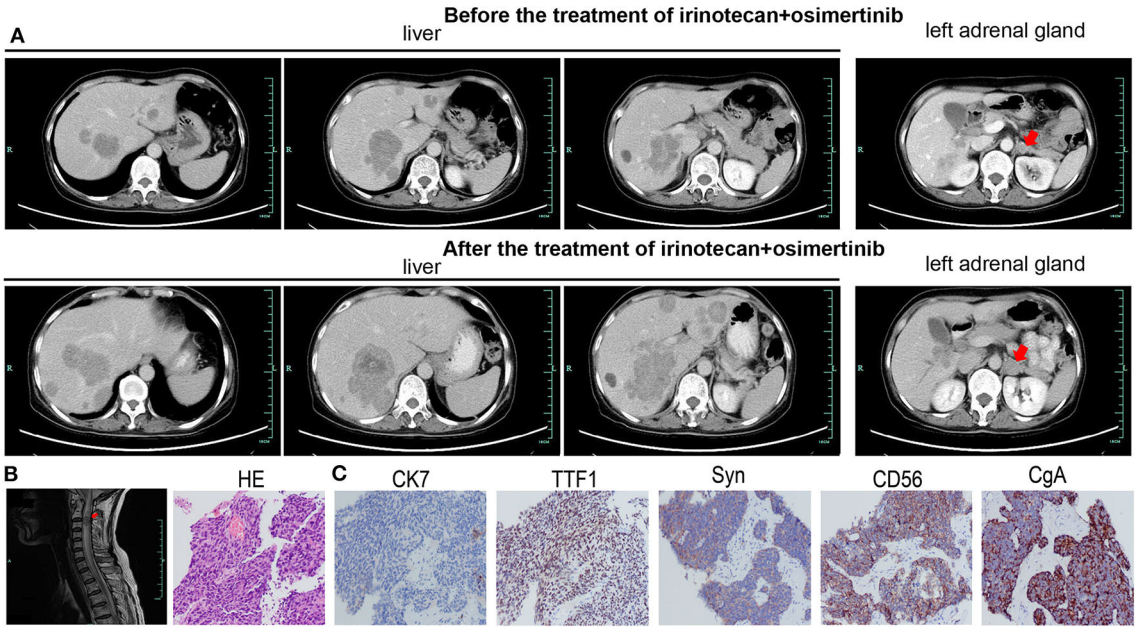

FIGURE 3 | The imageological and cytological examinations of tumor metastases. Tumor metastases in the liver **(A)**, left adrenal **(A)**, and spinal cord **(B)** before irinotecan plus osimertinib, and enlarged metastatic lesions in the liver **(A)** and left adrenal **(A)** after treatment. **(C)** The HE and IHC staining of biopsy specimens in the case. Typical for SCLC, IHC was strongly positive for TTF1, Syn, CD56, and CgA.

tomography computed tomography (PET-CT) identified areas of abnormal metabolism in the right lung, right hilum, and subcarinal lymph nodes (**Figure 2A**), as well as the left occipital lobe of the brain. The bone scan was negative for evidence of metastatic lesions. Two months after diagnosis, she was referred to our department. Brain MRI showed the metastatic tumor in the left occipital lobe dramatically increasing to 4.8 × 3.8 cm (**Figure 2B**). Meanwhile, four new masses were found in brain. As standard therapy for extensive-stage small cell carcinoma, the patient received etoposide and cisplatin (EP) chemotherapy with a poor clinical response. After four cycles of EP treatment, new metastatic lesions were detected in the right brain (**Figure 2C**). To control tumor progression, she received CyberKnife radiosurgery for the treatment of lung neoplasm, followed by a whole-brain radiotherapy (WBRT) for brain metastases. After WBRT, the patient displayed symptomatic disease progression with multiple new liver, left adrenal gland, and spinal cord metastases (**Figure 3A,B**). An ultrasound-guided percutaneous liver biopsy was performed. Re-biopsy of the metastases in the liver confirmed small cell

TABLE 1 | Genetic alterations in the case identified by NGS.

Genes	Alternations	Nucleotide change
ARID1B	p.S36F Missense mutation in exon 1	c.107C>T (p.S36F)
ASXL1	p.E332D Missense mutation in exon 11	c.996G>C (p.E332D)
EGFR	p.E746_A750del Non-shift code deletion mutation in exon 19	c.2235_2249delGGAATTAAGA GAAGC (p.E746_A750del)
KEAP1	p.A392T Missense mutation in exon 3	c.1174G>A (p.A392T)
MDM2	Amplification	-
PDCD1	p.R231 Truncation in exon 5	c.691C>T (p.R231*)
PGR	p.Q78H Missense mutation in exon 1	c.234G>T (p.Q78H)
PTEN	p.K237Cfs*17 7 Frameshift mutation in exon 7	c.709_715delAAGTTCA (p.K237Cfs*17)
RB1	Single copy number missing	-
ROS	p.G1809E Missense mutation in exon 33	c.5426G>A (p.G1809E)
SMAD4	c.1447+2dupT Shear mutation in Intron 11	c.1447+2dupT
TERT	Amplification	-

–, not applicable.

histology and revealed SCLC evident by positive expressions of CD56, TTF-1, CgA, and synaptophysin (**Figure 3C**). The tissue re-biopsy samples and blood samples were subjected to next-generation sequencing. As shown in **Table 1**, genotype disclosed an EGFR exon 19 deletion (19-Del) mutation and PTEN frameshift mutation (p.K237Cfs*17). She subsequently received two cycles of irinotecan in combination with 1 month of third-generation EGFR-TKI osimertinib. However, the tumor dramatically progressed with enlargement of liver metastases (**Figure 3A**). After that, her disease also failed to respond to anlotinib plus pembrolizumab and anlotinib plus Tiggio. Finally, with fast tumor progression, she died 1 month later.

DISCUSSION

SCLCs are often seen as aggressive cancers which are sensitive to chemotherapy and shrink at the initial stage and then relapse soon with chemoresistance. Currently, the exact mechanism underlying chemoresistance is still unknown. As previous studies have shown, the mutations in CSMD3/PCLO/RYR1/EPB41L3 may predict resistance to etoposide (16). However, none were detected in our case. In our case, PTEN mutation was detected. PTEN is a tumor suppressor in the PI3K/AKT pathway. The continuous activation of the PI3K/AKT pathway is a pivotal chemoresistance factor in SCLCs. PTEN could sensitize the tumor to chemotherapy including etoposide and cisplatin by inhibiting the PI3K/AKT signaling pathway (17, 18). The efforts to combine adenoviral PTEN gene therapy with cisplatin chemotherapy could enhance tumor suppression in SCLC (19). Thus, PTEN mutation may account for chemoresistance in our case by aberrant activation of the PI3K/AKT pathway.

In most previous studies, EGFR mutations in lung cancer, even in SCLC patients, are responsive to EGFR-TKIs (4, 11, 13, 20). It is still controversial whether EGFR-mutated SCLCs have adenocarcinoma components. Some researchers propose that EGFR mutations do exist but are just rare in SCLCs (4, 15). In our case, the patient received biopsy twice and the pathological examinations were almost identical. We did not find any pathological evidence supporting adenocarcinoma. Different from the favorable response to EGFR-TKIs in adenocarcinoma, the tumor in our case proved to be resistant to EGFR-TKI. The mechanisms of resistance to EGFR-TKIs are widely discussed. Le et al. discussed that the lack of response to EGFR-TKIs in EGFR-mutated *de novo* SCLC might be attributable to the lack of EGFR expression (21). Mutations of KRAS or BRAF might also be correlated with resistance to EGFR-TKIs in EGFR-mutated *de novo* SCLC patients (21, 22). Many researchers pointed out that EGFR and its downstream pathway were involved with primary resistance to EGFR-TKIs (23). Mutations in the EGFR downstream genes including PIK3CA and PTEN might confer resistance to EGFR-TKIs (22–27). A study using a genetic mouse model showed that PTEN inactivation advanced SCLC, suggesting that treatment targeting PTEN may be effective for a subset of SCLC patients (28). PTEN loss-of-function mutation resulted in poor PFS and OS in patients with EGFR mutations, and it was an independent predictor for short PFS in patients with EGFR-TKI treatment (23–25). Furthermore, in the 43 sequenced SCLC cases, one EGFR-mutated SCLC without PTEN mutation was responsive to EGFR-TKI gefitinib. Thus, our case suggests that PTEN may also play a key role in the poor outcome with EGFR-TKI treatment in EGFR-mutated SCLC patients.

Few studies have focused on the potential mechanisms of tumor resistance to both EGFR-TKIs and chemotherapy in EGFR-mutated *de novo* SCLC patients. Our case is the first one to propose that in this rare subtype of SCLCs, PTEN dysfunction may play a vital role in the instinct resistance to both chemotherapy and EGFR-TKIs. Another case resistant to both EGFR-TKIs and chemotherapy was reported by Varghese et al. (28). EGFR 19-Del and PIK3CA mutations coexisted in their case (28). Therefore, the aberrant activation of the PI3K/AKT pathway may be involved in the resistance of EGFR-mutated SCLC to both chemotherapy and EGFR-TKIs. Large sample size and further studies in EGFR-mutated SCLCs are needed to validate our findings.

CONCLUSION

We have reported a *de novo* SCLC case with EGFR 19-Del which was innately resistant to both chemotherapy and EGFR-TKI. The resistance to EGFR-TKIs and chemotherapy may be attributable to PTEN mutation. Moreover, we presented the frequencies of EGFR mutations and other genetic mutations in Chinese SCLC patients.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of the 903rd Hospital of PLA. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LW wrote this article. LW and FD were involved in diagnostic flow and patient follow-up. JS was a licensed pathologist and proposed the images of pathological examination. GD and YS contributed to the data collection and analysis of 43 pure SCLC cases using NGS technology. The manuscript was edited by XW. LW, FD, JS, GD, YS, YL, XH, LB, WW, and XG were involved in the interpretation of published data. All authors read and gave their final approval of the version to be published.

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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.2020.01048/full#supplementary-material

Supplemental Table 1 | The clinical characteristics of the 43 Chinese SCLC patients.

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Conflict of Interest: GD and YS were employed by the company Nanjing Geneseeq Technology Inc., Nanjing, China.

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

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CASE REPORT
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Next-Generation Sequencing at High Sequencing Depth as a Tool to Study the Evolution of Metastasis Driven by Genetic Change Events of Lung Squamous Cell Carcinoma

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Background: The aim of this study is to report tumoral genetic mutations observed at high sequencing depth in a lung squamous cell carcinoma (SqCC) sample. We describe the findings and differences in genetic mutations that were studied by deep next-generation sequencing methods on the primary tumor and liver metastasis samples. In this report, we also discuss how these differences may be involved in determining the tumor progression leading to the metastasis stage.

Methods: We followed one lung SqCC patient who underwent FDG-PET scan imaging, before and after three months of treatment. We sequenced 26 well-known cancer-related genes, at an average of ∼6,000 × sequencing coverage, in two spatially distinct regions, one from a primary lung tumor metastasis and the other from a distal liver metastasis, which was present before the treatment.

Results: A total of 3,922,196 read pairs were obtained across all two samples' sequenced locations. Merged mapped reads showed several variants, from which we selected 36 with high confidence call. While we found 83% of genetic concordance between the distal metastasis and primary tumor, six variants presented substantial discordance. In the liver metastasis sample, we observed three *de novo* genetic changes, two on the FGFR3 gene and one on the CDKN2A gene, and the frequency of one variant found on the FGFR2 gene has been increased. Two genetic variants in the HRAS gene, which were present initially in the primary tumor, have been completely lost in the liver tumor. The discordant variants have coding consequences as follows: FGFR3 (c.746C>G, p. Ser249Cys), CDKN2A (c.47_50delTGGC, p. Leu16Profs*9), and HRAS (c.182A>C, p. Gln61Pro). The pathogenicity prediction scores for the acquired variants, assessed using several databases, reported these variants as pathogenic, with a gain of function for FGFR3 and a loss of function for CDKN2A. The patient follow-up using imaging with 18F-FDG PET/CT before and after four cycles of treatment shows discordant tumor progression in metastatic liver compared to primary lung tumor.

Conclusions: Our results report the occurrence of several genetic changes between primary tumor and distant liver metastasis in lung SqCC, among which non-silent mutations may be associated with tumor evolution during metastasis.

Keywords: lung cancer, NGS, FGFR, HRAS, CDKN2, tumor evolution

BACKGROUND

Lung cancer is the most common type of cancer and the leading cause of cancer-related deaths for both men and women in the world (1, 2). There are two major types of lung cancer, namely, non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma. NSCLC can be further divided into three main subtypes: large cell lung carcinoma, squamous cell carcinoma, and adenocarcinoma. Lung SqCC accounts for almost 30% of NSCLC, while there are also several other types that occur less frequently (3, 4). Significant progress on cancer research was made possible by the development of next-generation sequencing (NGS) (5–8) and, subsequently, by extensive research efforts on several tumors and patients (9–11). The body of knowledge on cancer genetics has increased sizably, and, depending on cancer type, researchers have identified several target genes that could be involved in cancer development, to improve patient care and to enhance the survival rate (12–16). Clinical research efforts to provide a comprehensive landscape of molecular alterations of lung SqCC have successfully characterized this cancer at the genetic level (9, 17). Researchers from the Cancer Genome Atlas (TCGA) identified several genes that are aberrantly expressed and/or mutated in lung SqCC patients. Major alterations have been described in TP53, PI3K, CDKN2A, SOX2, CCND1, ERBB, MET, DDR2, and FGFR1 (18–29). In particular, identification of genetic amplification in FGFR1, mutations in ERBB family, and abnormalities in PI3K have raised considerable interest in view of applications to the therapeutic management of SqCC patients. Furthermore, in multiple clinical trials, some of these genetic alterations have been targeted and have shown promising progress in treating SqCC (LUME-Lung 1, LUX-Lung 8, BASALT-1 phase II, FLEX, SQUIRE, and others) (30–36). Unfortunately, the observed benefits have been sporadic and heterogeneous, leading to some disappointment (18). Recently, the understanding of the complex interactions between the immune system and cancer has led to the development of immune checkpoint inhibitors (ICI). The combination of chemotherapy and ICI, in the first-line treatment of metastatic SqCC, has shown higher overall survival (OS) and progression-free survival (PFS) than the use of chemotherapy alone (37–39). Unfortunately, several studies have shown that only a subset of patients could benefit from this therapeutic protocol, such as patients with wild-type EGFR or negative ALK rearrangements (40, 41). Moreover, in different PD-L1 expression subgroups, the benefit in OS and PFS is mixed (42).

Because of the heterogeneity of the tumor at genetic and molecular levels, treatment of SqCC continues to remain a challenge. There may be several reasons due to the existence of different genomic patterns in SqCC, and the genetic targets that have been found so far are probably not driver mutations and are therefore currently unactionable or "undruggable" mutations for this disease (18). Several available treatments do not exhibit efficacy in metastasis lung SqCC, leaving these patients with fewer and inefficient treatment options, a situation, which could explain the poor survival and the frequent relapse of these patients. Thus, the identification of potential targets in SqCC has become urgent and essential.

The spread of cancer cells from primary tumors to other vital organs is often associated with poor prognosis (43, 44). Given its potential impact on patient care, understanding the genetic differences between primary tumors and distant metastasis is critical and will have value in terms of treatment strategies. A better understanding of the dynamic of the molecular evolutionary process of the lung SqCC will provide greater insight toward the development of a suitable therapeutic guide (45, 46). In this study, we used tumor genetic data generated by NGS, to investigate if tumor heterogeneity occurs between primary tumor and liver metastatic sites in lung SqCC and to detect non-silent mutations, which may be associated with tumor evolution, and its evolutionary trajectory from primary tumor through relapsed disease.

CASE PRESENTATION

Patient

A 72-year-old African man was presented to our clinic in 2016, with a worsening dyspnea and chronic dry cough. Symptoms included fatigue and muscle atrophy, with pain in the chest and right rib cage. He suffered from cervical disc herniation and frequent reversible heart palpitation. The patient was a former smoker, consuming one pack per day for 16 years until he was 32 years old. In the previous two years, he had a significant weight loss of 20 kg. The physical exam shows a decrease in vesicular murmur and vocal vibrations of the right lung with no evidence of lymphadenopathy. The abdominal echography shows hepatomegaly. A suspicious mass for pulmonary neoplasm was found on chest X-ray. A CT scan detected a liver mass of 6 cm in the segment IV. Two biopsies were performed, one in the mediastinum (sample 69380-1-S7) and another in the liver (sample 69381-2-S8), and pathological examination confirmed the presence of squamous cell lung carcinoma, NSCLC at stage IVB (T4N2aM1c). The patient underwent FDG-PET scan imaging as part of the routine diagnosis and staging, before starting the chemotherapy treatment. Two hypermetabolic

masses were localized; the maximum standardized uptake value (SUVmax) was assessed for these masses. On 26th December 2016, he received the first cure with paclitaxel–carboplatin. On 31st December 2016, he was admitted in the hospital for dysenteric syndrome and hemorrhoids and was treated with loperamide, diosmectite, metronidazole, lidocaine hydrochloride, diosmin, and hesperidin. On 19th January and 9th February 2017, he received the second and third cure with paclitaxel and carboplatin, respectively. Subsequently, on 13th March 2017, a second FDG-PET scan was performed to assess tumor progression. On 16th March 2017, he received the fourth cure with paclitaxel–carboplatin. A complete blood count was performed before each treatment, which was normal. A poor tolerance to chemotherapy was observed, as characterized with the following: nausea, diarrhea, loss of appetite, overall condition deterioration, and neurological disorders. Thus, the combination of immune checkpoint inhibitor and chemotherapy was not recommended and PD-L1 expression level was not investigated. Tyrosine kinase inhibitors (TKIs) were suggested instead. The patient refused to continue with the treatment. Nevertheless, he gave his consent to conducting the genetic analysis and for the publication of the present case report.

Sample Processing

Microdissections from formalin-fixed paraffin-embedded (FFPE) specimens of these biopsies were performed, ensuring that a tissue area that contained a high percentage of tumor cells would be used for DNA extraction. gDNA was extracted using the QIAamp DNA Micro Kit (Qiagen), then the sequencing library was prepared and was submitted for sequencing on MiSeq (Illumina). We used an existing commercial gene panel test, the somatic Tumor Hotspot MASTR™ Plus with MID for sequencing library preparation (Multiplicom/Agilent). The assay developed for the identification of SNVs in 26 frequently mutated genes in solid tumors. This NGS assay was designed with the input from the French INCA centers (https://www.agilent.com/cs/library/datasheets/public/TUMOR%20HOTSPOT%20MASTR%20PLUS%205991-8378ENN.pdf). This panel targets the following 26 genes: ALK, PTEN, PIK3R1, EGFR, NRAS, STK11, ERBB4, ERBB2, AKT1, HIST1H3B, HRAS, PDGFRA, MAP2K1, CDKN2A, IDH1, H3F3A, IDH2, BRAF, PIK3CA, KIT, CTNNB1, KRAS, FGFR3, MET, FGFR2, and DDR2.

Data Analysis

We filtered the sequencing reads with a Q-score higher than 20, merged the read pairs, and mapped them on the human genome reference GRCh37/hg19. We filtered out duplicate reads, resulting from clonal amplification of the same fragments during library construction, and the regions which showed mapping coverage of less than 50×. We performed variant detection analysis for SNPs, insertions, and deletions using Sophia Genetics DDM platform v4.5. Only high-confidence variants were retained according to the following criteria: (1) occurring in a high-complexity region, (2) having high, or higher than expected, variant fraction (germline), (3) having a sequencing coverage >50 ×, (4) having >50 supporting reads, and (5) being aligned inside the target region. The copy number variation analysis was not investigated in the present study. Variants were ranked by concordance and discordance between the two samples, and annotation information such as coding consequences was taken forward for further analysis. For each variant, we also reported the variant frequency, defined as variation percent = (altNum/depth) × 100, where "altNum" is the number of reads containing the variant of interest and "depth" is the sum of number of reads containing the variant and the reads covering the reference allele. To obtain pathogenicity prediction scores, the target variants were submitted to the following databases made available by the Sophia Genetics DDM platform: COSMIC, Clinvar, OncoKB, cBioPortal, Varsome, Mutationassessor, Provean, FATHMM, MutationTaster, FATHMM-MK, MetaSVM, MetalR, LRT, SIFT, dbSNP, ClinVitae, and Provean. The protein structures encoded by each mutated gene were submitted to the SMART tool (47), to describe the biological consequence of each mutation on the functional domain of the protein.

RESULTS

Image Analysis With 18F-FDG PET/CT

Two intense hypermetabolic masses were revealed by 18F-FDG PET/CT (**Figure 1**). They were found into the antero-superior mediastinum and in the hepatic parenchyma. The SUVmax of the hypermetabolic masses was assessed before and after treatment. Before treatment, the SUVmax of the mediastinal mass was 7.3 and the hepatic mass was 9.4. After treatment, the mediastinal mass increased to 10.4, while the liver hypermetabolic mass decreased significantly to 3.9. While the primary lesion showed continuous tumor progression, the liver lesion showed significant improvement.

Sequencing Data Statistics

The mediastinal and liver samples were sequenced, resulting in 1,661,981 and 2,260,216 read pairs, respectively. Ninety percent of the total reads were above Q35, which is considered as a high threshold for read quality. The mapping of merged reads on the targets genes resulted in 1,628,242 reads (>97%) successfully mapped for the mediastinal sample and 2,134,774 reads (>94%) for the liver sample. In the present experiments, we achieved a high sequencing coverage of the target region. The mean target coverage in this study was around 6,000×. The target region coverage distribution for both samples is shown in **Figure 2C** and **Figure S1**.

Finding Common and Discordant Variants

In the lung sample, 12 out of the 26 genes sequenced presented with genetic changes, with 33 variants retained for high-confidence call and 28 discarded. In the liver sample, 13 genes presented with genetic changes, with 36 variants found and 24 discarded. The comparison between the distal metastasis and the primary tumor shows 30 shared variants and 6 distinct variants. When analyzing the variant frequency of the shared variants and using primary tumor data as baseline, we can observe several clusters (**Figure 2**).

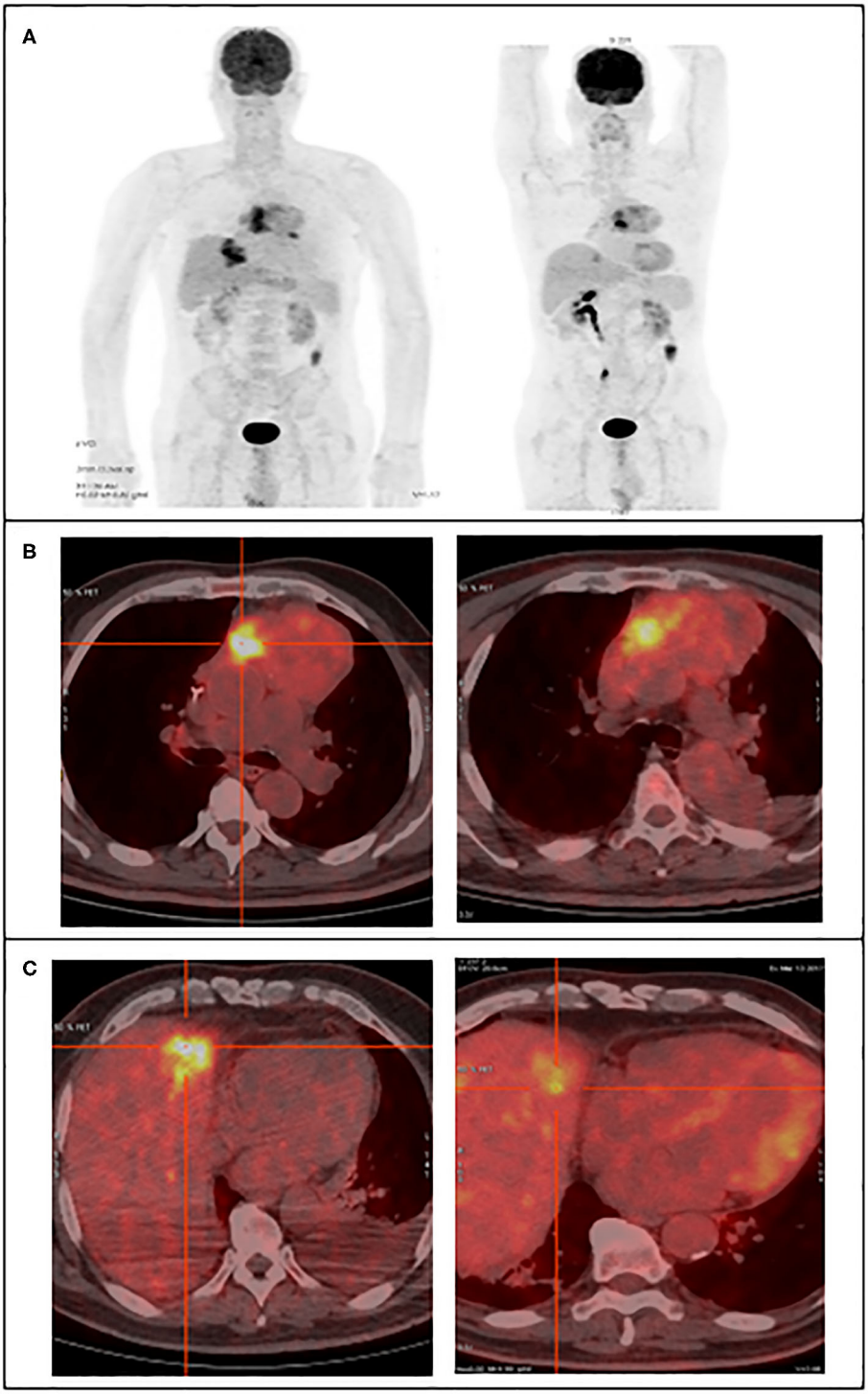
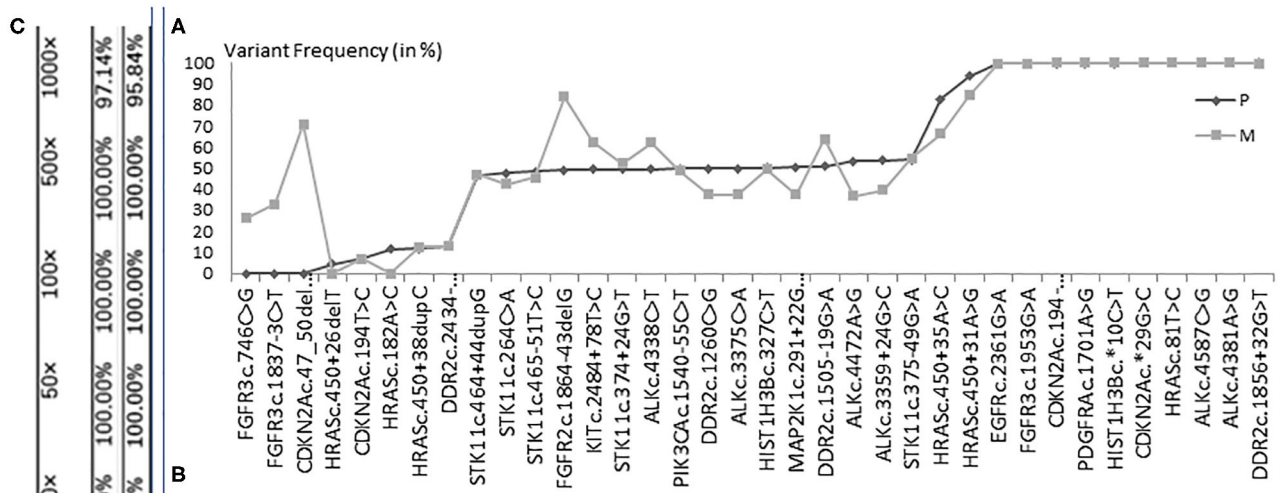


FIGURE 1 | 18F-FDG PET/CT images reveal the large hypermetabolic masses, in the superior mediastinum and the hepatic parenchyma, left figures before treatment and right figures post-treatment. **(A)** Maximum intensity projection image, **(B)** transaxial fusion image showing the mediastinal tumor, **(C)** transaxial fusion image showing the liver tumor.

We found 30 variants shared between primary tumor and metastasis, distributed across 12 genes, and having a similar frequency between the two samples. Among these variants, 6 have genetic changes in the coding sequence of the following genes: ALK (3 variants), CDKN2A (2), and PIK3CA (1) (**Figures 2**, **3**).



B

Discordant Variant information						69380-1-S7		69381-2-S8	
Gene	Type	Coding Consequence	Exon	c.DNA	Protein	Depth	Var (%)	Depth	Var (%)
FGFR2	INDEL	intronic	14	c.1864-43delG		7835	48.91	9356	83.79
FGFR3	SNP	missense	7	c.746C>G	p.Ser249Cys	>1000	0	1163	26.23
FGFR3	SNP	intronic	14	c.1837-3C>T		>1000	0	1941	32.72
CDKN2A	INDEL	frameshift	1	c.47_50delTGGC	p.Leu16Profs*9	>1000	0	9876	70.62
HRAS	SNP	missense	3	c.182A>C	p.Gln61Pro	1529	11.51	>1000	0
HRAS	INDEL	intronic	4	c.450+26delT		727	4.26	>1000	0

C

Sample ID	20×	30×	50×	100×	500×	1000×
69380-1-S7	100.00%	100.00%	100.00%	100.00%	100.00%	97.14%
69381-2-S8	100.00%	100.00%	100.00%	100.00%	100.00%	95.84%

FIGURE 2 | (A) The graphical representation of retained genetic variants and their frequency (var %), in primary tumor (P) and distal metastasis (M). **(B)** The illustration of the six discordant variants, among which three with coding consequences. Table **(C)** shows the percentage of target regions with certain coverage for both samples.

We found six discordant variants, covering the following genes: FGFR2, FGFR3, CDKN2A, and HRAS. Among these variants, only three variants had coding consequences as follows: a missense c.182A>C (p.Gln61Pro) on the HRAS gene, a frameshift c.47_50delTGGC (p.Leu16Profs*9) on CDKN2A gene, and a missense c.746C>G (p.Ser249Cys) on the FGFR3 gene. The HRAS (p.Gln61Pro) variant is found in primary lung tumor with a frequency of 11.5% and completely absent in the liver metastasis. The CDKN2A (p.Leu16Profs*9) and FGFR3 (p.Ser249Cys) variants are present only in the liver metastasis at a frequency of 70.6 and 26.2%, respectively (**Figures 2**, **3**). The full list of variants is publicly available in Clinvar database (www.ncbi.nlm.nih.gov/clinvar/), under the accession numbers SCV001250917 to SCV001250974.

Pathogenicity Prediction

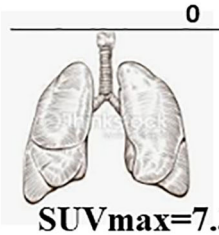
The pathogenicity prediction of the three discordant variants with coding consequences shows the following:

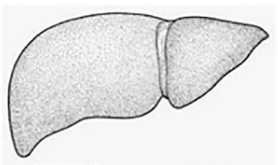
1/ The FGFR3 missense c.746C>G (p.Ser249Cys) is located between two functional domains, in a relatively conserved position, and is predicted to have a damaging, disease-causing, and high effect on protein function (**Figure 4**). This is the most frequently observed FGFR3 variant found in cancer studies and is reported as oncogenic with a gain of function. The pathogenicity prediction of this genetic variation confirmed its association with several cancers, including tumors of the lung and other tumors: the urinary bladder, the urinary tract, the head, the neck, the upper aero-digestive tract, the thymus, and the cervix. This genetic variant is reported as a recurrent hotspot mutation in these tumors. It could be either germline or somatic and is classified as a pathogenic allele.

2/ The CDKN2A, frameshift c.47_50delTGGC (p. Leu16Profs*9), is located at a relatively conserved position and predicted to have a high effect on protein function (**Figure 4**). The variant has a damaging, disease-causing effect on protein function and has been shown to be involved in pancreas carcinoma and thymoma. It has been reported as both somatic and germline mutation and has been classified as a pathogenic allele, associated with hereditary cancer predisposing syndrome.

3/ The variant HRAS, missense on c.182A>C (p. Gln61Pro), is located in the Ras domain and has been found to be expressed in the upper aero-digestive tract and in thyroid cancers (**Figure 4**). This variant is reported in both germline and somatic and is classified as a pathogenic allele. It has a

Shared Non-silent SNV		Lung Tumor		Liver Tumor	
Gene	Protein	Depth	Frequency (%)	Depth	Frequency (%)
ALK	p.Ile1461Val	11768	**99.89**	23882	**99.83**
ALK	p.Lys1491Arg	15235	**53.36**	29281	**36.72**
ALK	p.Asp1529Glu	8577	**99.88**	13954	**99.89**
CDKN2A	p.Leu65Pro	2692	**6.80**	3249	**6.74**
CDKN2A	p.Arg98*	9274	**1.78**	15545	**2.05**
PIK3CA	p.Val151Ala	13243	**1.50**	14806	**1.03**
Discordant Non-silent SNV					
HRAS	p.Gln61Pro	1529	**11.51**		**0**
CDKN2A	p.Leu16Profs*9		**0**	9876	**70.62**
FGFR3	p.Ser249Cys		**0**	1163	**26.23**





SUVmax=7.3 SUVmax=9.4

3 months later
(four courses of standard treatment
Paclitaxel-carboplatine)

SUVmax=10.4 SUVmax=3.9

FIGURE 3 | Summary of the current finding. The genetic variants found in primary lung tumor (left) and liver (right) metastasis sites in the lung SqCC patient. The shared and discordant non-silent SNV are mentioned with the frequency percentage.

damaging, disease-causing effect on protein function. We did not find relevant hits for this particular variant in OncoKB, cBioPortal, or My Cancer Genome.

DISCUSSION

Extensive clinical research efforts and advancements that have been made since the human genome was sequenced have greatly increased our knowledge on cancer genetics. Several genetic alterations have been shown to initiate and sustain specific deregulated cellular signaling pathways involved in various tumors. In the present study, using one lung SqCC patient, we screened 26 genes that are well known as potential driver cancer genes, using next-generation sequencing. Though there are many known gene panels containing more genes, we limited our analysis to the current panel, to achieve high sequencing coverage. The panel that we used has been previously validated as clinically relevant in a wide variety of solid tumors. Moreover, this multiple-gene panel focuses on actionable hotspot mutations, which have therapeutic as well prognostic values for different solid tumors, enabling a personalized medicine approach.

We performed in-depth investigation looking for SNVs and indels on the target genes. For each gene, both coding and noncoding regions were covered. We found that 83% of the retained variants are being shared by distant metastasis and primary tumor. Moreover, for each of these concordant variants, the variation frequency shows a strong concordance between the two tumor sites, with the presence of distinguished clusters, mainly containing both low- and high-frequency variants. Among the shared variants, we detected six non-silent SNVs, among which three variants at high frequency (36–99%) in the ALK gene, and at low frequency (1–6%): two in PIK3CA and one in CDKN2A. The variants CDKN2A (p.Leu65Pro) and ALK (p.Ile1461Val) were found in another patient also with lung SqCC, with the same variant frequency (data not shown). Somatic alterations of ALK gene are reported relatively rarely in squamous cell carcinoma (SqCC) (48), but, on the other hand, CDKN2A alterations are common and have been reported in

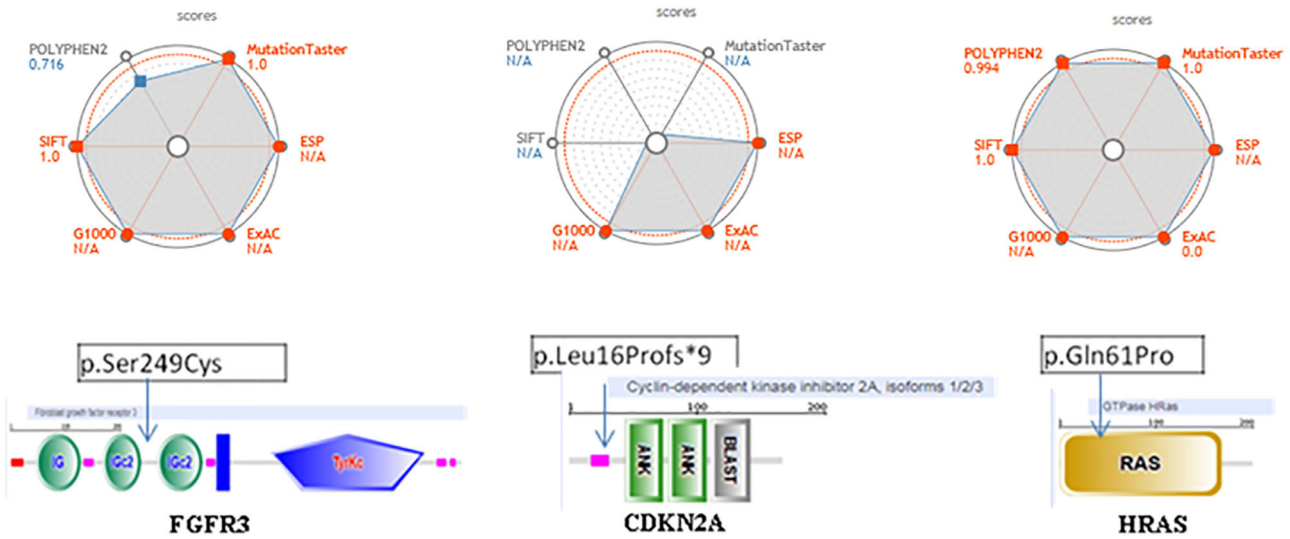


FIGURE 4 | Pathogenicity web plotting prediction scores for the three variants, FGFR3 (p.Ser249Cys) (left side), CDKN2A (p.Leu16Profs *9) (middle side), and HRAS (p.Gh161Pro) (right side). Values are scaled so that the most pathogenic scores are plotted toward the external circles. For POLYPHEN2, Mutation Taster and SIFT, those values approach 1; whereas for ESP, G1000, ExAC they approach. The greater the gray area, the variant is more likely pathogenic. ESP, G1000, ExAC empty values are considered as NIA. Score > 0.9 and population databases (if applicable) <0.1 are shown in red. In the bottom, we show the localization on these three of genetic changes events FGFR3, CDKN2A, and HRAS using SMART database.

recent genomic studies of lung SqCC (49). Interestingly, in one recent study, which included three patients with lung SqCC, the rate of concordance in somatic mutations between local metastasis in distinct lung lymph nodes and primary lung cancer varies from 15 to 82%, and some nodes have highly similar somatic mutation profiles (45). In another study which analyzed two lung SqCC patients, the rate of concordance in somatic mutations between distinct lung lymph nodes and primary lung cancer was identified, with 37% for one patient and 96% for the other (46). Spatial tumor heterogeneity in lung SqCC patients occurs frequently and differs significantly from one patient to another. Typically, in routine clinical practice a single biopsy is performed, to define a driver mutation and thus to suggest the optimal treatment. The occurrence of tumor heterogeneity, which was observed in the current and previous works, could make the use of a single solid biopsy ineffective. Liquid biopsy could be an actionable option to perform a serial monitoring of the tumor burden and get a comprehensive view on the genetic changes of the tumor cell diversification that could take place during distant metastasis.

In the present report, six genetic variations affecting four genes (FGFR2, FGFR3, CDKN2A, and HRAS) are shown to have a substantial discordance between the liver metastatic site and the primary tumor of the lung SqCC. Four genetic changes were gained on the genes FGFR2, FGFR3, and CDKN2A, and two genetic variants were lost on HRAS, in the metastatic tumor. Interestingly, after four cycles of treatment, a strong signal of resolution was detected at the liver metastasis through a significant sink over 60% of the tumor size. In contrast, the primary tumor showed a progression. These mixed outcomes after treatment may be related to the intra-tumor genetic heterogeneity of this lung SqCC patient. The discordant variants found in the current report have low frequency; it is possible that primary tumor cells with high-frequency genetic variants resist during the metastasis events, and a new clone harboring new low-frequency genetic variants emerges. These low-frequency variations might make metastatic tumor cells respond differently to chemotherapy treatment than the primary tumors.

Three of the discordant genetic changes have coding consequences, as follows: FGFR3 (p. Ser249Cys), CDKN2A (p. Leu16Profs*9), and HRAS (p. Gln61Pro). None of these non-silent mutations were found in the study of De Bruin or Um (45, 46). One explanation could be that the high sequencing-coverage depths, which were performed in this study, have resulted in identification of non-silent mutations. The advantage of performing whole-genome or exome sequencing would allow comprehensive understanding of the functional alterations found in lung cancer, but this approach could neglect rare or low-frequency variants. Sufficient base coverage of the targeted region, defined as the number of reads covering a base, is an important prerequisite for reliable variant detection from NGS data. In the present study, the cost of high sequencing coverage limited the number of assayed genes compared to the Bruin and Um studies. Nevertheless, the mean target coverage in these studies was ∼100×, while in our study all the target regions are covered in average ∼6,000× and at least 500× (**Figure S1**).

FGFR3 (fibroblast growth factor receptor 3) is a member of the fibroblast growth factor receptor family (FGFR). Alterations

in the FGFR kinase family are common in lung SqCC and comprise the most frequently altered tyrosine kinase family (3, 50, 51). The mechanism of activation of FGFR3 kinase was previously established for the observed variant FGFR3 (p. Ser249Cys). The presence of the mutant cysteine amino acid instead of serine residue allows, via disulfide bound, the receptor to dimerize abnormally, resulting in ligand-independent constitutive activation in the absence of the ligand and to structural perturbation of the dimers (49). Using cell culture and tumor xenograft models, researchers described the oncogenic nature of this observed FGFR3 mutation and showed that it could drive cellular transformation (49). Cancer cell lines harboring this variant have demonstrated sensitivity to the inhibition by FGFR inhibitors and that the cell transformation can be reversed (17, 52–55). The variant CDKN2A (p. Leu16Profs*9) is reported in somatic pancreas carcinoma and thymoma, also as a germline variant associated with hereditary cancer-predisposing syndrome. According to several databases, this variant is located at a relatively conserved position and is predicted to have a strong effect on protein function; the variant has a damaging, disease-causing effect on protein function (56, 57). The specific variant HRAS (p. Gln61Pro) is located at a medium-conserved position, reported as both germline and somatic mutation. This variant has been reported as having a medium effect on protein function and classified as a pathogenic allele. However, in the study of functional consequences at a specific mutation hotspot on the HRAS gene, the researchers show that Q61P and Q61E variants did not show increased transforming ability relative to HRAS wild-type (wt) mouse (58). Until recently, specialists just cataloged cancers as either wt or mutant for RAS (59), to predict resistance to targeted therapies, and no treatment anti-RAS therapies exist yet in the clinic (58). In Um's study, one genetic variant of HRAS (Q61R) was found, at low frequency, in only one SqCC patient, in the primary tumor as well as in one lung lymph node, but not in the other lymph node analyzed (45).

The metastatic event in lung cancer involves dissemination of cancer cells to anatomically distant organ sites, such as the liver and brain, and their subsequent adaptation to distant tissue microenvironments. Metastasis encompasses several biological processes, which consists of a complex series of well-organized steps: tumor cells exit their primary sites of growth by local invasion and intravasation, translocate systemically, survive in the circulation, arrest, affect the distant organ by extravasation, and adapt to survive and thrive in the initially healthy microenvironments of distant tissues (43, 44). Metastasis cascades are controlled by specific classes of genes, among which researchers have described metastasis initiation, progression, and virulence genes (60, 61). The relevant variants on FGFR3 and CDKN2A genes found in the present study are probably part of these virulence genes, which potentially act to promote metastasis, provide a selective advantage in secondary sites over the primary tumor, and enable the colonization of distant organs. Our case report opens the door to expand the number of tumor pairs to be examined. The analysis of more samples will better assess the clinical impact of the detected variants. It could also reveal more non-silent variants and unravel how these variants are involved in cancer evolution mechanisms and in the clinical outcome of SqCC patients. To this respect, TracerX is a large project aiming at analyzing tumoral heterogeneity in 850 NSCLC patients from several subtypes (http://www.cruklungcentre.org/Research/TRACERx). One of the challenges faced in oncology is that the genotype does not correlate with the phenotype, for some patients. Consequently, this could result in significant inter-patient variability of drugs response, even for patients harboring the same molecular alteration. The molecular analysis of this report is restricted to SNVs and indels. For future study, it is important to expand the genetic analysis to other alterations and to assess their correlation with gene expression and protein levels.

In this work, we shed light on cell molecular changes happening between the primary and metastasis tumors to the liver of lung SqCC, by targeting, at high sequencing depth, 26 well-known cancer-related genes. Our results show in lung SqCC the following: (1) the occurrence of genetic changes between primary tumor and distant metastasis to the liver, (2) the gain of function in the FGFR3 and probably the FGFR2 genes, and (3) the loss of function of CDKN2A. It would be interesting, for a more in-depth study, to investigate the prevalence of these variants in metastatic lung SqCC, in order to reveal the exact role of these variants in tumor evolution during each metastasis step and to assess the efficacy of treatments targeting these variants in the hope of opening opportunities for new efficient therapies for this disease.

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 below: https://www.ncbi.nlm.nih.gov/clinvar?term=SUB7198155%5BSubmitter%20Batch%5D.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR'S NOTE

Our major finding, we determined the genetic changes which occur between primary tumor and distal metastasis site in Lung SqCC.

AUTHOR CONTRIBUTIONS

HM and AO designed and carried out the experiments. HM and RI analyzed the data. HM, VB, RI, and AO contributed to the interpretation of the results. HM wrote the paper with input from

all authors. All authors discussed the results and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: HM and RI are cofounders of the diagnostic device company OncoDiag SAS, Paris, France. HM is also a genetic counselor at the Al-Azhar Oncology Center, Rabat, Morocco.

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CASE REPORT
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Real-World Treatment Patterns and Survival Outcome in Advanced Anaplastic Lymphoma Kinase (ALK) Rearranged Non-Small-Cell Lung Cancer Patients

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Introduction: Survival of ALK-rearranged NSCLC patients has dramatically improved by the use of multiple ALK-tyrosine kinase inhibitors (ALK-TKI). However, still little is known about the impact of drug sequencing and clinical features on survival in a real-world setting.

Methods: Patients with stage IV ALK-rearranged NSCLC treated at six centers in Switzerland and Italy were identified and standard clinical variables collected. OS curves were constructed using the Kaplan–Meier method and compared with the log-rank test. Multivariate Cox proportional hazard analysis was applied to determine the correlations between clinical features and OS. In four patients, biopsies were subjected to NGS.

Results: One-hundred and twenty-one patients with stage IV ALK-rearranged NSCLC diagnosed between 2011 and 2016 were included. With a median follow-up time of 39.5 months, the median OS from diagnosis of stage IV disease was 48.0 months. First-line treatment consisted of an ALK-TKI in 24% of patients, with crizotinib in 83% of them. Chemotherapy as first-line treatment did not influence OS ($p = 0.955$). The use of more than one ALK-TKI line positively correlated with OS ($p = 0.016$), as well as the use of alectinib or lorlatinib in any treatment line, as compared to the use of crizotinib ± ceritinib ($p = 0.022$). A never smoking history was an independent prognostic factor for OS ($p = 0.032$). Moreover, treatment with alectinib significantly improved OS.

Conclusions: Targeted treatment for ALK-positive NSCLC patients lead to prolonged OS. Smoking status was a negative independent prognostic factor in a multi-variate analysis. The use of alectinib or lorlatinib in any treatment line improved overall outcome.

Keywords: real world data, NSCLC, targeted therapy, overall survival, ALK-rearranged non-small cell lung cancer

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INTRODUCTION

Over the past years, several key improvements in the treatment of non-small cell lung cancer (NSCLC) have been made. In particular, the identification of genetic alterations responsible for initiation and maintenance of the malignant phenotype in several cancers including NSCLC has led to the development of targeted therapies. These new molecules specifically target the so-called oncogenic driver mutation and have improved patient outcomes, in terms of overall survival (OS), but also in terms of quality of life (QoL). Among different oncogenic driver mutations known in NSCLC, rearrangements of the *ALK* (anaplastic lymphoma kinase) gene have been a particularly successful target in terms of the development of new targeted therapies, achieving important improvements in survival (1–6). *ALK* gene rearrangements are present in about 5% of NSCLC patients (7). They were first identified in a resected adenocarcinoma specimen from a 62-year-old male smoker, and can involve different fusion partners presenting as either inversions or translocations (8, 9). Inversions on the short arm of chromosome 2, which juxtapose echinoderm microtubule-associated protein-like 4 (*EML4*) with *ALK* and produce EML4-ALK-fusion proteins are the most commonly observed rearrangement, but at least 27 fusion variants have been identified so far (2, 10).

Identification of an *ALK* rearrangement is of therapeutic relevance at the time of initiation of first-line treatment, as it confers sensitivity to ALK tyrosine kinase inhibitors (ALK-TKIs). Importantly, the rare occurrence of KRAS co-mutations leads to primary resistance to ALK-TKIs (11). These targeted therapies have resulted in major clinical advances over the last decade, with superior objective response rates (ORRs) and progression-free survival (PFS) compared to conventional chemotherapy in *ALK* rearranged patients, initially shown for the first generation ALK-TKI crizotinib (4, 12). Despite the initial efficacy of crizotinib, patients progress after a median time of about 12 months, with the brain being the most frequent site of progression or relapse (13). This is due to the development of resistance mechanisms, the most frequent one is the acquisition of point mutations in the rearranged *ALK* gene. Classically, second-generation ALK inhibitors such as alectinib or ceritinib have emerged as standard of care in crizotinib-resistant patients (14–17). Later, third-generation inhibitors such as lorlatinib or the second-generation inhibitor brigatinib were shown to overcome resistance to first and even second-generation ALK-TKIs (18–21). However, each ALK-TKI has a different spectrum of sensitivity to ALK-resistance mutations (22). In the light of these findings, identifying the various resistance mechanisms is becoming more important to select further treatments and overcome acquired resistance (2).

Here, we report the results of the transalpine ALK registry, a collaborative real-world study performed in five institutions in Switzerland and one in Italy. The goal was to collect outcome data of patients with ALK-rearranged NSCLC, with a particular focus on overall survival (OS) by the use of ALK-TKIs and their sequencing.

All the clinical ALK-rearranged NSCLC patients' data were retrospectively collected and analyzed. If available, biopsies both before starting ALK-TKI and at progression were subjected to next-generation sequencing (NGS) to further explore resistance mechanisms. All this study was approved by the local ethics committee (EK-ZH-2018-01919) and in accordance with the local laws and regulations.

MATERIALS AND METHODS

Study Design

This is a retrospective study study performed in collaboration among Swiss and Italian cancer centers. Data on patients with metastatic *ALK*-rearranged NSCLC on treatment with an ALK-TKI were collected by each participating center and assembled in a central database.

Patients Characteristics

Several demographic and background clinical characteristics were documented for each patient, such as age, gender, smoking status, histology, patterns of metastases, co-mutations, and treatments received in any line. Since the ALK-TKIs were utilized in different lines of systemic treatment, the OS was calculated from the date of diagnosis to the date of death or last time the patient was seen. Patients still alive at the time of data collection were censored at the date of the last available medical record. Several other clinical endpoints were also assessed: objective response rate (ORR) was defined as the proportion of patients achieving either a complete or a partial remission as best clinical response to an ALK-TKI according to the local radiologists' interpretation based on the response Evaluation Criteria for Solid Tumors (RECIST) 1.1 or clinically, when indicated. The ORR rate, as well as the progression-free survival, were not reported for the overall population due to the different treatment lines, in which ALK-TKI were administered.

The inclusion criteria were as follows: diagnosed with metastatic NSCLC, confirmation of an *ALK* gene rearrangement by standard diagnostic procedures used in the respective institution, as recorded in the patient's medical record, 18 years of age or older at diagnosis of *ALK*-rearranged NSCLC. If a biopsy at diagnosis and at progression was available, those samples were subjected to NGS.

Statistical Analyses

Descriptive statistics were used to summarize patients' demographic and treatment characteristics at diagnosis and subsequent recurrence or progression. All analyses were conducted using SigmaPlot statistical software (Version 12.5; San Jose, CA, USA). OS was analyzed using the Kaplan–Meier method and was reported as median with confidence limits (95% confidence intervals, CI), with statistical significance of survival differences assessed using a non-parametric log-rank test. In order to study any possible influence of main prognostic factors on the OS, multivariate analysis was performed according to the Cox proportional hazards survival model considering statistically significant $p < 0.05$. All statistical analyses were performed using two-sided tests. All data were analyzed for the pooled study sample comprising patients from Italy and Switzerland combined.

Molecular Analysis

In four patients, pre- and post-treatment biopsies were subjected to NGS using the Ion AmpliSeq Comprehensive Cancer Panel (CCP), or the Thermo Fisher Oncomine Focus Assay Panel (OFA). NGS was performed centrally at one of the sites (University Hospital Zurich). The variant calling was done using Ion Reporter (Thermo Fisher Scientific). Only variants predicted to be damaging by SIFT and PolyPhen were included. Additionally, the variant frequency cut-off was adjusted to the estimated tumor cell content in each specimen.

RESULTS

Patient Characteristics

Between January 2011 and June 2016, a total of 121 patients at six centers were identified. Break-apart fluorescence *in situ* hybridization was used in almost all cases to detect *ALK* rearrangement ($n = 119$). In the remaining two cases, ALK immunohistochemistry was performed. Baseline patients' characteristics are summarized in **Table 1**. Fifty-six were male and 65 female, median age was 52 years old (range 19–81). Histology was adenocarcinoma in 111 cases (92%). The majority of the patients were never smokers (56%, 58 patients, out of 104 assessable patients). Of 121 patients, 98 presented with stage IV disease (98/121; 92%), 37 (37/121; 31%) with brain metastases at the time of diagnosis; additionally, 14 patients (14/84; 17%) newly developed brain metastases under therapy. 115 received first-line treatment with either chemotherapy (ChT) ($n = 86$, 71%), or ALK-TKIs ($n = 29$, 24%). Seventy-five patients (62%) received ALK-TKIs after first-line treatment, 58 (48%) as second-line treatment. Forty-six patients (45%) received more than one ALK-TKI treatment line, including the use of alectinib or lorlatinib in 26 of them (25%). Seventeen patients (14%) have not received a treatment with ALK-TKI at the time of analysis (of these 17 patients, 6 patients started with first line chemo and were either still controlled/even in complete remission at censoring, or were converted to be treated in an oligo-metastatic approach and have never relapsed since then; eight patients have started with first line chemo and have died before ever having had the chance to get a TKI; form three patients only data on survival were available).

TABLE 1 | Characteristics of patients ($n = 121$).

Parameter	*n* (%)	
	Patients receiving TKI	Patients without TKI
	104	17
Region of origin		
Italy/Switzerland	42/62 (40/60)	1/16 (6/94)
Gender		
Female/Male	55/49 (53/47)	10/7 (58/42)
Age, median	52 (19–81 year)	61 (36–79)
Tobacco		
Never/former/current smokers	52/16/20 (50/15/19)[a]	6/8 (35/47)
Unknown	16 (15)	3 (18)
Histology		
Adenocarcinoma	95 (91)	16 (94)
Squamous/others	9 (8)	1 (6)
Stage at diagnosis		
I/II/III	3/2/15 (3/2/14)	0/2/1 (0/11/6)
IV	84 (81)	14 (82)
≥3 involved organs	46 (44)	5 (29)
BMs	33 (33)[a]	4 (23)
BMs treated with RT	24 (72)[b]	4 (100)
BMs new on treatment	13 (19)[c]	1 (8)
1st line CHT	75 (72)	11 (65)
1st line TKI	29 (24)	
Crizotinib/alectinib/ceritinib	24/3/2 (83/10/7)	
>1st line TKI	75 (62)	
2nd/3rd/4th/5th/6th/8th line	58/9/3/2/2/1 (48/7/2/2/2/1)	
One TKI line	57 (55)[d]	
>One TKI line	46 (45)[d]	
Use of alectinib/lorlatinib	26 (25)[e]	
Use of crizotinib ± ceritinib	78 (75)[e]	
No use of TKI	17 (14)	

[a] *Of 103 assessable patients.*
[b] *Of patients with BMs.*
[c] *Of patients without BMs at diagnosis.*
[d] *Of assessable patients.*
[e] *Of 103 assessable patients.*
BMs, brain metastases; CHT, chemotherapy; CI, confidence interval; OS, overall survival; RT, radiotherapy; TKI, tyrosine kinase inhibitor.

Outcome

With a median follow-up of 39.5 months (95% confidence interval [CI]: 32.1–77.7), median OS was 48.0 months (95% CI: 12.9–83.0). A non-significant trend in OS was observed between patients with or without brain metastases at diagnosis (median OS 34.8 vs. 72.4 months, respectively, $p = 0.323$), with no significant difference in OS between those treated or not with brain radiotherapy (median OS not reached vs. 24.6 months, respectively, $p = 0.567$). No significant difference in OS was observed between patients treated with ALK-TKIs or ChT as first-line treatment ($p = 0.955$). A significant difference in OS was observed in favor of patients treated with more than one treatment line of ALK-TKIs as compared to those treated only with one line of ALK-TKI (median OS of 85.7 vs. 34.8 months, respectively, $p = 0.016$) and whose ALK-TKIs included alectinib or lorlatinib (median OS of 85.7 vs. 37.3 months, respectively, $p = 0.022$; see **Table 2** and **Figure 1**). In multivariate analysis, never smoker status was the only independent prognostic factor associated with better OS (Hazard Ratio [HR] 0.499, 95% CI: 0.265–0.941, $p = 0.032$). A non-significant trend toward a better prognosis was observed for adenocarcinoma histology (HR 0.418, 95% CI: 0.175–1.002, $p = 0.051$; see **Table 3**).

Analysis of Resistance to ALK-TKIs

Case #1 was a male with a history of cisplatin/pemetrexed as a first-line therapy, with disease stabilization for 6 months. Upon progression, he was started on crizotinib with a partial remission (PR) as best response, which lasted for 7 months. He was first

TABLE 2 | Patients' outcome ($n = 121$).

Parameter	Median, months	95% CI	Log-rank *p*-value
Follow-up	39.5	32.1–77.7	
OS	48.0	12.9–83.0	
OS no BMs	72.4	33.0–111.7	0.323
OS BMs	34.8	NA	
Brain RT	NR	NA	0.567[a]
No brain RT	24.6	20.2–29.0	
1st line TKI	35.8	16.9–54.7	0.526
>1st line TKI	72.4	29.3–115.4	
1st line TKI	35.8	NA	0.955
1st line CHT	48.0	11.8–84.1	
One TKI line	34.8	21.6–48,0	0.016
>One TKI line	85.7	63.9–107.5	
Use of alectinib/lorlatinib	85.7	64.0–107.5	0.022
Use of crizotinib ± ceritinib	37.3	16.1–58.6	

[a] *On 37 patients with BM only.*

BMs, brain metastases; CHT, chemotherapy; CI, confidence interval; NA, not assessable; NR, not reached; OS, overall survival; RT, radiotherapy; TKI, tyrosine kinase inhibitor.

oligo-progressing and received a pulmonary lobectomy of the left lower lobe with continuation of crizotinib, but was switched to docetaxel three months later upon systemic progression. After two months he was progressing and switched to ceritinib for 5 months, but was then progressing in the brain. After that, alectinib was initiated, which achieved a PR for almost 1.5 years. He then progressed in the liver and a hepatic lesion was biopsied. This biopsy and the lobectomy specimen were subjected to NGS using the CCP and an ALK p.Ile1171Ser point mutation was detected in the liver biopsy, which was absent before, most likely explaining the resistance to alectinib (**Figure 2**).

Case #2 was a female patient who was diagnosed with stage IIIA adenocarcinoma of the lung. She was operated upfront and received an adjuvant chemotherapy with four cycles of cisplatin/pemetrexed, but relapsed 7 months later with metastases in retroclavicular lymph nodes. At that point, an ALK-translocation was diagnosed and she was started on crizotinib. Upon progression, she received cisplatin/pemetrexed again, later a re-challenge, followed by docetaxel and then ceritinib. Upon progression in the brain and liver she was started on lorlatinib. After an initial response she was progressing in the liver and in the bones. An NGS analysis performed on a biopsy of a progressing hepatic lesion using the OFA panel at that stage showed expression of the EGFR variant III with deletion of exons 2–7 (EGFRvIII, **Figure 2**). This EGFR variant has been discussed to be potentially immunogenic and was assessed in clinical trials in glioblastoma (23). The patient was started on nivolumab and responded for 6 months.

Case #3 was a male patient with a stage IV (bone) adenocarcinoma of the lung. After an initial course of palliative cisplatin/pemetrexed he was started on crizotinib and was responding for 14 months. He then presented with oligo-progressive disease (oligo-PD) in two locations (bone), on which he was irradiated while continuing crizotinib. However, 2 months later he was progressing in the primary tumor, which was biopsied. The initial diagnostic specimen and this re-biopsy were subjected to NGS using the CCP, which showed emergence of a BCL9 p.Arg1401_Pro1402delinsThrAla mutation (**Figure 2**). The primary tumor was then irradiated and the patient continued on crizotinib for more than 1.5 years until he deceased suddenly at home.

Case #4 was a female patient with stage IV (malignant pleural effusion) adenocarcinoma of the lung. She received one cycle of palliative cisplatin/pemetrexed, but due to side effects and her reduced general condition was switched to crizotinib shortly thereafter. She was progressing 7 months later, at which timepoint a broncho-alveolar lavage (BAL) was performed. Cells from this BAL and cells from the initial malignant pleural effusion were subjected to NGS using the CCP, which showed a newly emergent LPP p.Arg415Cys mutation. The patient was then started on ceritinib with a partial remission as best response, which was maintained for 6 months. She then deteriorated rapidly and eventually died due to a pulmonary infection and pulmonary PD.

DISCUSSION

Patients with *ALK*-fusion positive stage IV NSCLC represent a subgroup with favorable OS compared to patients with NSCLC and no actionable molecular alterations. Real-life data allow for evaluating the impact of treatment sequences, as well as giving detailed information about clinical features, which can be associated with patients outcome. Here we present the analysis of 121 *ALK*-fusion-positive patients treated in several institutions with comprehensive data and access to ALK-TKI through early access programs and/or clinical trials with ALK-TKI. In this cohort of ALK positive patients, the median OS was 48 months. This reflects the impressive efforts made in the development and access to ALK-TKIs when compared to NSCLC patients without actionable molecular alterations. Since the very first results with crizotinib in late and first-line, the treatment of these patients has dramatically changed (2). However, OS survival from the PROFILE-1014 differs based on the access to further ALK-TKI after crizotinib, with a 5 year survival rate of 75 vs. 28% in patients who received subsequent ALK-TKI or not, respectively (4, 24). Indeed, also in our cohort of patients, the major impact on survival was given by the access to subsequent ALK-TKIs with patients receiving subsequent ALK-TKI having a median OS of 85.7 vs. 34.8 months if they did not. These data closely resemble previous observations by Pacheco et al. (25) reporting a median overall survival of 80 months, where about 80% of the patients received subsequent ALK-TKIs and 89.6 months reported by Duruisseaux in patients receiving next-generation ALK-TKIs compared to 28.2 months when they did not (26). On the other hand a median OS of 49 months was reported by Gainor, as 70% received only ceritinib as second line ALK-TKI (17). Accessibility to drugs and performance status might play a role in the patients who underwent subsequent treatment lines, which is difficult to discriminate retrospectively.

We could not evaluate the impact of alectinib given in first vs. further lines of therapy due to the small number of

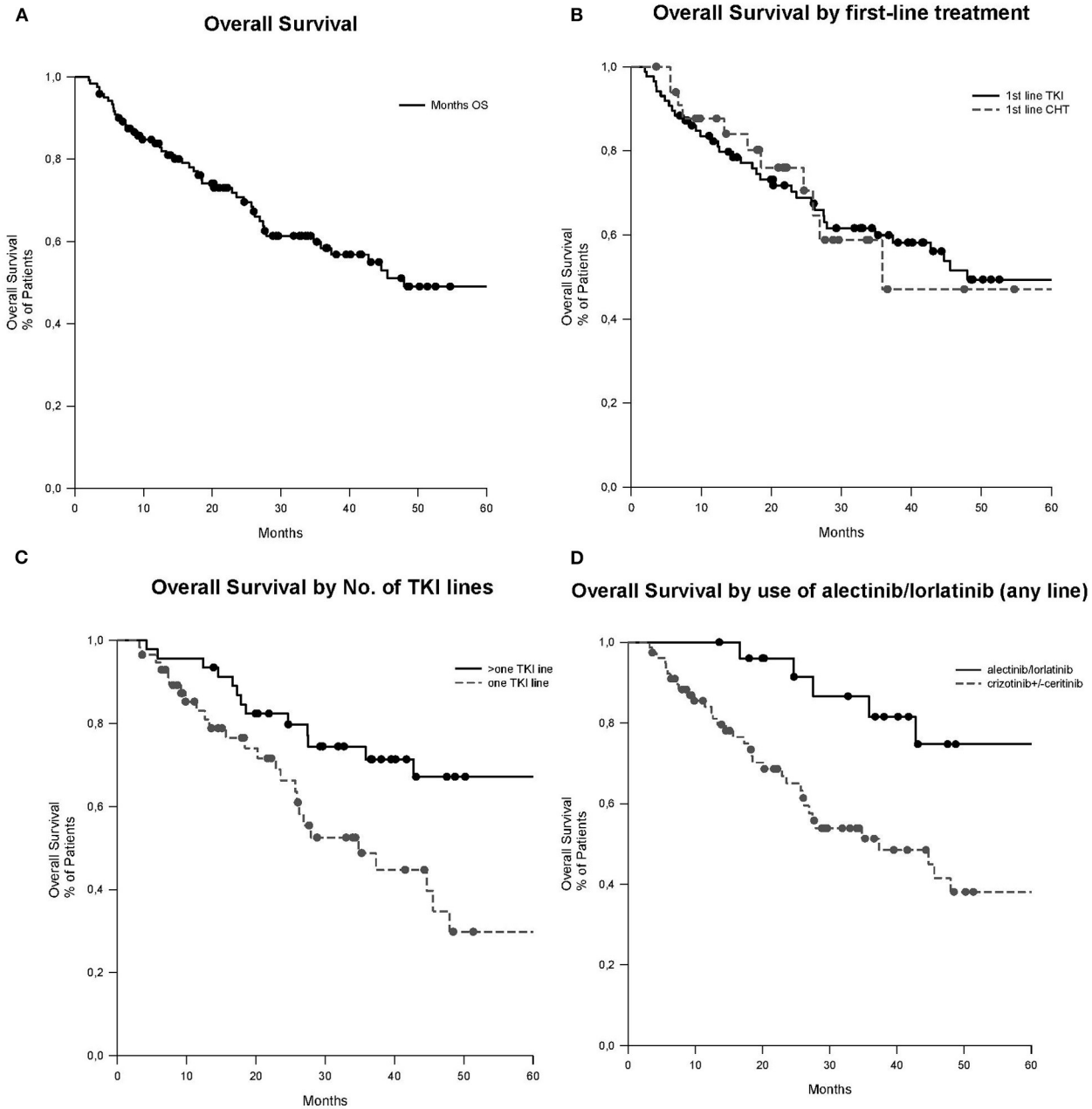

FIGURE 1 | OS curves (median follow-up of 39.5 months, 95% CI: 32.1–77.7). **(A)** OS of all patients, median 48.0 months (95% CI: 12.9–83.0). **(B)** OS according to the first-line of treatment, first-line TKI median 35.8 months (95% CI NA) vs. first-line ChT 48.0 months (95% CI: 11.8–84.1) ($p = 0.955$). **(C)** OS according to the number of lines of TKIs, >one line of TKIs median 85.7 months (95% CI: 63.9–107.5) vs. one line of TKI 34.8 months (95% CI: 21.6–48.0; $p = 0.016$). **(D)** OS according to the use of alectinib or lorlatinib in any line. Alectinib/lorlatinib median 85.7 months (95% CI: 64.0–107.5) vs. crizotinib ± ceritinib 37.3 months (95% CI: 16.1–58.6; $p = 0.022$). ChT, chemotherapy; CI, confidence interval; NA, not assessable; No., number; OS, overall survival; TKI, tyrosine kinase inhibitor.

patients receiving it in first-line ($n = 3$). In order to identify possible clinical features with impact on patients survival, we first evaluated the localization pattern and number of metastases at time of diagnosis. Clinical characteristics of this patient cohort did not differ from the ones reported in clinical trials, e.g., brain metastases were present in 30% of patients at time of diagnosis. It has been previously reported that liver and multiple-organ metastases might have a negative prognostic impact in patients with advanced adenocarcinoma of the lung (27). In our *ALK*-rearranged NSCLC cohort, however, the number or localizations of metastases did not have any impact on survival. In line with previous reports, the presence of brain metastases in patients with

TABLE 3 | Cox multivariate analysis on OS.

Parameter	HR	95% CI	*p*-value
Gender (female vs. male)	1.191	0.645-2.199	0.576
Tobacco (never vs. other)	0.499	0.265-0.941	0.032
Histology (adenocarcinoma vs. other)	0.418	0.175-1.002	0.051
Stage (IV vs. other)	2.187	0.860-5.563	0.100
Brain metastases (yes vs. no)	1.172	0.560-2453	0.673
Organ (3 or more vs. less)	1.653	0.795-3.437	0.178

CI, confidence interval; HR, Hazard Ratio; OS, overall survival.

ALK-fusion-positive NSCLC had no impact on patients outcome, and neither did radiotherapy of brain metastases. Only a positive smoking history was a significant prognostic factor for worse survival. About 45% of patients were current or former smokers, which underscores the importance of testing all patients for *ALK* gene rearrangements, irrespective of their smoking status. *ALK* testing was almost exclusively performed by FISH at time of diagnosis, and in four cases the analysis of resistance mechanisms to ALK-TKI was performed using a tissue biopsy. Molecular analysis included NGS, which allowed the detection of *ALK* mutations previously described to be associated with resistance

case	ALK-TKI	NGS results	relevant examples	# new variants post-treatment	# variants in total
1	Alectinib	pre-treatment biopsy / post-treatment biopsy 1 4 1	**ALK p.Ile1171Ser** post-treatment only (resistance to Alectinib)	1	6
2	Lorlatinib	initial biopsy / biopsy at relapse 2 2 2 OFA of biopsy at relapse EML4-ALK	**RAD50 p.Gln426Arg** in both biopsies (hereditary cancer pre-disposing syndrome) **KMT2A p.Gln1644His** in both biopsies (Methyltransferase, involved in cancer) OFA of re-biopsy (hepatic progression) EML4-ALK **EGFRvIII**	2	6
3	Crizotinib	pre-treatment biopsy / post-treatment biopsy 74 2 1	**BCL9 p.Arg1401_ Pro1402delinsThrAla** post-treatment only **NF1 p.Ala2818Thr** in both biopsies	1	77
4	Crizotinib	pre-treatment biopsy / post-treatment biopsy 71 1 9	**LPP p.Arg415Cys** post-treatment only (pathogenic in melanoma) **NOTCH1 p.Gly1088Ser** in both biopsies	9	80

FIGURE 2 | Summary of molecular aberrations in pre- and post-treatment biopsies.

to crizotinib and one patient with expression of an alternative splice variant of EGFR (EGFRvIII). This is the first report of such an alteration as a potential mechanism of resistance to lorlatinib. The EGFRvIII splice variant has been reported in solid tumors and has been reported to generate a highly immunogenic peptide that is currently being studied as target for immunotherapeutic approaches (23). In our patient, based on this result, a treatment with an anti PD-1 antibody (nivolumab) as monotherapy was initiated, leading to a partial response. This is of major interest as *ALK*-fusion-positive patients are usually excluded from clinical trials with immunotherapies due to reported lack of responses (28, 29). This discovery underlines the importance of detecting and understanding new mechanisms of resistance in this population of patients.

Taken together, an analysis of real-world data allowed for understanding the dynamics of tumor evolution in ALK-fusion-positive patients under specific ALK-TKIs. Understanding the resistance mechanisms that evolve during this treatment journey for each patient is key toward the general aim of precision medicine. In particular, access to further lines of ALK-TKIs had a significant impact on survival of a broad population of *ALK*-fusion-positive patients who represent every-day patients seen in clinical practice. The transalpine registry has fostered a collaborative effort between different institutions with the intent to learn more from each single case, to give access to drugs, and to search for resistance mechanisms to ALK-TKI; an issue still under debate with newly discovered implications for patients care.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Zurich Ethical committee, (EK-ZH-2018-091919) and all participants provided informed consent to participate in this study. Written informed consent was obtained from every patient for the publication of any potentially identifiable images or data included in this manuscript.

AUTHOR CONTRIBUTIONS

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

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Prevalence and Clinical Impact of Concomitant Mutations in Anaplastic Lymphoma Kinase Rearrangement Advanced Non-small-Cell Lung Cancer (Guangdong Association of Thoracic Oncology Study 1055)

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Background: In patients with anaplastic lymphoma kinase *(ALK)* rearrangement-positive advanced non–small-cell lung cancer (NSCLC), *ALK* inhibitors are now the standard treatment, but their clinical efficacy varies widely for each patient. In this multicenter retrospective study, we evaluated the clinical efficacy of crizotinib according to the *ALK* rearrangement variants and concomitant mutations present.

Patients and Methods: A total 132 patients with *ALK* rearrangement advanced NSCLC from 4 centers in Guangdong province, China were evaluated. All patients received crizotinib treatment and their *ALK* rearrangement status was identified by next-generation sequencing (NGS).

Results: The median progression-free survival (PFS) in patients with EML4-*ALK* rearrangement ($n = 121$), non-EML4-*ALK* rearrangement ($n = 5$), and EML4-*ALK* arrangement accompanied by non-EML4-*ALK* rearrangement ($n = 6$) was 12.8, 7.5, and 7.4 months, respectively, with no significant difference between them ($p = 0.1554$). Similarly, among patients with various EML4-*ALK* variants (variant 1, variant 3a/b, and other variants), the median PFS values were again comparable. According to baseline NGS data, the median PFS in patients who had *ALK* rearrangement only, *ALK* rearrangement and concomitant tumor-suppressor gene mutations, and *ALK* rearrangement and concomitant oncogene mutations was 14.2, 10.9, and 4.9 months, respectively; ($p = 0.0002$). A multivariable analysis indicated that concomitant oncogene mutations and tumor-suppressor gene mutations were both negative factors influencing the efficacy of crizotinib in *ALK* rearrangement NSCLC.

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Conclusion: Concomitant oncogene mutations and tumor-suppressor gene mutations had negative effects on the efficacy of crizotinib, while various *ALK* variants had a similar influence.

Keywords: *ALK* rearrangement, non–small-cell lung cancer, concomitant mutations, crizotinib, next-generation sequencing

INTRODUCTION

Lung cancer remains the leading cause of cancer deaths in China. In patients with non–small-cell lung cancer (NSCLC), anaplastic lymphoma kinase (*ALK*) gene rearrangement is detected in approximately 3–7% of cases (1). In 2007, Soda et al. (2) first identified the echinoderm microtubule-associated protein-like 4 *(EML4)*-*ALK* fusion oncogene in NSCLC. Currently, more than 20 *ALK* rearrangement variants have been discovered, the most frequent among which are variant 1 (E13:A20) and variant 3a/b (E6a/b:A20) (3). All variants contain the *ALK* tyrosine kinase domain and an oligomerization domain in the N-terminal fusion partner gene, which activate downstream pathways to control the proliferation and apoptosis of carcinoma cells. In addition, more non-*EML4* fusion variants have been discovered, including kinesin family member 5B (*KIF5B*) (4), kinesin light-chain 1 (*KLC1*) (5), cut-like homeobox 1 gene (*CUX1*) (6). Huntingtin-interacting protein 1(*H1P1*) (7), translocated promoter region (*TPR*) (8), baculoviral inhibition of apoptosis protein repeat-containing 6 (*BIRC6*) (9), and S1 RNA binding domain 1 (*SRBD1*) (10). These variants have all shown clinical responses to *ALK* inhibitors.

Since the first-generation *ALK* tyrosine kinase inhibitor (TKI) crizotinib (11, 12) was introduced, the development of targeted therapy has greatly improved the survival time and quality-of- life of patients with *ALK* rearrangement advanced NSCLC. In addition, second- and third-generation *ALK* TKIs, including ceritinib (13), alectinib (14), brigatinib (15), and lorlatinib (16), have also shown significant efficacy in these patients. However, despite their efficacy in patients with *ALK* rearrangement, all patients inevitably develop resistance to treatment and clinical efficacy varies widely for each patient. To date, a series of studies have investigated whether different *ALK* variants may affect the clinical response in patients who receive *ALK* inhibitors, and whether they are associated with resistance mechanisms. Lin et al. (17) have previously reported that *ALK* G1202R is significantly more common with variant 3 than variant 1 (57 vs. 30%; $p = 0.023$).

With the rapid development of next-generation sequencing (NGS), more and more *ALK* concomitant genes have been found. Epidermal growth factor receptor (*EGFR*) mutations are the most common mutations in NSCLC, there have been a series of studies showing that concomitant mutations are associated with inferior efficacy of *EGFR* TKI therapy (18, 19). In *ALK* rearrangement advanced NSCLC, it is still unclear whether concomitant mutations are negative predictive factors for *ALK* TKI therapy. Some retrospective studies and case reports have reported the poor efficacy of crizotinib treatment for *ALK* rearrangement NSCLC co-occurring with *TP53, KRAS* and *EGFR* mutations (20, 21). Therefore, we performed a retrospective multicenter study to explore the factors affecting the efficacy of crizotinib according to baseline next-generation sequencing data in patients with *ALK* rearrangement-positive advanced NSCLC.

MATERIALS AND METHODS

Patients

Between January 2012 and June 2019, a total of 132 patients with *ALK* rearrangement advanced NSCLC from 4 medical centers across Guangdong province, China were evaluated. All patients had been histologically diagnosed with NSCLC, and with clinical stage IIIB, IV or recurrent disease according to the 7th American Joint Committee on Cancer (AJCC) staging system. The *ALK* rearrangement status was identified by next-generation sequencing. Clinicopathologic parameters including age, sex, histological type, clinical stage, ECOG performance status, smoking history, and gene status were collected prior to administering crizotinib therapy. The treatment progression-free survival (PFS) was defined as the time from initiation of crizotinib to the date of radiographically-confirmed progressive disease (PD) or death, whichever occurred first. The objective response rate (ORR) was defined as the percentage of patients with a complete response (CR) or partial response (PR), and the disease control rate (DCR) was defined as the percentage of patients with CR, PR, or stable disease (SD). The patients' clinical response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1.

This study was approved by Guangdong Association of Thoracic Oncology (GASTO ID:1055). All patients signed informed consent to participate in the study.

Gene Analysis

All patient samples were identified as *ALK* rearrangement by next-generation sequencing. NGS-detected samples included formalin-fixed, paraffin-embedded (FFPE) tumor tissues ($n = 100$), malignant plural effusions ($n = 10$), or plasma ($n = 22$). Genomic DNA was extracted from FFPE samples, malignant plural effusions, or plasma samples, sheared into fragments and then subjected to end-repairing, A-tailing, and ligation with indexed adapters sequentially, followed by size selection using beads. Finally, libraries were amplified by PCR and purified for target enrichment. Libraries were sequenced on Illumina Hiseq platforms (425-gene panel or 1021-gene panel) and the Illumina NovaSeq6000 platform (543-gene panel). The sequencing depth was at least 500X mean coverage, and NGS detected genomic alterations included single-nucleotide variation (SNV), insertion/deletions (Indel), copy number variation (CNV), and gene rearrangement.

Statistical Analysis

The patients' baseline characteristics, concomitant mutations, and *ALK* variants were compared using χ^2 or Fisher's exact test. PFS curves were estimated using the Kaplan-Meier method. Differences between ALK rearrangement variants and concomitant mutations were calculated with the log-rank test. Variables with $p < 0.2$ in the univariate Cox regression analysis were included in the multivariate Cox proportional hazards regression model to identify independent risk factors, which were expressed as hazards ratios (HR) with 95% confidence intervals (CI). All statistical analyses were performed using SAS™ 9.4 software, and R software (version 3.6.3). The statistical significance level was defined as a two-sides $p < 0.05$.

RESULTS

Baseline Characteristics of the Patients

The baseline characteristics of the 132 patients with *ALK* rearranged NSCLC that were evaluated in this study are shown in **Table 1**. The patients' median age was 51 years (range 26–82 years), 55.3% were female, and 87.9% had adenocarcinoma. All patients received crizotinib therapy, of whom 95 patients (72.0%) received it as first-line treatment while 37 patients (28.0%) received it as second- or further-line treatment. In terms of clinical stage, 10 patients (7.6%) had stage IIIB disease, while 109 (82.6%) and 13 (9.8%) had stage IV or recurrent disease, respectively. Thirty-one patients (23.5%) had only lung or pleural metastasis (M1a). The most common distant metastatic site was bone (35.6% of patients), followed by brain (30.3%) and liver metastases (19.7%). At the end of the study, 71 patients (53.8%) had confirmed progressive disease (PD) or had died. Overall survival (OS) data are not yet mature.

TABLE 1 | Baseline characteristics of the patients ($n = 132$).

Characteristics	No. of patients (%)
Median age, years (range)	51 (26–82)
Sex	
Male	59 (44.7)
Female	73 (55.3)
Histological type	
Adenocarcinoma	116 (87.9)
Non-adenocarcinama	16 (12.1)
Smoking history	
Never	104 (78.8)
Current/former	28 (21.2)
Stage at initiation of crizotinib	
IIIB	10 (7.6)
IV	109 (82.6)
Recurrent	13 (9.8)
EGOG PS	
0–1	122 (92.4)
≥2	10 (7.6)
Distant metastases	
CNS	40 (30.3)
Liver	26 (19.7)
Bone	47 (35.6)
Clinical type	
Central	41 (31.1)
Peripheral	91 (68.9)
Line of crizotinib treatment	
First	95 (72.0)
≥ Second	37 (28.0)

Data are median values (range) or number of patients (%).
CNS, central nervous system; ECOG PS, Eastern Cooperative Oncology Group performance status.

ALK Rearrangement Variants and Clinical Efficacy of Crizotinib

Among the 132 patients, 121 had *EML4-ALK* rearrangement, 5 patients had rare non-*EML4-ALK* rearrangement, including Lintergenic-*ALK*, KIF5B-*ALK*, ACTR3BP5-*ALK*, STRN-*ALK* and KLC1-*ALK* (one patient each), and 6 patients had *EML4-ALK* rearrangement accompanied by non-*EML4-ALK* rearrangement (detailed information on the non-*EML4-ALK* rearrangement and *EML4-ALK* rearrangement accompanied by non-*EML4-ALK* rearrangement variants and their best responses to crizotinib are shown in **Supplementary Table 1** and **Supplementary Figure 1**). In terms of *EML4-ALK* rearrangement, the most common variant was variant 1 (E13:A20), which accounted for 37.1% of patients (49/132), followed by variant 3a/b (E6:A20) and variant 2 (E20:A20), which accounted for 30.3% (40/132) and 11.4% of patients (15/132), respectively (**Figure 1A**). The distant metastatic sites showed no significant correlation with the *ALK* variant type (**Supplementary Table 2**).

When comparing the efficacy of crizotinib, we considered two approaches. Firstly, we categorized patents into three subgroups: those with *EML4-ALK* rearrangement, non-*EML4-ALK* rearrangement, and *EML4-ALK* rearrangement accompanied by non-*EML4*-ALK rearrangement. The median PFS for patients with *EML4-ALK* rearrangement was 12.8 months (95% CI 11.2–16.8); for patients with non-*EML4-ALK* rearrangement the median PFS was 7.5 months (95% CI 1.0-NE), and for *EML4-ALK* rearrangement accompanied by non-*EML4-ALK* rearrangement, it was 7.4 months (95% CI 3.8–16.0), with no significant difference between them ($P = 0.1554$) (**Figure 2A**). The ORR in the three subgroups was 54.5, 60.0, and 66.7%, respectively, again with no significant differences between them (**Table 2**).

Secondly, according to the *EML4-ALK* rearrangement, we divided patients into variant 1, variant 3a/b, and other variant groups. The baseline characteristics of these three groups were well-balanced (**Supplementary Table 2**). The median PFS was similar in the three groups. In the variant 1 group the median PFS was 12.2 months (95% CI 9.2–23.5); in the variant 3a/b group, it was 12.3 months (95% CI 7.5–14.2); and in the group with other variants, it was 16.0 months (95% CI 8.0–19.4) ($P = 0.2597$) (**Figure 2B**). Similarly, no correlation was observed between *EML4-ALK* variants and the ORR with crizotinib treatment (**Table 2**). Similar results were observed in subgroups with baseline CNS metastases (**Supplementary Figure 2A**).

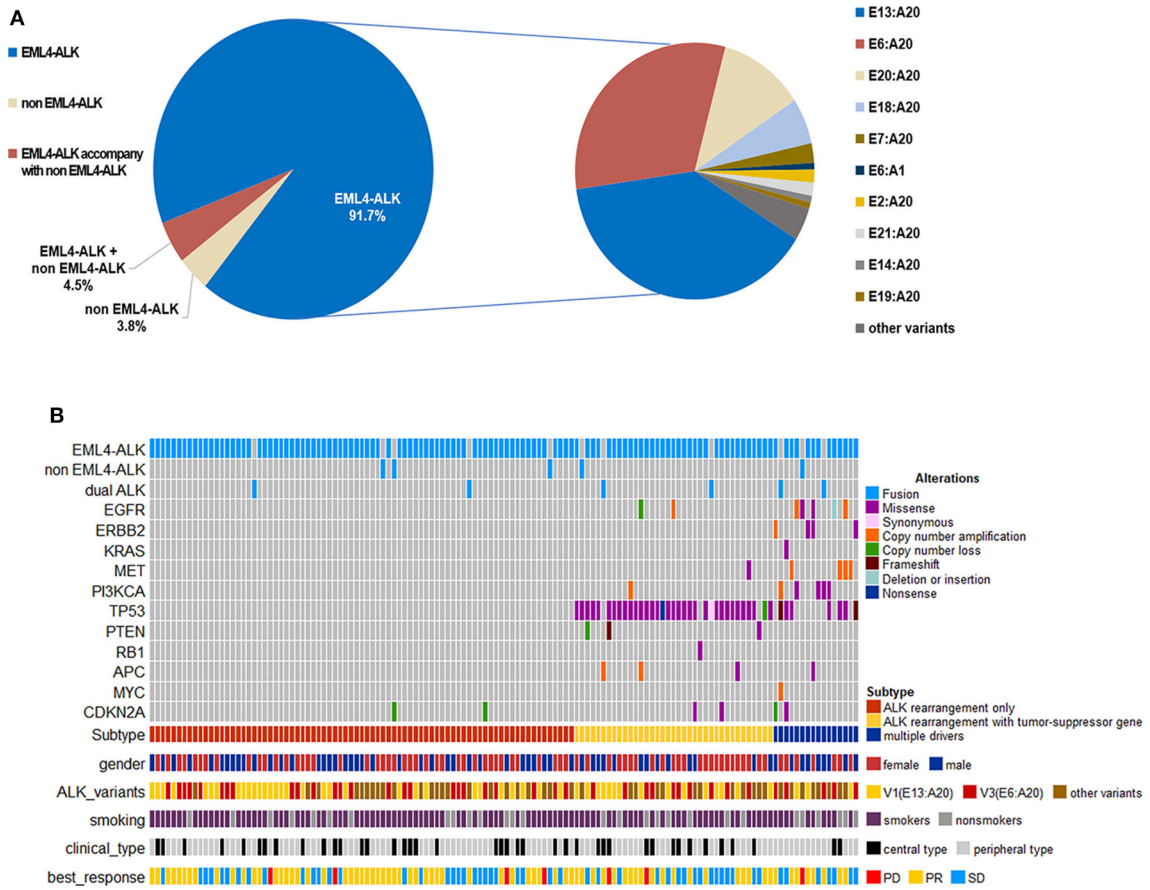


FIGURE 1 | (A) Frequency of *ALK* variants in the study cohort ($n = 132$). **(B)** Distribution of concomitant mutations stratified by subgroups according to baseline NGS sequencing and their clinical features.

Prevalence and Clinical Impact of Concomitant Mutations

Among the 132 patients, 12.1% (16/132) patients had concomitant oncogene mutations (*EGFR, ERBB2, KRAS, BRAF, MET, RET, ROS1, or PIK3CA*), including 3 (2.3%) patients with *EGFR* mutations, 4 (3.0%) with *ERBB2* mutations, 1 (0.76%) with *KRAS* mutations, 4 (3.0%) with *MET* amplification, and 4 (3.0%) with *PI3KCA* mutations. *BRAF, RET,* and *ROS1* mutations were not found because of the limited sample size. In addition, we found that 32.6% of patients (43/132) had tumor-suppressor gene mutations (*TP53, PTEN, APC*, or *RB1*), the most common of which was *TP53* mutation (39/132) (**Figure 1B**). There was no significant correlation between *ALK* rearrangement variants and concomitant mutations (**Supplementary Table 2**). However, concomitant mutations were significantly associated with poor efficacy of crizotinib. In patients with and without oncogene mutations, median PFS values were 4.9 months (95% CI 3.3–8.6) and 12.9 months (95% CI 11.9–16.8), respectively (HR 2.825; 95% CI 1.559–5.118; $P = 0.0003$) (**Figure 2C**). In patients with and without tumor-suppressor gene mutations, median PFS values were 7.9 months (95% CI 5.2–13.1) and 14.2 months (95% CI 11.9–17.6), respectively (HR 2.094; 95% CI 1.270–3.453; $P = 0.0031$) (**Figure 2D**). Similarly, in patients with baseline CNS metastases, concomitant mutations also had a negative effect on the clinical efficacy of crizotinib (**Supplementary Figures 2B,C**). No significant differences in the objective response rate (ORR) were observed according to concomitant mutations (**Table 2**).

In the univariate analysis which included age, gender, histological diagnosis, smoking status, ECOG, central nervous system metastases, clinical type, treatment line of crizotinib therapy, clinical stage, oncogenes, tumor-suppressor genes and *ALK* variants, we found that smoking status ($P = 0.048$), treatment line of crizotinib therapy ($P = 0.047$), oncogenes ($P = 0.0003$) and tumor-suppressor genes ($P = 0.0031$) were significantly associated with PFS. *ALK* variants tended to be associated with PFS ($P = 0.254$). When we included these factors in a multivariate Cox regression analysis, concomitant oncogene mutations (HR 2.615 [95% CI 1.398–4.889]; $P = 0.0026$) and tumor-suppressor gene mutations (HR 2.122 [95% CI 1.264–3.564]; $P = 0.0044$) both remained independent negative factors affecting the efficacy of crizotinib for patients with *ALK* rearrangement NSCLC (**Figure 3B**). However, the impacts of crizotinib treatment line and smoking status became less significant in the multivariate analysis.

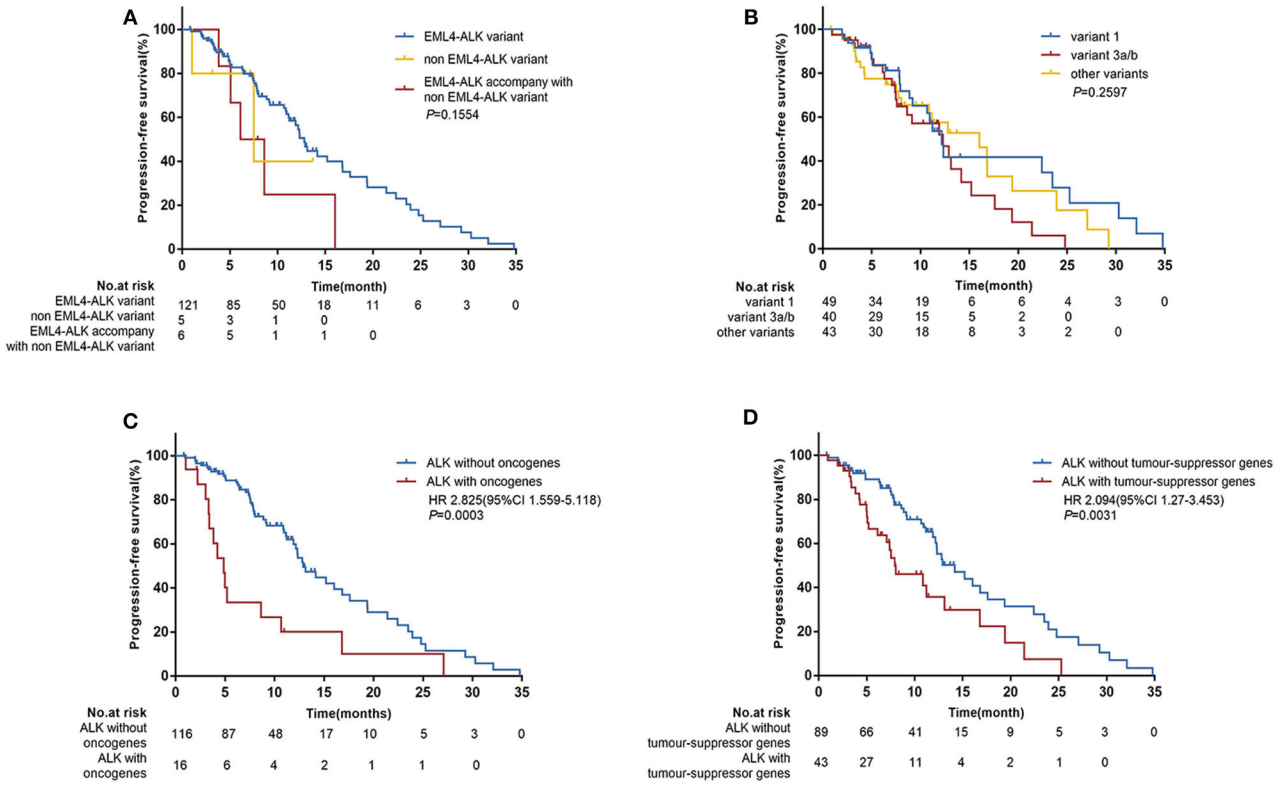


FIGURE 2 | Progression-free survival (PFS) according to baseline next-generation sequencing (NGS) data. **(A)** Patients were categorized into three subgroups: EML4-*ALK* rearrangements ($n = 121$), non-EML4-*ALK* rearrangements ($n = 5$), and EML4-*ALK* rearrangements accompanied by non-EML4-*ALK* rearrangements ($n = 6$). **(B)** Patients with different EML4-*ALK* variants: variant 1 ($n = 49$), variant 3a/b ($n = 40$), and other variants ($n = 43$). **(C)** Patients with oncogene mutations ($n = 16$) vs. patients without oncogene mutations ($n = 116$). **(D)** Patients with tumor-suppressor gene mutations ($n = 43$) vs. patients without tumor-suppressor gene mutations ($n = 89$). HR, hazard ratios; CI, confidence interval; *p*-values were calculated using the log-rank test.

In further analyses, we divided patients into three groups according to their concomitant mutations: patients with *ALK*-rearrangement only ($n = 81$), patients with *ALK* rearrangement and concomitant tumor-suppressor gene mutations ($n = 35$), and patients with *ALK* rearrangement and concomitant oncogene mutations irrespective of tumor-suppressor gene mutations ($n = 16$). The median PFS values in these three groups were 14.2 months (95% CI 12.2–19.4), 10.9 months (95% CI 7.4–19.4), and 4.9 months (95% CI 3.3–8.6), respectively; ($P = 0.0002$) (**Figure 3A**).

Progression Patterns and Resistance Mechanisms to Crizotinib

At the data cut-off time, a total of 71 patients (53.8%) had confirmed progressive disease (PD). Among these patients, 26 (36.6%) had isolated central nervous system (CNS) progression. Patients with CNS metastases at baseline were more likely to have isolated CNS progression compared with patients without CNS metastases at baseline (61.5 vs. 22.2%, respectively; $P < 0.001$). The patients with isolated CNS progression seemed to have inferior PFS values with crizotinib treatment compared with patients with progression at other sites; however, the difference between them was not significant (6.4 months [95% CI 4.9–10.9] vs. 9.2 months [95% CI 7.5–12.3], respectively; $P = 0.5129$) (**Supplementary Figure 3**). There was also no correlation between progression sites and different *ALK* rearrangement variants (**Supplementary Table 2**).

Twenty-five patients underwent repeat biopsies to detect acquired resistance mechanisms to crizotinib. Among these patients, 20 (80.0%) remained *ALK* rearrangement, but in 5 patients *ALK* rearrangement wasn't detected in their tissues. Secondary *ALK* mutations were identified in 8 (32.0%) patients. All secondary *ALK* mutations were detected in patients with *ALK* rearrangement present (**Figure 4C**) but there was no significant correlation for the *ALK* variants (variant3a/b, 25.0% vs. non-variant 3a/b, 35.3%; $P = 0.607$). The median PFS was significantly prolonged in patients with *ALK* rearrangement absent compared with patients with *ALK* rearrangement present (21.4 months [95% CI 6.3–34.8] vs. 10.8 months [95% CI 7.4–16.0]; $P = 0.0453$) (**Figure 4A**). Patients in whom secondary *ALK* mutations were detected showed inferior survival compared with those in whom secondary *ALK* mutations were not detected, although the difference was not statistically significant (PFS, 9.0 months [95% CI 4.9–16.0] vs. 12.9 months [95% CI 7.6–21.4]; $P = 0.1063$) (**Figure 4B**).

TABLE 2 | Clinical responses according to *ALK* variants and concomitant mutations detected.

Variable (No.)	ORR *n* (%)	*p*-value	DCR *n* (%)	*p*-value[a]
EML4-*ALK* (121)	66 (54.5)	0.824	115 (95.0)	0.284
Non-EML4-*ALK* (5)	3 (60.0)		4 (80.0)	
EML4-*ALK* accompanying non-EML4-*ALK* (6)	4 (66.7)		6 (100.0)	
Variant 1 (49)	27 (55.1)	0.875	47 (95.9)	0.822
Variant 3a/b (40)	21 (52.5)		38 (95.0)	
Other variants (43)	25 (58.1)		40 (93.0)	
Oncogenes present (16)	8 (50.0)	0.649	15 (93.8)	0.857
Oncogenes absent (116)	65 (56.0)		110 (94.8)	
Tumor-suppressor genes present (43)	21 (48.8)	0.299	41 (95.3)	0.816
Tumor-suppressor genes absent (89)	52 (58.4)		84 (94.4)	

Clinical responses were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1.

[a]*p-values calculated using* χ^2 *or Fisher's exact test.*

DCR, disease control rate; EML, echinoderm microtubule-associated protein-like 4; ORR, objective response rate.

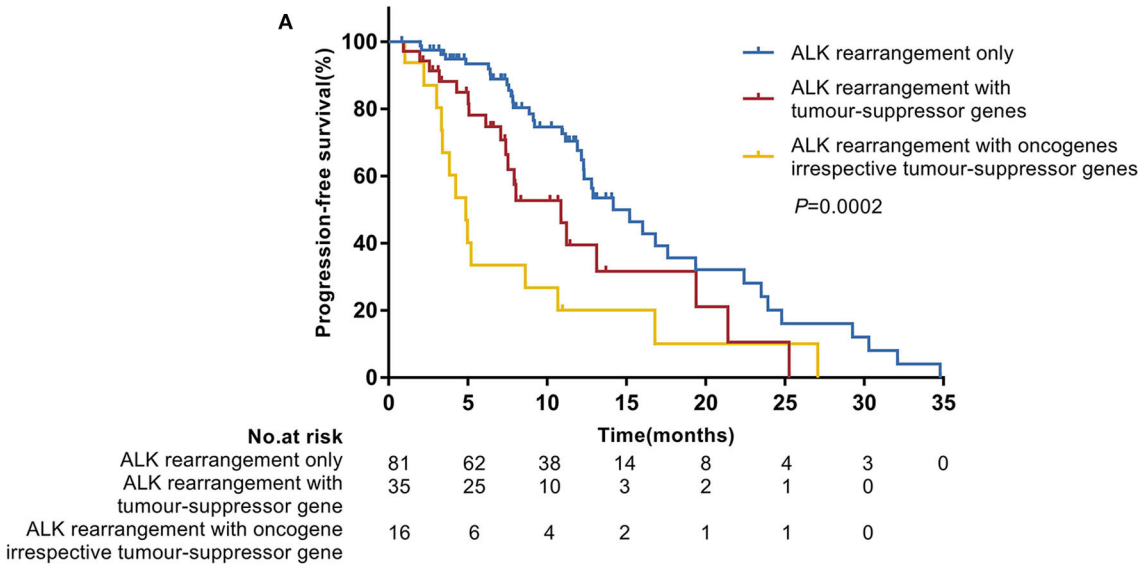
DISCUSSION

To the best of our knowledge, the present study is the first large-sample size study to comprehensively investigate the correlation between concomitant mutations and the efficacy of *ALK* inhibitors according to next-generation sequencing data in patients with *ALK* rearrangement NSCLC. We showed that concomitant mutations, irrespective of oncogenes or tumor-suppressor genes, had a negative effect on the efficacy of crizotinib in patients with *ALK* rearrangement NSCLC. However, patients with different *ALK* variants had similar clinical responses to crizotinib.

In our study, we reported a relatively large data set in which the prevalence of different *ALK* variants was evaluated and we compared the clinical efficacy of crizotinib between the different *ALK* variants. Consistent with previous studies, *EML4* was the most common fusion partner, but we also reported several rare fusion partners and dual fusion partners. When we evaluated clinical responses, patients with the rare fusion variants were found to have a similar median PFS with crizotinib treatment compared with *EML4-ALK* variants. In the case of dual fusion partners, all 6 patients had *EML4-ALK* rearrangement accompanied by a non-*EML4-ALK* rearrangement, and which was the major driver fusion gene was unclear. When we evaluated clinical responses, patients with the rare rearrangement variants or *EML4-ALK* accompanied by a non-*EML4-ALK* rearrangement were found to have a similar median PFS with crizotinib treatment compared with *EML4-ALK* variants. However, the sample size of these rare *ALK* variants was small, which limits conclusive data on the crizotinib sensitivity of rare *ALK* variants. Given the low occurrence rate of *ALK* in lung cancer, multicenter participation and predefined subgroup analysis of these rare *ALK* variants may be worth considering in future studies. In terms of *EML4-ALK* rearrangement, the most common variants were variant 1 (E13:A20), followed by variant 3a/b (E6:A20) and variant 2 (E20:A20), as has been reported in a series of other studies. Although the correlation between *ALK* variants and clinical efficacy has been investigated in several studies, a consensus has not yet been reached. Yoshida et al. (22) reported that variant 1 was associated with superior efficacy to crizotinib than other variant types, and Woo et al. (23) found that variant 3a/b, which has a stable *EML4-ALK* fusion protein, was associated with a significantly shorter PFS with *ALK* inhibitors than other variants. However, Mitiushkina et al. (24) found no difference in the treatment response between various *ALK* variants. Furthermore, in the prospective, phase III ALEX trial, there was a similar survival benefit with crizotinib and alectinib treatment for the different variants (25). In the present study, we found that various *EML4-ALK* variants had similar PFS values and response rates with crizotinib treatment, consistent with previous phase III ALEX study (25). The same results were observed in the subgroup with baseline CNS metastases.

For *EGFR*-mutated NSCLC, a series of studies have investigated the correlation of concomitant mutations and efficacy to *EGFR* TKIs. Hong et al. (19) reported that co-alteration mutations are associated with resistance to *EGFR* TKIs, and *EGFR* 21 L858R had a significantly higher incidence of co-alterations than *EGFR* 19 deletion. A prospective phase II study [the BENEFIT study (18)] also revealed that patients with an *EGFR* mutation only had superior responses to first-generation *EGFR* TKIs than those with oncogenes and tumor-suppressor genes present, or both (18). A similar conclusion was reported for *ROS1* fusion in that concomitant mutations were observed to be frequent in patients with *ROS1* fusion and these concomitant mutations had negative impacts on overall survival (26).

For *ALK* rearrangement NSCLC, several studies have found that *ALK* rearrangements are not absolutely exclusive with other driver mutations. Won et al. (27) reported that 4.4% of patients with *ALK*-positive NSCLC have *EGFR* concomitant mutation using Sanger sequencing, and this rose to 15.4% of patients when using NGS. Ulivi et al. (21) found that 1.6% and 2.5% of patients ($n = 380$) who harbor double *EML4-ALK* and *EGFR* mutations and *EML4-ALK* and *KRAS* mutations have a poor prognosis. Regarding the tumor-suppressor gene, Wang et al. (20) had previously reported that 38.1% of patients (8/22) with *ALK* rearrangement NSCLC had *TP53* mutations, which reduced responsiveness to crizotinib and worsened the prognosis. However, all current studies of *ALK*-positive patients have been small-sample size and didn't use NGS to comprehensively investigate the baseline genetic mutations and the clinical response. In our study, we found concomitant mutations were common in patients with *ALK* rearrangement, and not related to *ALK* variants. Concomitant mutations are heterogeneous and may have different impacts on crizotinib efficacy. It seemed that concomitant oncogene mutations had a worse negative effect than concomitant tumor-suppressor gene mutations (HR 2.615 vs. 2.122, respectively), in multivariable analyses, and both remained poor independent factors for clinical efficacy of crizotinib after adjusting for *ALK* variants and patient characteristics.



B

Baseline risk factors	Univariate analysis HR(95% CI)	Univariate analysis P value	Multi variate analysis HR(95% CI)	Multi variate analysis P value
Age (<65)	1.334(0.692-2.569)	0.390	-	-
Gender	0.990(0.611-1.605)	0.969	-	-
Histological	0.726(0.387-1.359)	0.317	-	-
smoking	1.394(1.003-1.937)	0.048	1.194(0.850-1.677)	0.3069
ECOG	0.827(0.527-1.297)	0.408	-	-
CNS metastase	1.253(0.766-2.050)	0.369	-	-
clinical type	1.042(0.621-1.746)	0.877	-	-
treatment line	0.610 (0.375-0.993)	0.047	0.614(0.375-1.006)	0.0529
stage	0.758 (0.401-1.434)	0.395	-	-
oncogenes	2.825(1.559-5.118)	0.0003	2.615(1.398-4.889)	0.0026
tumour-suppressor genes	2.094(1.270-3.453)	0.0031	2.122(1.264-3.564)	0.0044
variants	1.122(0.921-1.367)	0.254	1.217(0.998-1.485)	0.0524

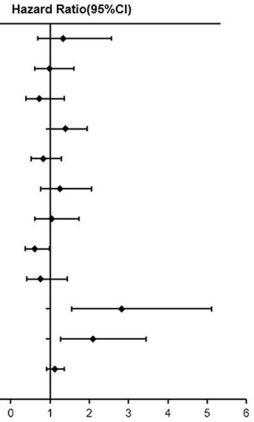


FIGURE 3 | (A) Progression-free survival (PFS) according to concomitant mutations. **(B)** Hazard ratios (HR) were evaluated by Cox regression. CI, confidence interval; ECOG, Eastern Cooperative Oncology Group performance status.

In our study, the PFS was 4.9 months with crizotinib treatment in patients with concomitant oncogene mutations, which was inferior than that in previously reported phase III studies [7.7 months for chemotherapy-pretreated (11) and 10.9 months for treatment-naive patients (12)]. Our findings support previous views of high intratumor molecular heterogeneity in *ALK* rearrangement NSCLC, and the activation of bypass signaling pathways may induce the primary resistance to crizotinib in these patients. The status of these concomitant mutations should be considered when defining targeted treatments for *ALK* rearrangement patients as patients carrying these genomic aberrations may not benefit from crizotinib monotherapy. Our findings based on a small sample-size of patients with oncogene mutations remains to be verified and expanded in future studies. In *EGFR* mutation NSCLC, present studies have revealed that *EGFR* TKIs combined with chemotherapy (28, 29) or antiangiogenic (30) therapy may have better efficacy than monotherapy with *EGFR* TKIs. However, few studies have investigated the effectiveness and safety of combination therapies and it is not clear whether dual targeted TKI inhibitors for patients with concomitant oncogene mutations or combined with chemotherapy or antiangiogenic therapy may provide the better benefit for patients with concomitant tumor-suppressor gene mutations. In addition, there is a lack of evidence for first-line treatment with next-generation *ALK* inhibitors in patients with concomitant mutations. Kron et al. (31) found that patients with *ALK/TP53* co-mutations had a worse PFS with next-generation *ALK*-inhibitors after crizotinib treatment compared with patients with *TP53* with wild-type mutations (5.4 vs. 9.9 months, respectively; $P = 0.039$). The impact on efficacy of next-generation *ALK* inhibitors according to baseline NGS analysis needs to be further investigated in multicenter studies.

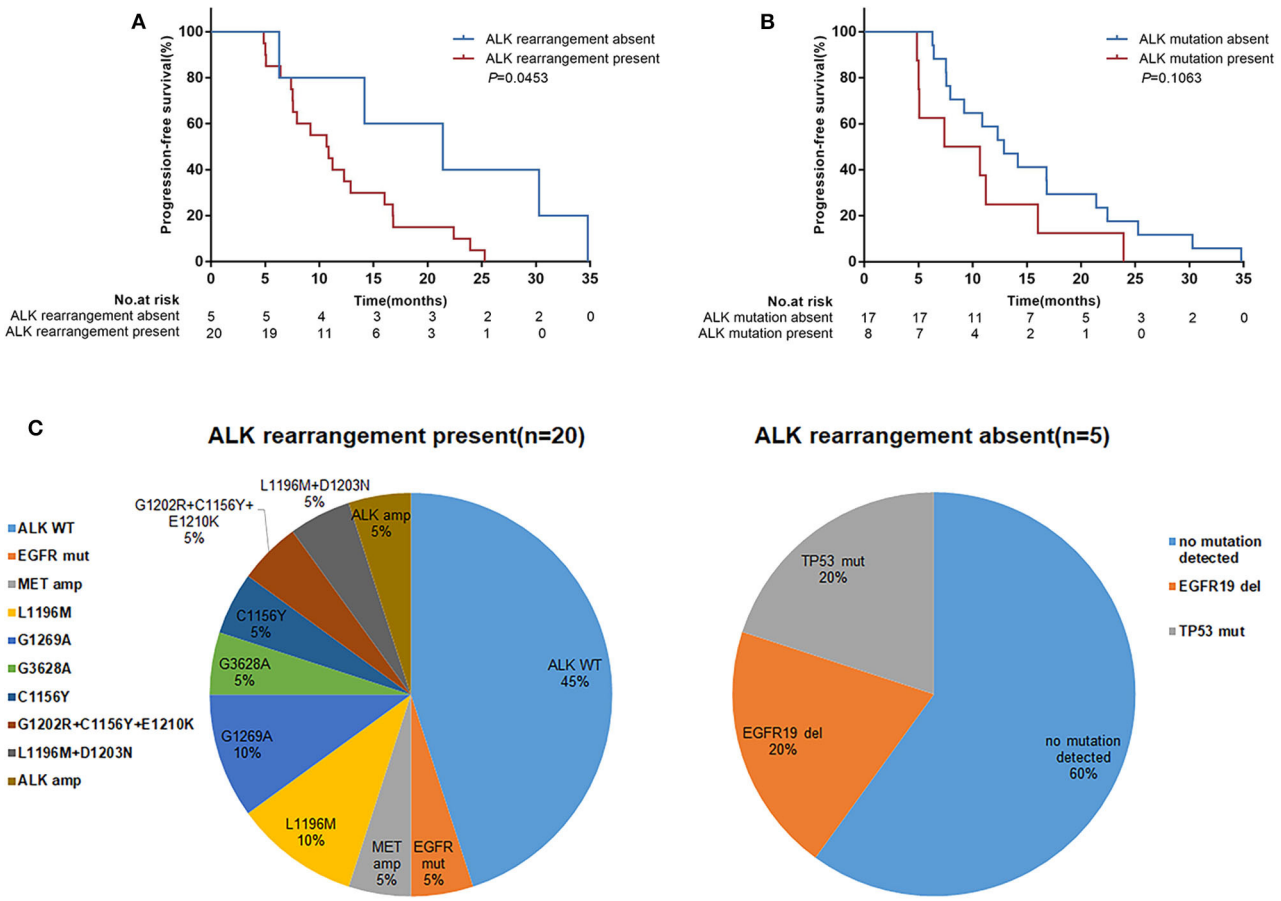


FIGURE 4 | Progression-free survival (PFS) according to *ALK* resistance mechanisms detected in repeat tumor biopsies following disease progression on crizotinib treatment. **(A)** Patients with *ALK* rearrangement absent ($n = 5$) vs. *ALK* rearrangement present ($n = 20$); **(B)** Patients with *ALK* mutations absent ($n = 17$) vs. *ALK* mutations present ($n = 8$). *p*-values were calculated using the log-rank test. **(C)** Distribution of *ALK* resistance mutations after disease progression on crizotinib treatment by *ALK* rearrangement present or absent. WT, wild-type; amp, amplification; mut, mutation; del, deletion.

In our study, there were 25 patients who received repeat biopsies to detect the resistance mechanisms. It seemed that patients with *ALK* rearrangement absent have a longer PFS with crizotinib treatment than patients with *ALK* rearrangement present. This may be explained by tumor cells harboring *ALK* rearrangement decreasing or disappearing after effective therapy. Increasing evidence has shown that dynamic molecular changes are associated with clinical efficacy. The BENEFIT study (18) found that patients with clearance of an *EGFR* mutation after 8 weeks had a significantly prolonged PFS with first-line gefitinib treatment compared with patients with persisting *EGFR* mutations. Pailler et al. (32) also showed that a decrease in the number of circulating tumor cells (CTCs) and an *ALK*-copy number gain with crizotinib treatment was associated with a longer PFS ($P = 0.025$). The present study suggested that dynamic detection of *ALK* rearrangement may predict efficacy to crizotinib, but larger sample size prospective studies are needed for further analysis.

Our study has several limitations. Firstly, it was a retrospective study and still had a limited sample size, particularly for non-*EML4-ALK* rearrangement variants, dual *ALK* rearrangement variants and oncogene mutations, therefore, the results should be interpreted with caution. Multicenter studies based on next-generation *ALK* inhibitors will be conducted in future to validate and expand our findings. Secondly, we used three different gene panels in our studies, which were mainly based on patients' clinical characteristics and financial situation, although all contained lung cancer-related genes. The NGS-detected samples included tumor tissues and liquid biopsies, which may have different sensitivities for mutation detection. Recent studies have shown that sensitivity of *EGFR* ctDNA is lower for tumor tissues (33, 34), while the data for *ALK* rearrangement assessment using ctDNA is relatively limited compared with *EGFR* mutations. McCoach et al. (35) demonstrated that cfDNA NGS testing is a surrogate tool for detecting *ALK* alterations in newly diagnosed patients, as well as for resistant mutations in patients progressing on targeted therapy. Thirdly, the OS of patients according to *ALK*

variants and concomitant mutations were not mature and further follow-up observation is required.

CONCLUSION

The present study found that concomitant mutations have a significant negative effect on the efficacy of crizotinib in patients with *ALK* rearrangement advanced NSCLC, but that various *ALK* variants may have a similar influence. The status of concomitant mutations should be considered when defining targeted treatment for *ALK* rearrangement patients. Our findings need further validation and expansion in future studies.

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 below: The National Omics Data Encyclopedia [accession: OEP001055, https://www.biosino.org/node/project/detail/OEP001055].

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Guangdong Association of Thoracic Oncology (GASTO ID: 1055). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ML contributed to study conception and design, analysis of the data, and wrote the manuscript. XH, CZ, and WF contributed to study conception and design and acquisition of data. GJ, HL, SY, and JC contributed to acquisition of data. LC contributed to study conception and design and overall review. All authors reviewed the manuscript and approved the final version submitted for publication.

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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.2020.01216/full#supplementary-material

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Survival Benefit and Genetic Profile of Pemetrexed as Initial Chemotherapy in Selected Chinese Patients With Advanced Lung Adenocarcinoma

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Objective: This study investigated survival in selected Chinese patients with advanced lung adenocarcinoma who received initial chemotherapy with pemetrexed. We also explored the relationship between genetic biomarkers and pemetrexed efficacy.

Methods: We retrospectively collected patients ($n = 1{,}047$) enrolled in the Chinese Patient Assistance Program from multiple centers who received pemetrexed alone or combined with platinum as initial chemotherapy and continued pemetrexed maintenance therapy for advanced lung adenocarcinoma from November 2014 to June 2017. The outcomes were duration of treatment (DOT) and overall survival (OS). Clinical features were analyzed for their influence on the treatment effect and prognosis. Next-generation sequencing (NGS) was performed to identify genetic biomarkers associated with the efficacy of pemetrexed.

Results: The median DOT was 9.1 months (95% CI: 8.5–9.8), and the median OS was 26.2 months (95% CI: 24.2–28.1). OS was positively correlated with DOT ($r = 0.403$, $P < 0.001$). Multivariable analysis showed that smoking status and Eastern Cooperative Oncology Group (ECOG) performance status (PS) were independently associated with DOT; smoking status, ECOG PS, targeted therapy, and *EGFR/ALK/ROS1* status were independently associated with OS. NGS in 22 patients with available samples showed genes with high mutation rates were: *TP53* (54.5%), *EGFR* (50.0%), *MYC* (18.2%), and *PIK3CA* (13.6%). When grouped based on progression-free survival (PFS) reported in the PARAMOUNT study, the DOT > 6.9 months set was associated with *PIK3CA*, *ALK*, *BRINP3*, *CDKN2A*, *CSMD3*, *EPHA3*, *KRAS*, and *RB1* mutations, while *ERBB2* mutation was observed only in the DOT ≤ 6.9 months set.

Conclusion: This study shows that initial chemotherapy with pemetrexed is an effective regimen for advanced lung adenocarcinoma in selected Chinese patients. There is no specific genetic profile predicting the benefit of pemetrexed found by NGS. Biomarkers predicting the efficacy of pemetrexed need further exploration.

Keywords: pemetrexed, lung adenocarcinoma, Chinese, next-generation sequencing, chemotherapy

INTRODUCTION

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death all over the world (1). About 80–85% of human lung cancers belong to the category of non-small cell lung cancer (NSCLC). These patients usually present with locally advanced (stage IIIB) or metastatic disease (stage IV) (2). Immunotherapy plus chemotherapy have become a standard first-line treatment for patients with no oncogenic driver alterations in advanced NSCLC. Nevertheless, which chemotherapy regimen combined with immunotherapy agents, will achieve optimal outcomes for these patients is unknown. In addition, in developing countries, including China, immunotherapy is often too expensive for many patients, although these drugs are recommended by the available guidelines (3). Therefore, chemotherapy is still indispensable (4–6). Some randomized controlled trials and real-world studies abroad demonstrated

that pemetrexed-cisplatin induction and pemetrexed maintenance therapy is an effective and well-tolerated regimen in stage IIIB-IV patients with non-squamous NSCLC (7–14), but there is still a lack of large sample size evidence to report the survival benefit of pemetrexed as the initial chemotherapy in Chinese patients with advanced lung adenocarcinoma.

Moreover, heterogeneity in response to pemetrexed has been observed, some patients experience long progression-free survival (PFS) or overall survival (OS), whereas others are having short PFS and OS (15). Identifying patients who are unlikely to benefit from pemetrexed would avoid unnecessary treatment and allow alternative therapy that might achieve better outcomes. Although we know that histologically non-squamous NSCLC is a predictive factor for the efficacy of pemetrexed, this still cannot help us screen out the patients who will really benefit from pemetrexed (16). Previous studies showed that thymidylate synthase (TS), folate receptor alpha (FRA), epidermal growth factor receptor (*EGFR*) gene mutation, anaplastic lymphoma kinase (*ALK*) gene rearrangement, and plasma microRNA levels might be predictive markers for pemetrexed (17–22), but the predictability of these biomarkers has not been reproducible due to the lack of prospective studies. Therefore, it is necessary to explore novel and reliable biomarkers to predict the effect of pemetrexed.

Next-generation sequencing (NGS) based on tumor tissue or liquid biopsy has begun to play a role in genomic profiling. Its high-throughput nature makes testing of thousands of genes or even the whole genomes possible with a small amount of DNA, allowing this method to identify actionable genomic alterations (23).

In order to reduce the financial burden and provide timely treatment for Chinese patients, the Chinese Primary Health Care Foundation launched a patient assistance program (PAP) for advanced non-squamous NSCLC patients with pemetrexed as maintenance therapy; those patients receive a 100% discount after a self-funded four-cycle induction pemetrexed therapy, starting from October 1, 2014. The patients who do not complete the four-cycle induction pemetrexed therapy are not

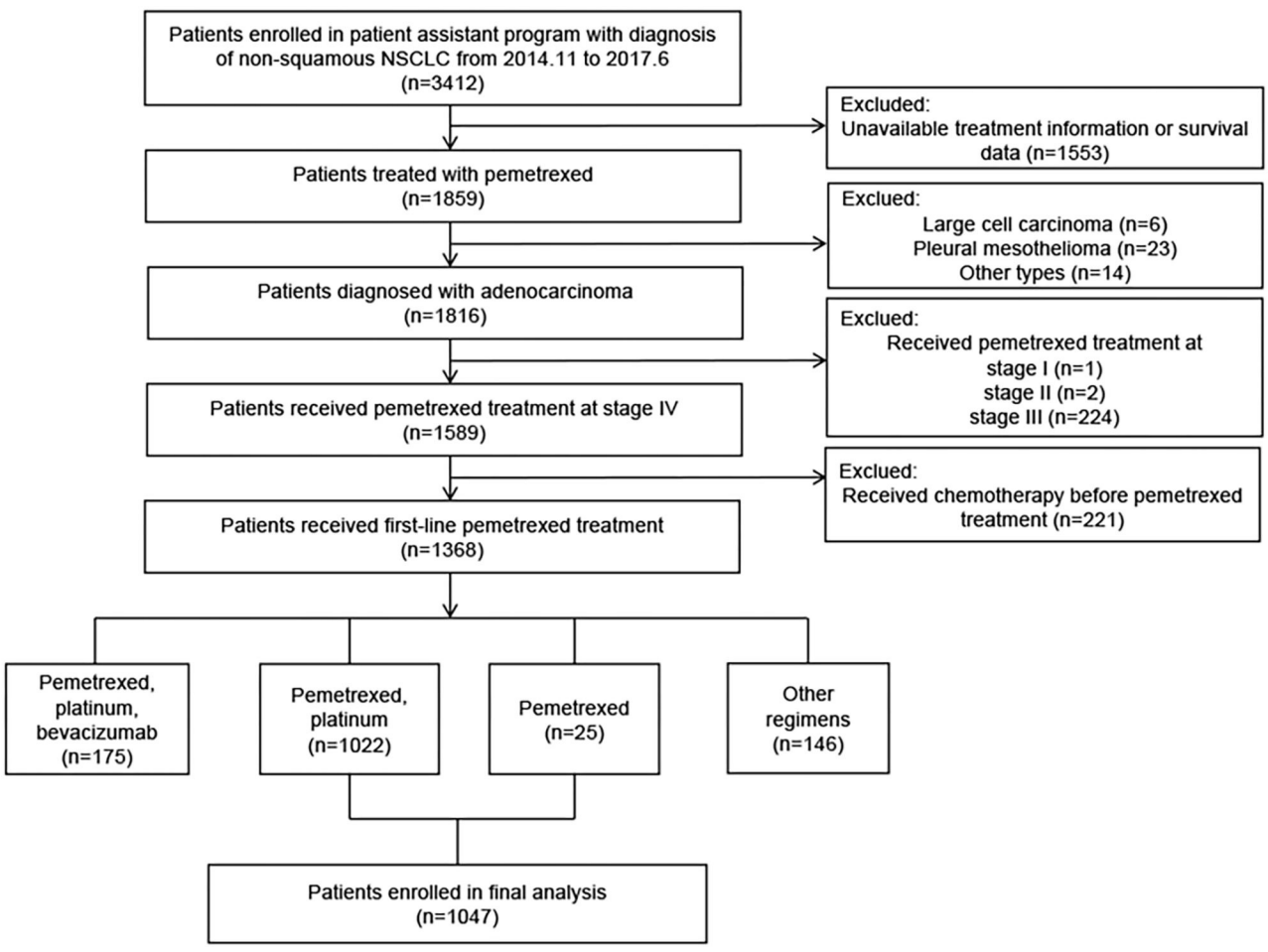

FIGURE 1 | Flowchart of patients' screening for inclusion in the study.

eligible for the PAP. Following the implementation of the PAP, we observed the duration of treatment (DOT) with pemetrexed and OS among these selected patients and compared the genomic differences between the patients with long and short duration of pemetrexed treatment to explore potential predictive biomarkers.

TABLE 1 | Demographic and clinical characteristics of patients with advanced lung adenocarcinoma.

Characteristics	Patients (n = 1,047)
Sex, *n* (%)	
Male	594 (56.7%)
Female	453 (43.3%)
Age (years)	
Median (range)	59 (24–93)
<65, *n* (%)	758 (72.4%)
≥65, *n* (%)	289 (27.6%)
Smoking status, *n* (%)	
Non-smoker	608 (58.1%)
Smoker	439 (41.9%)
ECOG PS, *n* (%)	
0–1	984 (94.0%)
2	63 (6.0%)
Targeted therapy before pemetrexed treatment, *n* (%)	
Yes	149 (14.2%)
No	898 (84.8%)
Gene status before pemetrexed treatment, *n* (%)	
***EGFR* Mutation**	
Positive	266 (25.4%)
Negative	568 (54.3%)
Unknown	213 (20.3%)
***ALK* Rearrangement**	
Positive	59 (5.6%)
Negative	580 (55.4%)
Unknown	408 (39.0%)
***KRAS* Mutation**	
Positive	11 (1.1%)
Negative	147 (14.0%)
Unknown	889 (84.9%)
***ROS1* Fusion**	
Positive	18 (1.7%)
Negative	233 (22.3%)
Unknown	796 (76.0%)
Chemotherapy regimen, *n* (%)	
Pemetrexed plus platinum	1,022 (97.6%)
Pemetrexed monotherapy	25 (2.4%)
Best tumor response, *n* (%)	
PR	477 (45.6%)
SD	570 (54.4%)

ECOG PS, Eastern Cooperative Oncology Group performance status; PR, partial response; SD, stable disease.

METHODS

Study Design

This was a retrospective study of data from multiple centers across China. The patients were funded by the PAP to receive pemetrexed and visited their treating hospitals from November 2014 to June 2017. The PAP is offered by the Chinese Primary Health Care Foundation. All data were extracted from the patients' medical records, and telephone follow-up was conducted by physicians across more than 200 tertiary hospitals in China. The study protocol was approved by the Research Ethics Committee of Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, School of Medicine, South China University of Technology (No. GDREC2017303H). This study was conducted in accordance with the Good Clinical Practice (GCP) principles. Written informed consent was obtained from all included patients.

Study Population

The patients meeting the following criteria were included: Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2; lung adenocarcinoma; stage IV (according to the American Joint Committee on Cancer staging system, 7th edition); pemetrexed as initial chemotherapy; received four cycles of pemetrexed monotherapy or pemetrexed plus platinum as induction chemotherapy with no disease progression according to Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 (24): complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD); and at least one cycle of pemetrexed maintenance therapy from PAP.

The exclusion criteria were: history of chemotherapy; combination with other antitumor drugs such as bevacizumab; disease progression before the completion of four cycles of induction pemetrexed chemotherapy; or unavailable treatment information or survival data.

Data Source

The demographic and clinical characteristics of the patients were extracted from their medical records and entered into the Medical Record Abstraction Form (MERAF) by designated hospital staff. These characteristics included sex, age, smoking status, disease stage at diagnosis, the time at diagnosis, disease stage at pemetrexed treatment, NSCLC histological type, gene status, ECOG PS, the time of pemetrexed treatment initiation, first-line anticancer treatment regimen, cycles, the best response to pemetrexed treatment, the time of progressive disease administered with pemetrexed, other treatment after

TABLE 2 | The gene aberration rates in patients with definitive results.

Gene aberration	Positive, *n*	Negative, *n*	Mutation rate, %
EGFR	266	568	31.9%
ALK	59	580	9.2%
ROS1	18	233	7.2%
KRAS	11	147	7.0%

pemetrexed, survival status, and the time to death. Patients who had smoked ≥100 cigarettes in their lifetime were defined as smokers (25). The last time of pemetrexed treatment was obtained through the electronic PAP system. Survival data were collected by the follow-up registration system from each site and by telephone follow-up. The response was assessed based on imaging examination reports and medical case notes.

Outcomes

The outcomes were the DOT of pemetrexed and OS. DOT was defined as the time from the initiation to the last pemetrexed chemotherapy. OS was defined as the time from the initiation of pemetrexed chemotherapy to death or the last follow-up, whichever came first. The last follow-up was conducted on March 31, 2018.

Grouping

The median PFS was 6.9 months for patients with advanced non-squamous NSCLC who received maintenance therapy with pemetrexed after induction chemotherapy with pemetrexed plus cisplatin according to the double-blind, phase 3, randomized controlled trial "PARAMOUNT" (9). Since the PARAMOUNT study provided high-level evidence for maintenance therapy with pemetrexed, we selected their median PFS as the cutoff for grouping. In the present study, according to their PFS, patients with NGS detection whose DOT was ≤6.9 months were assigned to the short duration group, whereas the long duration group included patients with DOT of >6.9 months.

Next-Generation Sequencing

Patients with available blood samples at Guangdong Provincial People's Hospital underwent NGS. Plasma samples were obtained after disease progression in patients with initial pemetrexed chemotherapy. The NGS tests targeted at least 139 genes related to lung cancer and were performed in two clinical testing centers (Burning Rock Biotech Ltd and Nanjing Geneseeq Technology Inc.). First, DNA was extracted from blood. Then, the NGS library was prepared, and DNA was profiled using a capture-based sequencing panel. Finally, sequence data were analyzed and compared between the long and short duration groups.

Statistical Analysis

SPSS 22.0 (IBM Corp., USA) was used for all statistical analyses. Descriptive statistics were used to describe the enrolled patients. DOT and OS were assessed using the Kaplan-Meier method. We also performed the Pearson correlation test to evaluate the correlation between DOT and OS. The factors associated with DOT and OS were analyzed by performing univariable and multivariable analyses using Cox proportional hazards models, including the following covariables: age, sex, smoking status, ECOG PS, and gene status. Multivariable analysis of OS also included the factor: targeted therapy. Variables with $P < 0.1$ in the univariable analyses were included in the multivariable analysis. Two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Demographics and Clinical Characteristics

A total of 3,412 patients were screened. Of these, 1,047 patients from 44 hospitals with advanced lung adenocarcinoma who received pemetrexed treatment were included in the analyses. The screening flowchart and clinical characteristics of the patients are presented in **Figure 1**, **Table 1**, respectively.

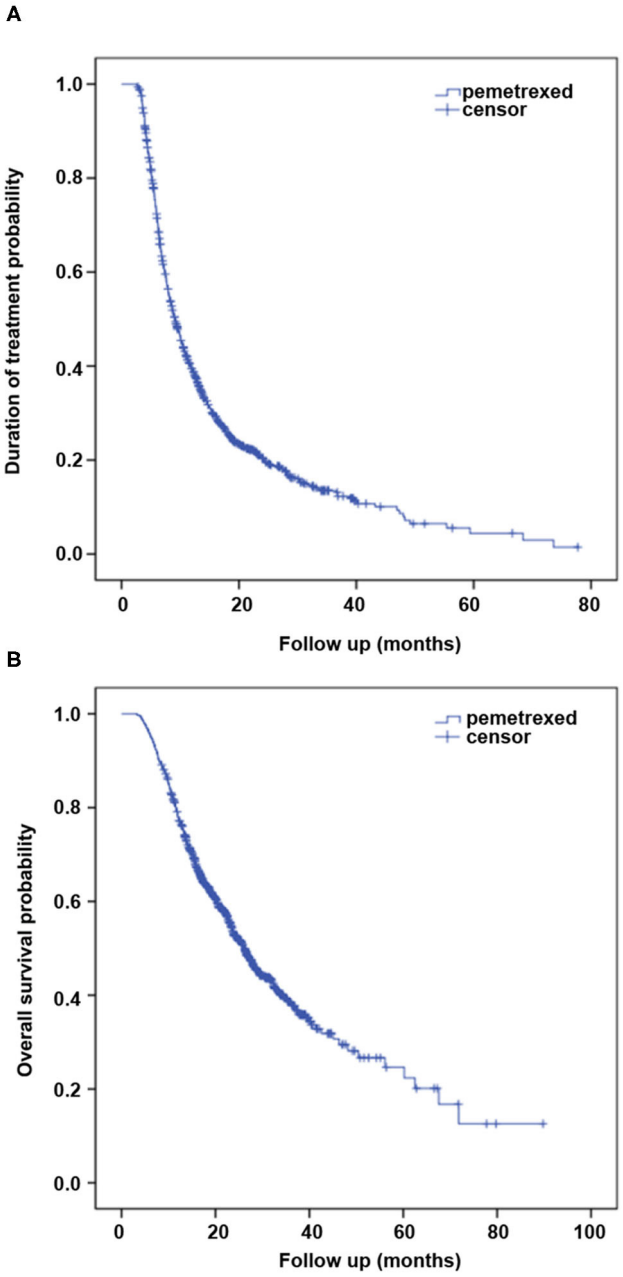

FIGURE 2 | Kaplan–Meier curves of **(A)** duration of treatment (DOT) and **(B)** overall survival (OS) for 1,047 patients treated with pemetrexed.

TABLE 3 | Univariable and multivariable analysis of duration of pemetrexed treatment.

Variable	Univariable analysis				Multivariable analysis			
	HR	95% CI		*P*	HR	95% CI		*P*
		Lower	Upper			Lower	Upper	
Age (≥65 vs. <65 years)	1.045	0.896	1.219	0.574	-	-	-	-
Sex (female vs. male)	0.889	0.772	1.022	0.099	-	-	-	0.642
Smoking status (smoker vs. non-smoker)	1.163	1.011	1.338	0.034	1.167	1.015	1.342	0.031
ECOG PS (2 vs. 0–1)	2.105	1.626	2.726	<0.001	2.112	1.631	2.735	<0.001
***EGFR* Mutation**								
(+ vs. -)	1.071	0.909	1.261	0.413	-	-	-	-
(unknown vs. -)	0.976	0.814	1.171	0.795	-	-	-	-
***ALK* Rearrangement**								
(+ vs. -)	0.716	0.520	0.987	0.041	-	-	-	0.056
(unknown vs. -)	1.000	0.866	1.155	0.996	-	-	-	0.943
***KRAS* Mutation**								
(+ vs. -)	1.754	0.919	3.348	0.088	-	-	-	0.117
(unknown vs. -)	1.069	0.878	1.301	0.509	-	-	-	0.788
***ROS1* Fusion**								
(+ vs. -)	0.604	0.343	1.063	0.080	-	-	-	0.096
(unknown vs. -)	1.010	0.855	1.192	0.911	-	-	-	0.762

HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status.

The gene status before pemetrexed treatment was analyzed, including *EGFR*, *ALK*, Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and c-ros oncogene 1 (*ROS1*). The gene aberration rates in patients with definitive results were 31.9% (266/834) for *EGFR* mutation, 9.2% (59/639) for *ALK* rearrangement, 7.0% (11/158) for *KRAS* mutation, and 7.2% (18/251) for *ROS1* fusion (**Table 2**).

Survival Outcomes

The median follow-up of all patients was 19.1 months. The median DOT was 9.1 months (95% confidence interval [CI]: 8.5–9.8) for 811 patients who had stopped pemetrexed treatment at the last follow-up (**Figure 2A**). Among the 536 patients who had died, the median OS was 26.2 months (95%CI: 24.2–28.1) (**Figure 2B**). Moreover, a positive correlation was observed between DOT defined in the present study and OS evaluated by Pearson correlation test ($r = 0.543$, $P < 0.001$).

DOT and OS were both longer than the PFS and OS observed in the PARAMOUNT, JMII, and S110 trials. Clinical characteristics and efficacy of pemetrexed in the present study were compared with these trials (**Supplemental Table 1**).

Factors Associated With Duration of Treatment and Overall Survival

When the variables were analyzed by univariable analysis, sex, smoking status, ECOG PS, and gene status (*ALK*, *KRAS*, *ROS1*) were revealed as significant factors ($P < 0.1$) associated with DOT. These parameters were included in the multivariable Cox regression analysis. As a result, only smoking status (hazard ratio [HR], 1.167; 95%CI, 1.015–1.342; $P = 0.031$) and ECOG PS (HR, 2.112; 95% CI, 1.631–2.735; $P < 0.001$) were independent factors influencing DOT of pemetrexed (**Table 3**). Kaplan–Meier curves of DOT about smoking status and ECOG PS factors are shown in **Figure 3**.

Multivariable analysis in the Cox proportional hazards model revealed the independent factors influencing OS, including smoking status (HR, 1.323; 95% CI, 1.111–1.574; $P = 0.002$), ECOG PS (HR, 2.984; 95% CI, 2.286–3.894; $P < 0.001$), targeted therapy (HR, 0.697; 95% CI, 0.556–0.875; $P = 0.002$), and *EGFR/ALK/ROS1* status (HR, 0.609; 95% CI, 0.552–0.863; $P < 0.001$) (**Table 4**). **Figure 4** presents the Kaplan–Meier curves of OS about these significant factors: smoking status, ECOG PS, *EGFR/ALK/ROS1*, and targeted therapy factors.

Genetic Differences Between the Long and Short Duration Groups Using on Next-Generation Sequencing

Among 1,047 patients, 117 patients received initial chemotherapy with pemetrexed at Guangdong Provincial People's Hospital. Twenty-two plasma samples from 22 patients were collected to perform NGS. Their DOT ranged from 4.5 to 27.1 months. Thirteen patients were assigned to the long duration group (DOT $>$ 6.9 months), whereas the short duration group (DOT $\leq$ 6.9 months) included nine patients. Their clinical characteristics are presented in **Supplemental Table 2**. The demographics and clinical characteristics of the patients were similar between the two groups in regard to age, sex, smoking status, and best response.

A total of 30 intersection genes were analyzed in these 22 patients, and the genes with high mutation rate included *TP53*, *EGFR*, *MYC*, and *PIK3CA*, accounting for 54.5% (12/22), 50.0% (11/22), 18.2% (4/22), and 13.6% (3/22), respectively

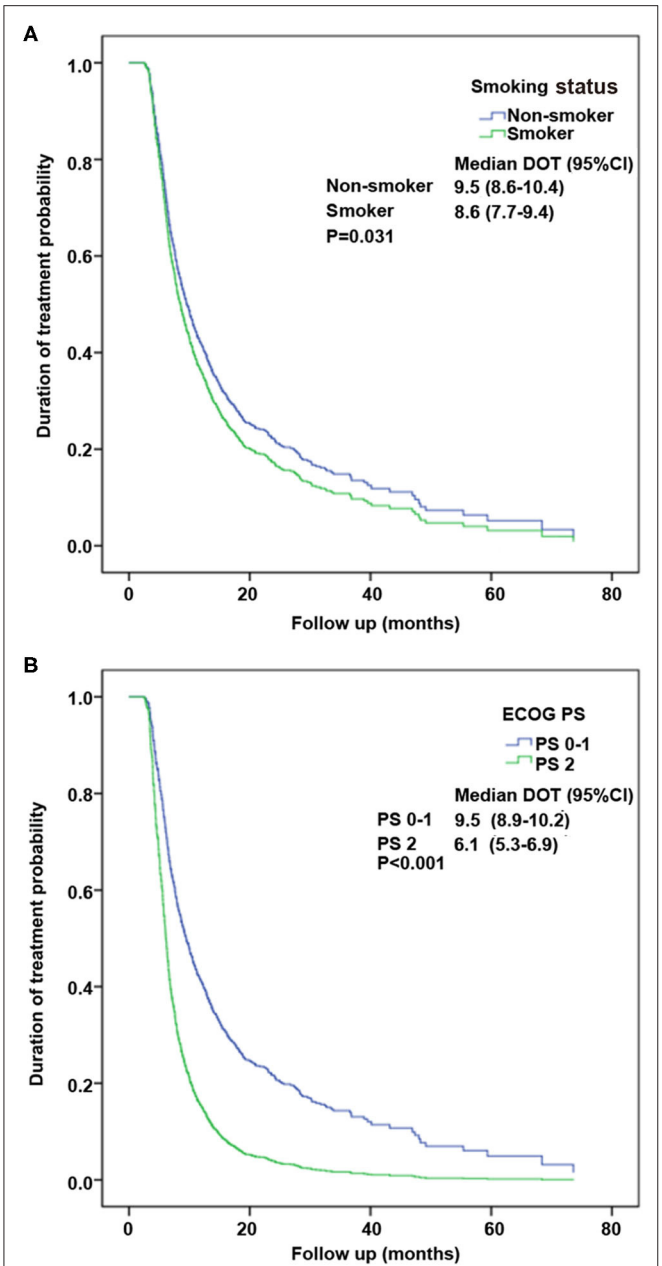

FIGURE 3 | Kaplan–Meier curves of the duration of treatment (DOT) about the significant factors by multivariate analysis. **(A)** Performance status (PS) factor. **(B)** Smoking status factor.

(**Figure 5A**). We also compared the difference of 12 genes that were mutated in more than one patient between the two groups (**Figure 5B**). Three genes (*TP53*, *EGFR*, and *MYC*) appeared recurrently in the two groups. Genes that were mutated in the long duration group but not in the short duration group included *PIK3CA*, *ALK*, *BRINP3*, *CDKN2A*, *CSMD3*, *EPHA3*, *KRAS*, and *RB1*. *ERBB2* mutation was detected only in the short duration group.

DISCUSSION

Previous randomized controlled trials demonstrated that pemetrexed is effective and well-tolerated for patients with advanced non-squamous NSCLC (9–11). The PARAMOUNT trial was a relatively large randomized controlled trial that investigated whether continuation maintenance therapy with pemetrexed improved PFS after induction therapy with pemetrexed plus cisplatin in 539 patients randomly assigned to receive continuation maintenance therapy with pemetrexed plus best supportive care ($n = 359$) or with placebo plus best supportive care ($n = 180$) (9). The median PFS of pemetrexed during the induction plus maintenance period was 6.9 months, and the OS was 16.9 months. Therefore, that study provides high-level evidence for maintenance therapy with pemetrexed. Nevertheless, 94% of the patients enrolled in the PARAMOUNT study were Caucasian. Yang et al. reviewed the JMII and S110 studies to supplement the efficacy and safety data from PARAMOUNT on pemetrexed maintenance therapy in East Asian patients with non-squamous NSCLC (26). The median PFS during the entire period (induction plus maintenance) in the JMII and S110 studies was 5.7 months (95% CI, 4.4–7.3 months) and 6.83 months (95% CI, 5.78–7.98 months), which was consistent with that observed in the PARAMOUNT study (26). The median OS during the entire period was 20.2 months (95% CI, 16.7 to not available) in the JMII trial, whereas, in the S110 trial, the median OS could not be estimated due to a high censor rate (72.9%). Although the two studies suggested the efficacy of pemetrexed induction and maintenance therapy in East Asian patients, their samples were small. In our study, we collected a total of 1,047 patients from PAP throughout China, including 44 tertiary hospitals. It is the largest study performed so far to investigate the efficacy of pemetrexed initial chemotherapy in Chinese patients with advanced lung adenocarcinoma. The results showed that the median DOT of pemetrexed induction plus maintenance therapy was 9.1 months (95% CI: 8.5–9.8), and the median OS was 26.2 months (95% CI: 24.2–28.1), which were longer than that observed in clinical trials above. It added some evidence based on large samples showing that patients with lung adenocarcinoma can benefit from pemetrexed initial chemotherapy, especially for East Asians. This also provides a basis for which chemotherapy regimen may be the most appropriate combination with immunity inhibitors such as PD-1/PD-L1 inhibitor in the age of immunotherapy.

In the present study, the DOT was longer than the PFS observed in the PARAMOUNT, JMII, and S110 trials. One reason may be that our outcome DOT is different from PFS and is defined as the time from the start to the last treatment of pemetrexed. In clinical trials, the PFS is a common endpoint. Patients in clinical trials stop pemetrexed treatment once evaluated to have disease progression, according to RECIST. Nevertheless, in routine clinical practice, some patients still have a high likelihood of responding to maintenance therapy despite RECIST suggesting disease progression. Indeed, *EGFR* mutated patients with gradual and local progression after EGFR-tyrosine kinase inhibitors (TKIs) treatment failure still show

TABLE 4 | Univariable and multivariable analysis of overall survival.

Variable	Univariable analysis				Multivariable analysis			
	HR	95% CI		*P*	HR	95% CI		*P*
		Lower	Upper			Lower	Upper	
Age (≥65 vs. <65 years)	1.242	1.031	1.495	0.022	-	-	-	0.093
Sex (female vs. male)	0.731	0.614	0.870	<0.001	-	-	-	0.267
Smoking status (smoker vs. non-smoker)	1.416	1.194	1.680	<0.001	1.323	1.111	1.574	0.002
ECOG PS (2 vs. 0–1)	2.934	2.254	3.821	<0.001	2.984	2.286	3.894	<0.001
***EGFR/ALK/ROS1* Status**								
(+ vs. -)	0.569	0.463	0.699	<0.001	0.609	0.552	0.863	<0.001
(unknown vs. -)	1.023	0.822	1.274	0.838	0.979	0.785	1.221	0.852
Targeted therapy (with vs. without)	0.599	0.485	0.740	<0.001	0.697	0.556	0.875	0.002

HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status.

persistent symptom benefit to continuing EGFR-TKIs treatment (27). In clinical practice, there are also some patients who receive pemetrexed treatment with gradual and local progression and showing persistent clinical benefit, although the disease was evaluated as progression according to RECIST. Although more details regarding the PFS and treatment beyond progression were not available, the DOT of 9.1 months in the present study was consistent with the PFS of 9.4 months reported by a real-world study from USA (12). Moreover, because of toxicity or other reasons, some patients may quit pemetrexed treatment when their tumors had not enlarged. Therefore, DOT may reflect the efficacy and safety of pemetrexed treatment more practically and objectively in clinical practice. The other reason may be that the present study was conducted in selected Chinese patients who might have a good prognosis and good response to pemetrexed since they completed the four-cycle induction therapy.

OS was also longer in the present study than that observed in the PARAMOUNT and JMII trials. Selected Chinese patients who received at least four cycles of pemetrexed treatment were enrolled in our study, which resulted in the longer OS besides the DOT. We also compared the clinical characteristics in the present study with those of the PARAMOUNT and JMII trials. Race and druggable target gene mutations were different between this study and the PARAMOUNT trial. Our study focused on Chinese patients, while 94% of patients were Caucasian in the PARAMOUNT trial, and we know that there are differences in genetic profile between the two races (28). *EGFR* mutations are more common in Asian populations than Caucasians (29). The rate of *EGFR* mutation in the present study is lower than that reported by Wu et al. (30) due to diverse gene detection methods with different sensitivity from 44 tertiary hospitals throughout China and certain percentages of patients with unknown *EGFR* status. Even so, *EGFR* mutated rate in our study was different from the PARAMOUNT trial. The multivariable analysis also showed that the *EGFR/ALK/ROS1* status and targeted therapy were independently associated with OS. This was similar to a previous study which reported that patients with an oncogenic driver mutation and who received a targeted therapy had survival benefits compared with those without oncogenic mutation or those who did not receive targeted therapy (31). The JMII trial was performed to evaluate the efficacy of pemetrexed and carboplatin, followed by pemetrexed maintenance therapy in chemotherapy-naïve patients with advanced non-squamous NSCLC in Japan. Out of 109 patients, 106 were evaluable for efficacy analysis. Although the median OS was 20.2 months (95% CI, 16.7 to not available), among 60 patients who received continuation maintenance with pemetrexed, the median OS from the beginning of induction treatment was not calculable. Besides genetic differences, more effective anticancer agents such as immunotherapy after pemetrexed treatment failure have been available from 2014 to 2018 when our study was conducted (32). The PARAMOUNT trial was carried out between 2008 and 2010, and the OS data cutoff date was in 2012 when antitumor treatment was more limited. The JMII trial was carried out between 2009 and 2010. All of these situations are potential reasons why the OS in our study was longer than that of previous clinical trials.

The multivariable analysis showed that the smoking status and ECOG PS were independently associated with DOT and OS, which was consistent with some previous studies (33–35). These factors might be potential clinical predictors for the effect of pemetrexed. Nevertheless, the differences of pemetrexed response among the patients could not be completely predicted by these clinical factors. This study tried to find potential genetic factors using NGS. A heterogeneous genetic profile was observed between the two groups by NGS. Mutations in *PIK3CA*, *ALK*, *BRINP3*, *CDKN2A*, *CSMD3*, *EPHA3*, *KRAS*, and *RB1* were only observed in the long duration group, whereas *ERBB2* mutation was only observed in the short duration group. But, these genes were not specific genetic profile benefit to pemetrexed treatment and may not predict the efficacy of pemetrexed. Further studies are needed to explore the predictor of the benefit to pemetrexed treatment in the future.

There are several limitations to our study. First, the present study excluded the patients who received pemetrexed for <4 cycles due to disease progression. This cannot represent the whole pemetrexed-treated patients in clinical practice in a real-world condition. Second, there are certain percentages

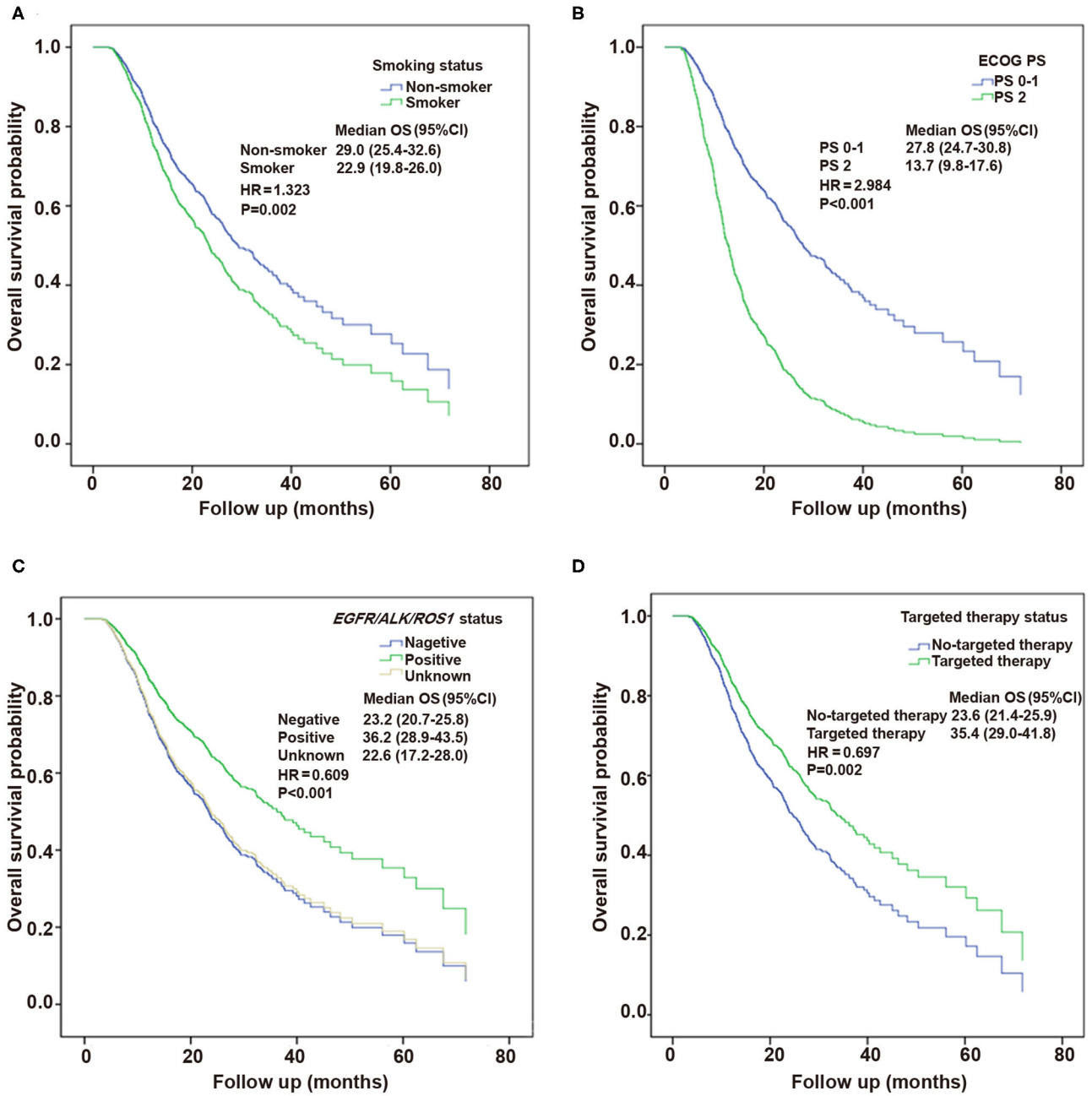

FIGURE 4 | Kaplan–Meier curves of overall survival (OS) about the significant factors by multivariate analysis. **(A)** Smoking status factor. **(B)** Performance status (PS) factor. **(C)** *EGFR/ALK/ROS1* mutation factor. **(D)** Targeted therapy factor.

of patients with unknown *EGFR, ALK, and ROS1* statuses, and the rate of patients with druggable target gene mutations received targeted therapy before pemetrexed treatment is low. Moreover, the exploration and comparison of gene profiles by NGS were conducted in a small sample of patients. All of these might make a bias to the conclusion. Third, more details regarding the PFS and treatment during beyond progression were not collected to prove the effectiveness of continuing pemetrexed chemotherapy beyond progression according to RECIST criteria. PFS and treatment beyond progression will have to be examined.

CONCLUSION

This study shows that initial chemotherapy with pemetrexed is an effective regimen for advanced lung adenocarcinoma

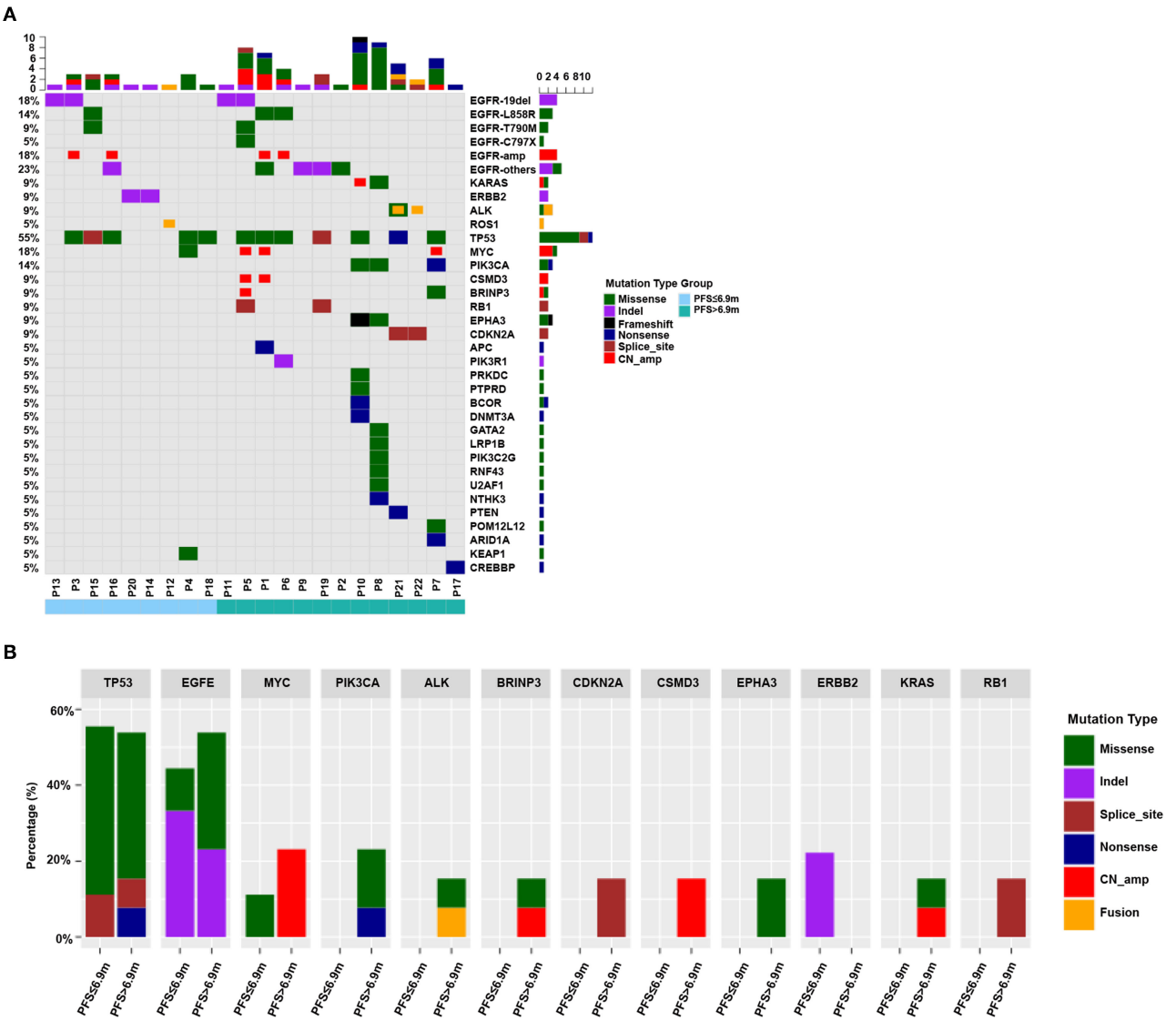

FIGURE 5 | (A) Mutational frequency of 30 intersection genes analyzed in 22 patients by next-generation sequencing (NGS). The genes with high mutation rate included *TP53*, *EGFR*, *MYC*, and *PIK3CA*. **(B)** The differences of 12 genes mutational frequency between PFS > 6.9 months and PFS ≤ 6.9 months groups. *TP53*, *EGFR*, and *MYC* appeared recurrently in the two groups. *PIK3CA*, *ALK*, *BRINP3*, *CDKN2A*, *CSMD3*, *EPHA3*, *KRAS,* and *RB1* occurred only in the PFS > 6.9 months group whereas *ERBB2* mutation was detected only in the PFS ≤ 6.9 months group.

in selected Chinese patients. There is no specific genetic profile predicting the benefit of pemetrexed found by NGS. Biomarkers predicting the efficacy of pemetrexed need further exploration. More studies are needed to find a clinical treatment strategy of that chemotherapy combines with immunotherapy or targeted therapy.

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 at: The National Omics Data Encyclopedia (http://www.biosino.org/node/project/detail/OEP001054) and the European Genome-phenome Archive (https://www.ebi.ac.uk/ega/studies/EGAS00001004546).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, School of Medicine, South China University of Technology. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QZ and Y-LW conceived the idea and designed the experiments. L-HG and M-FZ analyzed the data together. L-HG wrote the manuscript. All authors, except QZ and Y-LW, were involved in the acquisition of data. All authors participated in the interpretation of the study results, drafting, critical revision, and approval of the final version of the manuscript.

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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.2020.01568/full#supplementary-material

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ORIGINAL RESEARCH
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Dual Primary Cancer Patients With Lung Cancer as a Second Primary Malignancy: A Population-Based Study

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Background: Research on patients with lung cancer as a second primary malignancy (LCSPM) remains limited. This study aims to determine the clinical characteristics, prognosis, and temporal relationship of other cancers to lung cancer in these patients.

Methods: This study retrospectively analyzed 3465 patients with dual primary cancers from the 5253 patients with LCSPM retrieved from the Surveillance, Epidemiology and End Results (SEER) database from 2010 to 2015.

Results: 2285 eligible patients were further analyzed in this study cohort with 59.3% of 1-year OS, 34.7% of 3-year OS, and 25.2% of 5-year OS. The most common first primary cancer (FPC) in dual primary cancer patients with LCSPM was prostate cancer, followed by female breast cancer and urinary bladder cancer. In the entire study population, the median interval between the two primary malignancies was 21 months (range: 3.5–52 months). Age, sex, FPC location, surgery, stage, and histology of lung cancer were regarded as independent prognostic factors for these patients. The prognosis of patients with urinary bladder cancer as FPC was the worst in the univariate ($p = 0.024$) and multivariate ($p < 0.001$) Cox analyses. Lung cancer-directed surgery could significantly improve long-term survival (HR = 0.22, $p < 0.001$). Additionally, the C-index of the established nomogram with acceptable calibration curves was 0.760 (95% CI: 0.744–0.776) in the training cohort and was 0.759 (95% CI: 0.737–0.781) in the validation cohort, showing an ideal model discrimination ability. The corresponding decision curve analysis (DCA) indicated the nomogram had relatively ideal clinical utility.

Conclusions: Cancer patients still have the risk of developing a new primary lung cancer. Close, lifelong follow-up is recommended for all these patients. Early detection for surgical treatment will significantly improve the prognosis of dual primary cancer patients with

LCSPM. The nomogram developed to predict 1-, 3-, and 5-year OS rates has relatively good performance.

Keywords: lung cancer as a second primary malignancy, multiple primary cancers, lung cancer, nomogram, Surveillance, Epidemiology and End Results

INTRODUCTION

Lung cancer poses a serious threat to public health due to its high morbidity and mortality. Nevertheless, little attention has been paid to multiple primary cancers (MPC) involving lung cancer. With the advancement of medical technology and the extension of survival time of cancer patients, more and more cancer patients develop one or more new primary malignant tumors in the same or other organs during follow-up. MPC involving lung cancer is common clinically. Depending on incomplete statistics, the incidence of MPC involving lung cancer ranges from 0.9% to 26.3% (1–4). However, research on MPC involving lung cancer is still limited. People still do not have a clear idea of these patients. When patients have multiple primary malignancies at the same time, it is complicated for clinicians to judge the prognosis of these patients. Although the TNM staging system is the most widely used method for evaluating prognosis, it still has some limitations, especially for patients with multiple primary malignancies (they tend to have special biological characteristics different from single primary malignancy). Thus, it is necessary to learn more about this particular disease and seek more refined methods to predict the survival of these patients. Nomogram, which has been widely used to evaluate the prognosis of cancer patients in recent years owing to its convenience and accuracy (5, 6), may be a good choice for this purpose. This study is to conduct a retrospective analysis based on the clinical information of LCSPM patients to understand the common site distribution of the first primary cancer (FPC) and time interval between two primary malignancies and to determine the prognostic factors and to develop a nomogram that can predict the survival in order to provide certain evidence for guiding clinical practice.

MATERIALS AND METHODS

Data Source and Variable Selection

The clinical information of LCSPM patients was extracted from the SEER database between 2010 and 2015. We accessed the database using SEER*Stat 8.3.5 software (http://seer.cancer.gov/seerstat/). These data from the SEER database were open to the public for research purposes. This study was also approved by the Institutional Research Committee of Zhongnan Hospital of Wuhan University. We mainly studied the dual primary cancer patients with LCSPM, so cases with three or more primary malignancies were excluded from this study. Given there were still no uniform diagnostic criteria for multiple primary lung cancer (MPLC) and it was difficult to determine whether the second tumor lesion was primary or metastatic, this study also excluded patients with lung cancer as the first primary malignant tumor. The detailed patient selection process is summarized in **Figure 1**. The collected variables included age at diagnosis, sex, "race record," "ICO-O-3 Hist/behav, malignant," "month since index" (the time interval between two primary cancers), "Derived AJCC Stage (7th ed)," "COD to site recode," "Survival months," "Vital status record (study cutoff used)," "Rx Sumn-Surg Prim Site(1998+)," and "years of diagnosis."

Statistical Analysis

In this study, overall survival (OS) was calculated from the diagnosis date of the second primary malignancy (lung cancer) to the date of the last follow-up or death in the SEER database. The OS of all variables were calculated using the Kaplan-Meier method. Survival curves were compared with the log-rank test. Simple random sampling was performed in version 3.6.0 of R software, and patients were randomly divided into a training cohort and a validation cohort at a ratio of 7 to 3. In the training cohort, the Cox proportional hazards model was utilized to estimate OS hazard ratio (HR) for prognostic factors, including age, sex, race, histology, location of FPC, the time interval between two primary cancers, AJCC stage, year of diagnosis, and surgery. All variables were first subjected to univariate Cox analysis, and then variables with $p < 0.05$ were subjected to multivariate Cox analysis. Based on these independent prognostic factors, Kaplan-Meier survival analysis was further performed, and a prognostic nomogram was also constructed to predict 1-, 3-, and 5-year OS rates. The nomogram was developed with the "rms" package in R. In order to evaluate the predictive accuracy of the nomogram, the concordance index (C-index) was calculated by the bootstrap method with 100 resamples, and calibration curves were also drawn simultaneously. Statistics of C-index are generally between 0.5 and 1.0, and the higher the C-index, the higher the predictive value (7). Additionally, decision curve analysis (DCA) widely recommended by many researchers (8, 9), was also used to evaluate the clinical utility of the nomogram in this study.

RESULTS

Clinical and Pathological Characteristics

In total, 5253 patients with MPC involving lung cancer were extracted from the SEER database, and 3465 (66.0%) dual

Abbreviations: MPC, multiple primary cancers; MPLC, multiple primary lung cancers; LCSPM, lung cancer as a second primary malignancy; FPC, first primary cancer; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; OS, overall survival; HR, hazard ratio; SEER, Surveillance, Epidemiology, and End Results.

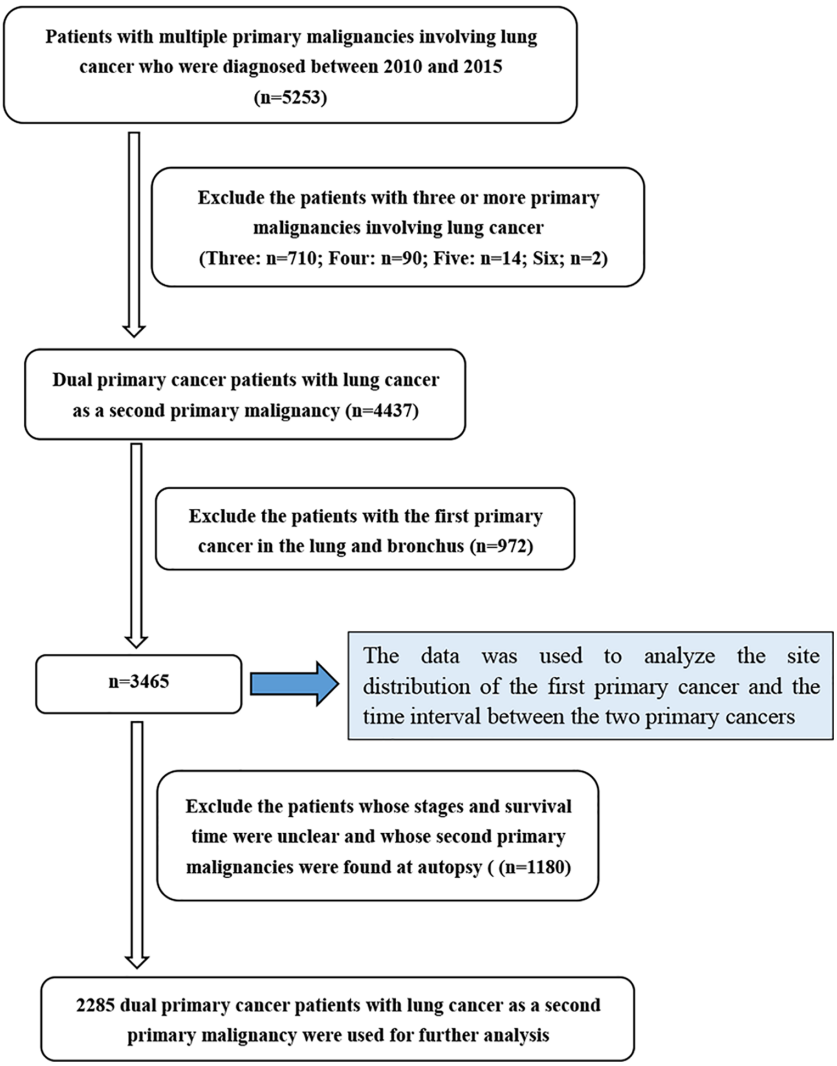

FIGURE 1 | Flow chart detailing the selection of the patients in this study.

primary cancer patients with LCSPM were used to analyze FPC site distribution and the time interval between the two primary cancers. Furthermore, of the 3465 patients, 2285 had complete information and were randomly divided into a training cohort (n=1601) and a validation cohort (n=684) according to 7:3. Their clinicopathological characteristics are presented in **Table 1**. As can be seen from the table, in the training and validation cohorts, the majority of patients were aged over 65, male, and white. Among these patients, adenocarcinoma had the highest frequency, followed by squamous cell carcinoma (in the entire cohort, 37% of patients presented with adenocarcinoma, 22.3% with squamous cell carcinomas, and 9.3% with small cell cancer). Additionally, patients with stage I lung cancer had a significant proportion in the training and validation cohorts, accounting for 37.6% and 35.2%, respectively. However, in the training and validation cohorts, only 34.6% and 32.4% of patients received lung cancer-directed surgery, respectively. Nonsurgical patients numbered significantly more than surgical patients, accounting for more than 50% in the two cohorts. Additionally, more and more cancer patients were diagnosed with primary lung cancer as the years of diagnosis increased. The incidence rate increased from 3.5% in 2010 to 27.4% in 2015. Given this, we explored the clinicopathological characteristics of patients from 2010 to 2015 (**Table 2**). In every single year, the proportion of men was more than that of women, and adenocarcinoma was still the most common histological type, followed by squamous cell carcinoma. In addition, the proportion of patients with stage I was higher than that of patients with other stages (stage II, III, and IV), and the number of nonsurgical patients was also more than that of surgical patients, and the proportion of nonsurgical patients appeared to be increasing year by year.

TABLE 1 | Demographic and clinicopathological characteristics of the training and validation cohorts.

Variables	Entire cohort (n=2285) (N, %)	Training cohort (n=1601) (N, %)	Validation cohort (n=684) (N, %)
Age (years)			
<65	622 (27.2)	436 (27.2)	186 (27.1)
>= 65	1663 (72.8)	1165 (72.8)	498 (72.9)
Sex			
Female	877 (38.4)	616 (38.4)	261 (38.1)
Male	1408 (61.6)	985 (61.6)	423 (61.9)
Race			
White	1862 (81.5)	1303 (81.4)	559 (81.7)
Black	252 (11.0)	175 (10.9)	77 (11.2)
Other	171 (7.5)	123 (7.7)	48 (7.1)
Histology of lung cancer			
Adenocarcinoma	863 (37.8)	618 (38.6)	245 (35.8)
Squamous cell carcinomas	510 (22.3)	366 (22.9)	144 (21.1)
Small cell cancer	213 (9.3)	142 (8.8)	71 (10.4)
Others	699 (30.6)	475 (29.7)	224 (32.7)
Location of FPC			
Prostate	486 (21.3)	331 (20.6)	155 (22.6)
Female Breast	308 (13.5)	210 (13.1)	98 (14.4)
Urinary Bladder	238 (10.4)	174 (10.9)	64 (9.3)
Others	1253 (54.8)	886 (55.4)	367 (53.7)
Stage of lung cancer			
Stage I	843 (36.9)	602 (37.6)	241 (35.2)
Stage II	217 (9.5)	159 (9.9)	58 (8.5)
Stage III	414 (18.1)	298 (18.7)	116 (16.9)
Stage IV	811 (35.5)	542 (33.8)	269 (39.4)
Surgery			
No	1511 (66.1)	1048 (65.4)	463 (67.6)
Yes	774 (33.9)	553 (34.6)	221 (32.4)
Interval (months)			
<24	1391 (60.9)	986 (61.5)	405 (59.2)
24 - 47	695 (30.4)	479 (30.0)	216 (31.5)
48 - 72	199 8.7)	136 (8.5)	63 (9.3)
Year of diagnosis			
2010	81 (3.5)	59 (3.6)	22 (3.3)
2011	228 (10.0)	166 (10.4)	64 (9.3)
2012	344 (15.1)	239 (14.9)	105 (15.4)
2013	436 (19.1)	301 (18.8)	135 (19.7)
2014	569 (24.9)	394 (24.7)	175 (25.6)
2015	625 (27.4)	442 (27.6)	183 (26.7)

The Site Distribution of FPC and the Time Interval Between Two Primary Cancers

Among the 5253 LCSPM patients, 4437 were dual primary cancers, and 710 were triple primary cancers, 90 were four primary cancers, 14 were five primary cancers, and 2 were six primary cancers (**Figure 1**). There were 76 sites of the FPC, and the most common site was prostate (20.8%), followed by female breast (13.4%) and urinary bladder (11.0%) (**Figure 2** and **Table 3**), for which median interval time was, respectively, 26, 52, and 24 months. Compared with the longest interval of 52 months for female breast cancer patients, patients with pancreatic cancer had the shortest median interval (3.5 months) for developing a primary malignant tumor in the lung (**Table 3**). Additionally, for the entire study population, the median time interval was 21 months (range: 3.5–52 months) as shown in **Table 3**. The time interval of most patients was less than 24 months in the training cohort (61.5%) and validation cohort (59.2%). The proportion of these patients with interval time over 48 months was less than 10% in the two cohorts (**Table 1**).

Prognosis Factors for Overall Survival

After a univariate Cox analysis of 1601 patients in the training cohort, the results showed that age, gender, histology, AJCC stage, FPC location, and surgery were all related to the survival prognosis of these patients (Log-rank test, all $p < 0.05$; **Table 4**). The same finding was also observed in the multivariate Cox analysis. The abovementioned factors were all regarded as independent prognostic factors on which the Kaplan-Meier survival analysis was also further performed as shown in **Figure 3**. It can be seen from **Table 4** and **Figure 3** that the prognosis of patients over 65 years old was worse than that of patients under the age of 65 (HR = 1.18, $p = 0.024$) and 3-year OS rates were 33.6% and 39.3%, respectively (log-rank test, $p = 0.023$). Men were associated with a worse 3-year OS compared to women (30.4% vs. 42.8%, $p < 0.001$). The later the stage of lung cancer, the worse the prognosis (log-rank test, $p < 0.001$). Lung cancer-directed surgery could significantly improve long-term survival (HR = 0.22, $p < 0.001$). The prognosis of patients with urinary bladder cancer as FPC was the worst in the Kaplan-Meier survival analysis, univariate, and multivariate Cox analysis (log-rank test, all $p < 0.05$). The prognosis of patients with squamous cell carcinoma was between small cell lung cancer (SCLC) and adenocarcinoma, and 3-year OS rates were 30.7%, 11.8%, and 37.0%, respectively (log-rank test, all $p < 0.05$).

Considering the great difference in biological behavior and prognosis between NSCLC and SCLC, we separately analyzed the survival of these patients. Age, gender, AJCC stage, FPC location, and surgery were all regarded as related to the survival prognosis of NSCLC patients (log-rank test, all $p < 0.05$; **Figure 4** and **Table S1**). However, for patients with SCLC as a second primary malignant tumor, age, gender, and FPC location did not affect the prognosis, and surgery alone was considered to be an independent prognostic factor for patients (**Figure 4** and **Table S1**). In addition, in the univariate Cox analysis, we found that the time interval between two primary cancers was not related to the long-term survival of NSCLC and SCLC patients (all $p > 0.05$). There was also no correlation between the prognosis and the year of diagnosis (all $p > 0.05$).

Development and Validation of the Prognosis Nomogram

A prognosis nomogram was developed to predict 1-, 3-, and 5-year OS rates on the basis of 1601 patients in the training cohort. The established nomogram included all statistically significant prognostic factors in the Cox proportional hazard model, involving age, gender, histology, AJCC stage, FPC location, and surgery (**Figure 5**). According to the different classifications of each characteristic, points were projected upward to get the score of each item. The total points were calculated by adding all the

TABLE 2 | Clinicopathological characteristics of dual primary cancers patients with LCSPM between 2010 and 2015.

Variables	2010 (n = 81)	2011 (n = 230)	2012 (n = 344)	2013 (n = 436)	2014 (n = 569)	2015 (n = 625)
Age (years)	68.72 (11.44)	70.50 (10.03)	69.11 (10.60)	70.18 (9.72)	70.41 (9.60)	70.53 (9.60)
Sex						
Female	37 (45.68)	90 (39.13)	127 (36.92)	148 (33.94)	212 (37.26)	263 (42.08)
Male	44 (54.32)	140 (60.87)	217 (63.08)	288 (66.06)	357 (62.74)	362 (57.92)
Histology of lung cancer						
Adenocarcinoma	28 (34.57)	89 (38.70)	128 (37.21)	176 (40.37)	208 (36.56)	234 (37.44)
Squamous cell carcinomas	16 (19.75)	53 (23.04)	77 (22.38)	93 (21.33)	125 (21.97)	146 (23.36)
Small cell cancer	8 (9.88)	15 (6.52)	37 (10.76)	36 (8.26)	52 (9.14)	65 (10.40)
Others	29 (35.80)	73 (31.74)	102 (29.61)	131 (30.05)	184 (32.34)	180 (28.80)
Location of FPC						
Prostate	17 (20.99)	46 (20.0)	78 (22.67)	92 (21.10)	125 (21.97)	128 (20.48)
Female Breast	17 (20.99)	29 (12.61)	39 (11.34)	55 (12.61)	72 (12.65)	96 (15.36)
Urinary Bladder	7 (8.64)	28 (12.17)	34 (9.88)	47 (10.78)	67 (11.78)	55 (8.80)
Others	40 (49.38)	127 (55.22)	193 (56.10)	242 (55.50)	305 (53.60)	346 (55.36)
Interval (months)	4.11 (2.44)	8.32 (4.99)	14.93 (9.11)	19.42 (12.34)	24.21 (15.63)	30.41 (19.84)
Stage of lung cancer						
Stage I	36 (44.44)	89 (38.70)	126 (36.63)	150 (34.40)	204 (35.85)	238 (38.08)
Stage II	8 (9.88)	25 (10.87)	33 (9.59)	44 (10.09)	41 (7.21)	66 (10.56)
Stage III	11 (13.58)	45 (19.57)	77 (22.38)	71 (16.28)	107 (18.80)	103 (16.48)
Stage IV	26 (32.10)	71 (30.87)	108 (31.40)	171 (39.22)	217 (38.14)	218 (34.88)
Surgery for lung cancer						
No	47 (58.02)	141 (61.3)	213 (61.92)	283 (64.91)	395 (69.42)	432 (69.12)
Yes	34 (41.98)	89 (38.7)	131 (38.08)	153 (35.09)	174 (30.58)	193 (30.88)

Continuous variables (age and interval) are presented as mean and standard deviation, and categorical variables are presented as numbers and percentages.

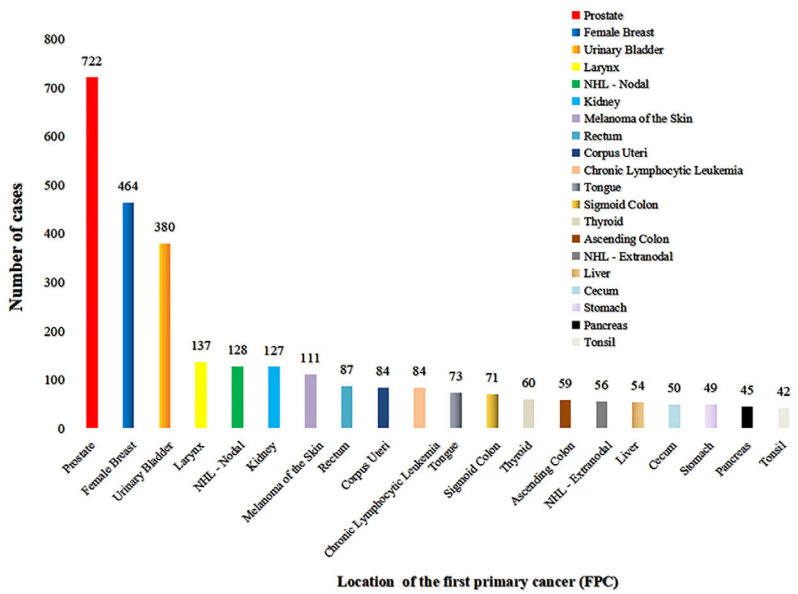

FIGURE 2 | The site distribution of FPC. There were 76 sites of FPC, and the most common site was the prostate (722), followed by female breast (464), and urinary bladder (380) (excluding patients with the first primary cancer in the lung and bronchi). Only the location distribution of more than 40 cases was shown here.

points, and then the survival rate of patients were calculated by projecting the total points downward. The higher the score was, the worse the survival prognosis was. This nomogram can be used to predict the survival rate of different patients according to their own conditions, thereby improving the efficiency and accuracy of prediction. In this study, the established nomogram was verified by the bootstrap method with 100 resamples in the training (n=1601) and validation (n=684) cohorts. The C-index of internal validation was 0.760 (95% CI: 0.744–0.776), and that of external validation was 0.759 (95% CI: 0.737–0.781). The corresponding calibration curves of 1-, 3-, and 5-year OS rates in training and validation cohorts are also shown

TABLE 3 | Location of the first primary cancer (FPC) and median interval between two primary cancers.

Location of FPC	N (%)	Median interval (months)
Total	3465 (100)	21
Prostate	722 (20.8)	26
Female Breast	464 (13.4)	52
Urinary Bladder	380 (11.0)	24
Larynx	137 (3.95)	9.5
NHL - Nodal	128 (3.69)	17.5
Kidney	127 (3.67)	11
Melanoma of the Skin	111 (3.20)	40.5
Rectum	87 (2.51)	23
Corpus Uteri	84 (2.42)	11.5
Chronic Lymphocytic Leukemia	84 (2.42)	37.5
Tongue	73 (2.11)	18.5
Sigmoid Colon	71 (2.05)	24.5
Thyroid	60 (1.73)	29
Ascending Colon	59 (1.70)	31.5
NHL - Extranodal	56 (1.62)	18.5
Liver	54 (1.56)	30.5
Cecum	50 (1.44)	16
Stomach	49 (1.41)	15
Pancreas	45 (1.30)	3.5
Others	625 (18.1)	8.5

in **Figures 6** and **S1**, from which we can see that all calibration curves are close to the ideal 45° dotted line. This indicates that the predicted value of the model had good consistency with the actual observed value. In addition, all DCA curves in training and validation cohorts also indicated the model had relatively ideal clinical utility (**Figures 6** and **S1**).

DISCUSSION

In recent years, with the continuous advancement of medical technology and the improvement of patient compliance, many cancer patients have been diagnosed with new primary malignant tumors in their lungs. In the past, a large number of studies have focused on single primary lung cancer or multiple primary lung cancer (MPLC), but there are few studies on lung cancer patients with other primary malignancies. To date, little is known about the regularity of the time interval between two primary malignancies and the prognosis of dual primary cancer patients with LCSPM. Thus, this study retrospectively analyzed the clinical characteristics of 3465 dual primary cancer patients with LCSPM extracted from the SEER database between 2010 and 2015, intending to improve the understanding of these diseases and provide a certain reference for future clinical work.

During the follow-up of cancer patients, clinicians tend to focus more on the organ where the primary tumor is located and other organs where the tumor is more likely to metastasize, which will inadvertently ignore the risk of developing a primary malignancy in other organs. Lung cancer, a malignant tumor with a high incidence rate and mortality rate, poses a serious threat to public health. Thus, it is of great clinical significance to clarify the common sites of FPC in LCSPM patients to improve the effectiveness of follow-up and vigilance of cancer patients. Through analysis of 185 patients with MPC involving lung cancer from Guangdong Lung Cancer Research Institute from 2005 to 2013, Li et al. found that colorectal cancer, esophageal cancer, and thyroid cancer were the tumors that most frequently accompanied lung cancer (10). Liu et al. also reported that the most common tumors associated with lung cancer were upper aerodigestive tract cancer, colorectal cancer, and cervical cancer (1). In this study, we found that, in 3465 dual primary cancer patients with LCSPM, the most common organ of FPC was prostate, followed by female breast, and urinary bladder, accounting for 20.8%, 13.4%, and 11.0%, respectively. Obviously, the findings of these studies were significantly different. We believe that, in addition to the different sample size, the reasons for this phenomenon might also be related to geographical environment (China/American), ethnic differences, and research design (different from them, the cases with FPC in the lung and bronchus were excluded in our study). Despite the differences, all the findings suggest that cancer patients were still at risk of developing new primary malignant tumors in their lungs. Thus, cancer patients, as well as clinicians, should pay close attention to the changes of the lung or other organs and be alert to the occurrence of lung cancer or other malignant tumors during follow-up. Of course, we should also note that periodic follow-up to find a new primary tumor in the lung is a kind of cancer screening for high-risk populations. These patients usually have a long history of smoking, exposure to chemicals, family history of lung cancer, etc.

Definitely, understanding the time interval between two primary cancers can assist clinicians to develop better follow-up strategies for cancer patients. Li and his colleagues found that the median interval between two primary cancers in MPC patients was 41.2 months (10). Liu et al. also observed that, when lung cancer was the second primary cancer, the interval time between the two primary malignancies was 46 months (1). The findings were longer than that of our study (the median interval was 21 months in our study), which may be related to the inclusion criteria and sample size of the study. Because there was no recognized diagnostic criteria for MPLC, our study excluded the cases with lung cancer as FPC and included 3465 dual primary cancer patients with LCSPM from the SEER database with significantly more cases than other studies (there were only 185 cases in Li's study and 142 cases in Liu's study). To the best of our knowledge, this is one of the largest studies on this topic. In daily clinical practice, how long and how often to follow up after the diagnosis of FPC is a matter in hand. Our study found that the median interval between the FPCs (prostate cancer, female breast cancer, and urinary bladder cancer) and lung cancer (the second primary cancer) were 26 months, 52 months, and 24 months, respectively. Additionally, for the entire study cohort, the median interval between the FPC and second primary cancer (lung cancer) was 21 months, the shortest interval was 2 months, and the longest was 81 months. This indicates that patients with cancer are still at the risk of developing another new primary malignancy in the lungs. Close, lifelong follow-up was recommended for all cancer patients not only to detect recurrence or metastasis, but also to detect early-stage primary tumors in the lungs or other organs.

In this study, we observed that age, sex, histology, stage, and surgery were all closely related to the prognosis of these patients. Advanced age (> 65 years old) and being male were independent

TABLE 4 | Univariate and multivariate Cox analysis for these patients in the training cohort.

Variables	Univariate Cox analysis HR (95% CI)	P value	Multivariate Cox analysis HR (95% CI)	P value
Age (years)				
<65	Reference		Reference	
>= 65	1.18 (1.02-1.36)	0.024	1.25 (1.08-1.45)	0.003
Sex				
Female	Reference		Reference	
Male	1.45 (1.27-1.65)	<0.001	1.28 (1.08-1.51)	0.004
Race				
White	Reference		—	
Black	0.98 (0.80-1.20)	0.865	—	
Other	1.07 (0.85-1.35)	0.558	—	
Histology of lung cancer				
Adenocarcinoma	Reference		Reference	
Squamous cell carcinomas	1.21 (1.02-1.42)	0.024	1.21 (1.03-1.43)	0.022
Small cell cancer	2.13 (1.73-2.62)	<0.001	1.34 (1.08-1.65)	0.007
Others	0.87 (0.74-1.02)	0.089	1.13 (0.96-1.32)	0.147
Location of FPC				
Prostate	Reference		Reference	
Female Breast	0.67 (0.53-0.84)	<0.001	1.21 (0.91-1.61)	0.199
Urinary Bladder	1.29 (1.03-1.61)	0.024	1.53 (1.23-1.92)	<0.001
Others	0.85 (0.72-0.99)	0.046	1.35 (1.13-1.61)	<0.001
Stage of lung cancer				
Stage I	Reference		Reference	
Stage II	1.74 (1.35-2.23)	<0.001	1.80 (1.44-2.32)	<0.001
Stage III	2.70 (2.23-3.29)	<0.001	1.80 (1.46-2.21)	<0.001
Stage IV	6.36 (5.39-7.51)	<0.001	3.90 (3.24-4.70)	<0.001
Surgery				
No	Reference		Reference	
Yes	0.22 (0.18-0.25)	<0.001	0.36 (0.30-0.44)	<0.001
Interval (months)				
<24	Reference		—	
24 - 47	1.08 (0.94-1.24)	0.277	—	
48 - 72	0.86 (0.65-1.13)	0.276	—	
Year of diagnosis (year)				
2010	Reference		—	
2011	1.03 (0.74-1.45)	0.852	—	
2012	1.06 (0.76-1.47)	0.741	—	
2013	1.14 (0.83-1.58)	0.424	—	
2014	1.06 (0.77-1.47)	0.702	—	
2015	0.88 (0.63-1.22)	0.441	—	

risk factors for patients. Compared with nonsurgical treatment, lung cancer-directed surgery could significantly improve OS of these patients, with 3-year OS rates of 18.0% and 66.0%, respectively. SCLC had the worst prognosis. The later the stage of lung cancer, the worse the prognosis. This was also in line with the findings of other studies (11, 12). Massard et al. (11) reported that the survival of LCSPM patients was associated with the stage of lung cancer. Kim et al. (12) also found advanced lung cancer stage was a poor prognostic factor for patients with MPC involving lung cancer. In addition, some retrospective research has demonstrated that patients with MPC involving lung cancer tended to have the better long-term survival than ordinary lung cancer population (1, 4, 13). However, so far there are few studies on whether the prognosis of LCSPM is related to another primary malignancy. This study found that the 3-year OS of LCSPM patients with urinary bladder cancer as FPC was significantly lower than that of patients with other primary malignancies as FPC. It should be noted that lung cancer here referred only to NSCLC, and the prognosis of dual primary cancer patients with SCLC as a second primary malignancy had no relation to the FPC. Kim et al. (12) observed that cancer patients with another primary malignancy in the head and neck tended to have a worse prognosis than these patients with another primary malignancy elsewhere. Unfortunately, due to so few cases (less than 1.3%) with FPC in the head and neck, our study did not separately compare the prognosis of these patients with those of other patients, which may result in different results.

Additionally, our study found that, since 2010, more and more cancer patients were diagnosed with another new primary tumor in their lungs. This trend was mainly related to the following points. First, the age of the population was prolonged. Second, more and more chemicals were coming into contact. The third was the influence of bad habits, such as cigarettes. The fourth were the advances in imaging technology and the increasing pace of life. Finally, an important factor was the increasing awareness of early lung cancer screening. Several studies (14, 15) have demonstrated

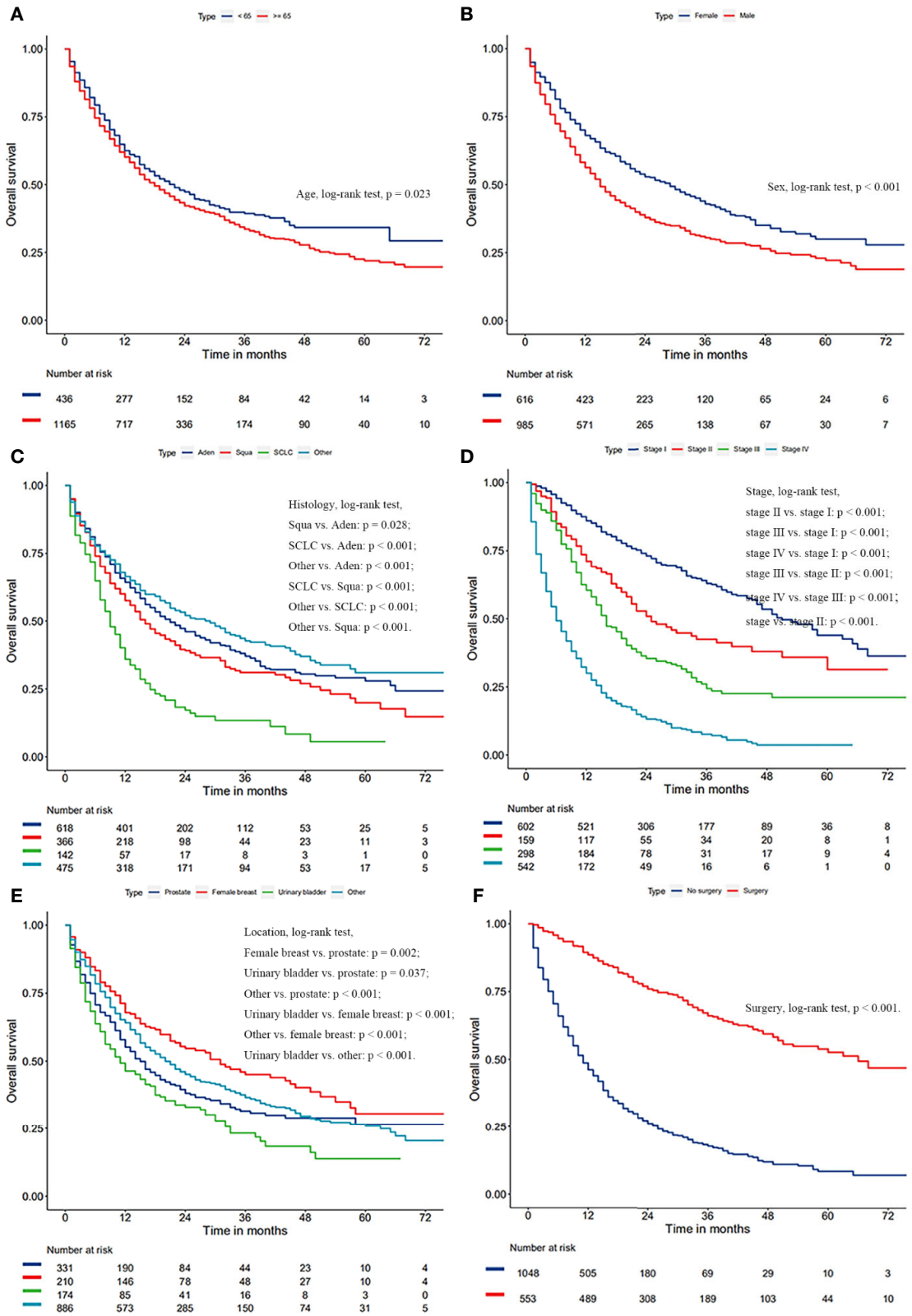

FIGURE 3 | Kaplan-Meier survival curves of overall survival based on age **(A)**, gender **(B)**, histology of lung cancer **(C)**, AJCC stage of lung cancer **(D)**, surgery **(E)**, and location of FPC **(F)**.

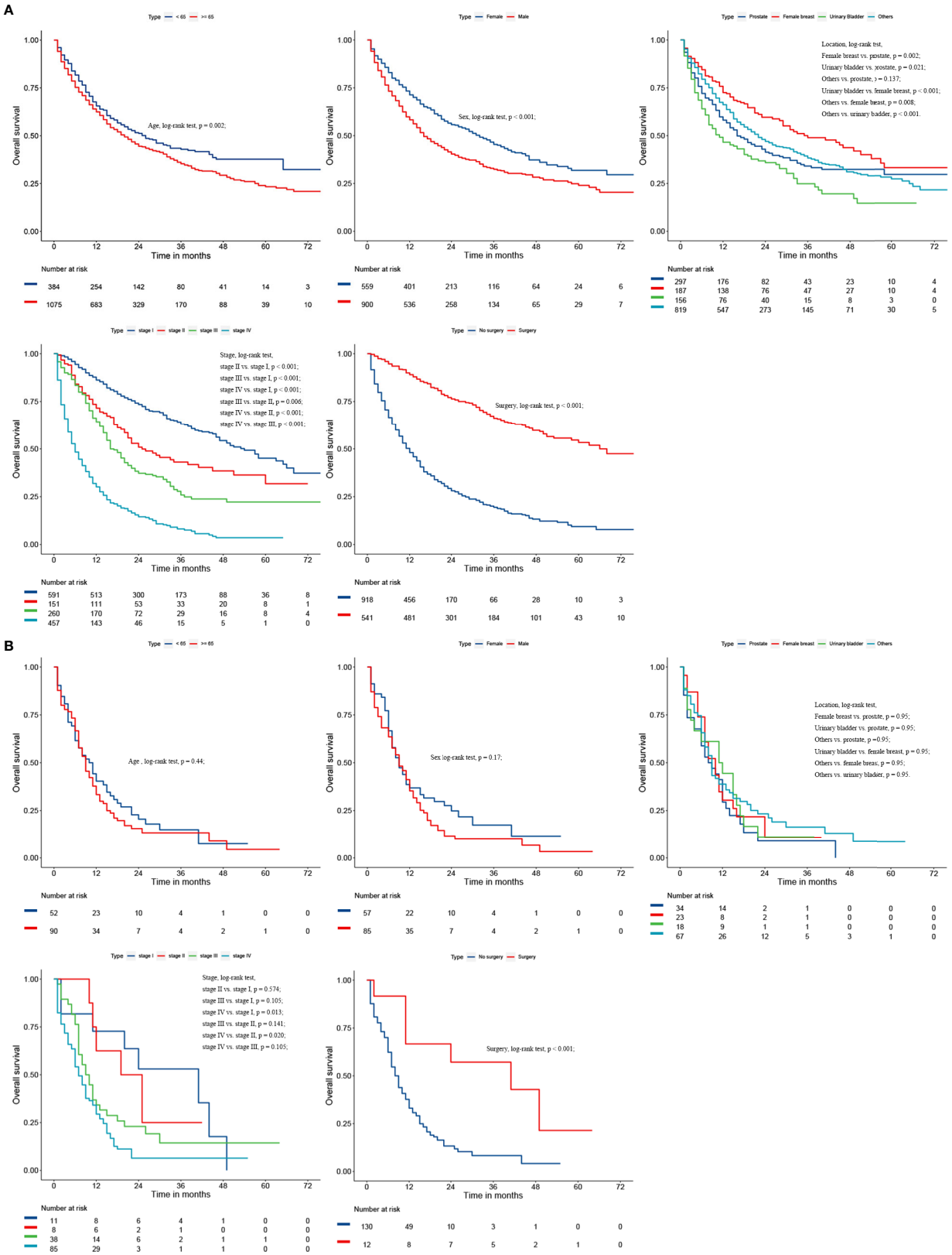

FIGURE 4 | Kaplan-Meier survival curves of overall survival for NSCLC **(A)** and SCLC **(B)**.

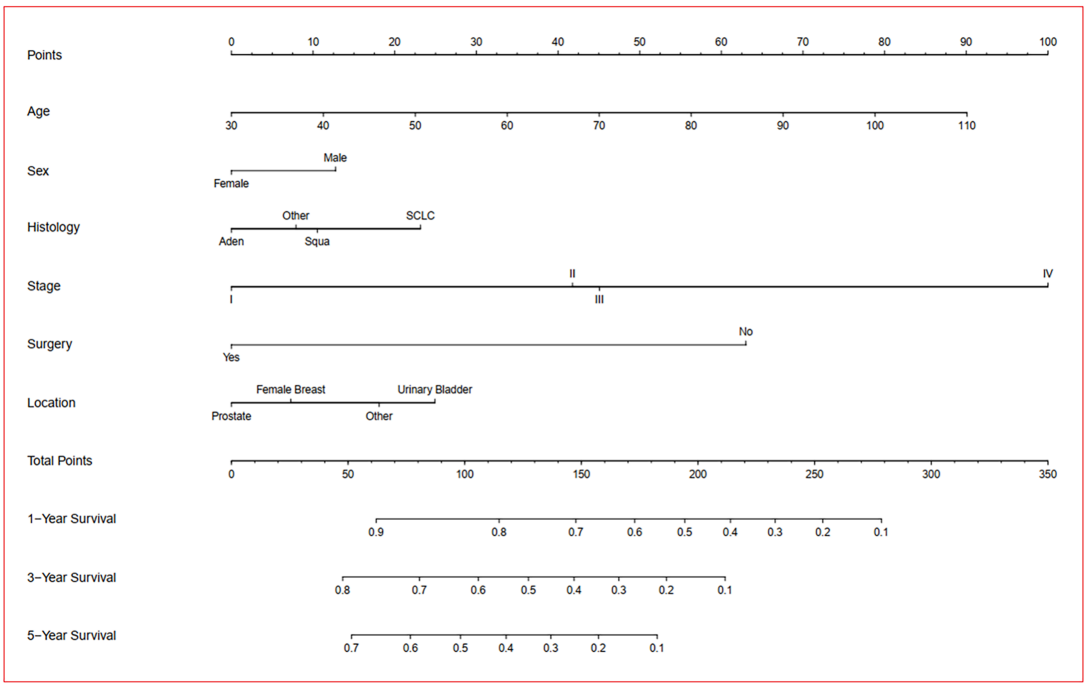

FIGURE 5 | Prognostic nomogram of overall survival in dual primary cancer patients with LCSPM. Nomogram to predict 1-, 3-, and 5-year OS rates of the patients. The factors of age, sex, histology, stage, location of FPC, and surgery were included in the model. Aden: adenocarcinoma; Squa: squamous cell carcinomas; SCLC: small cell lung cancer.

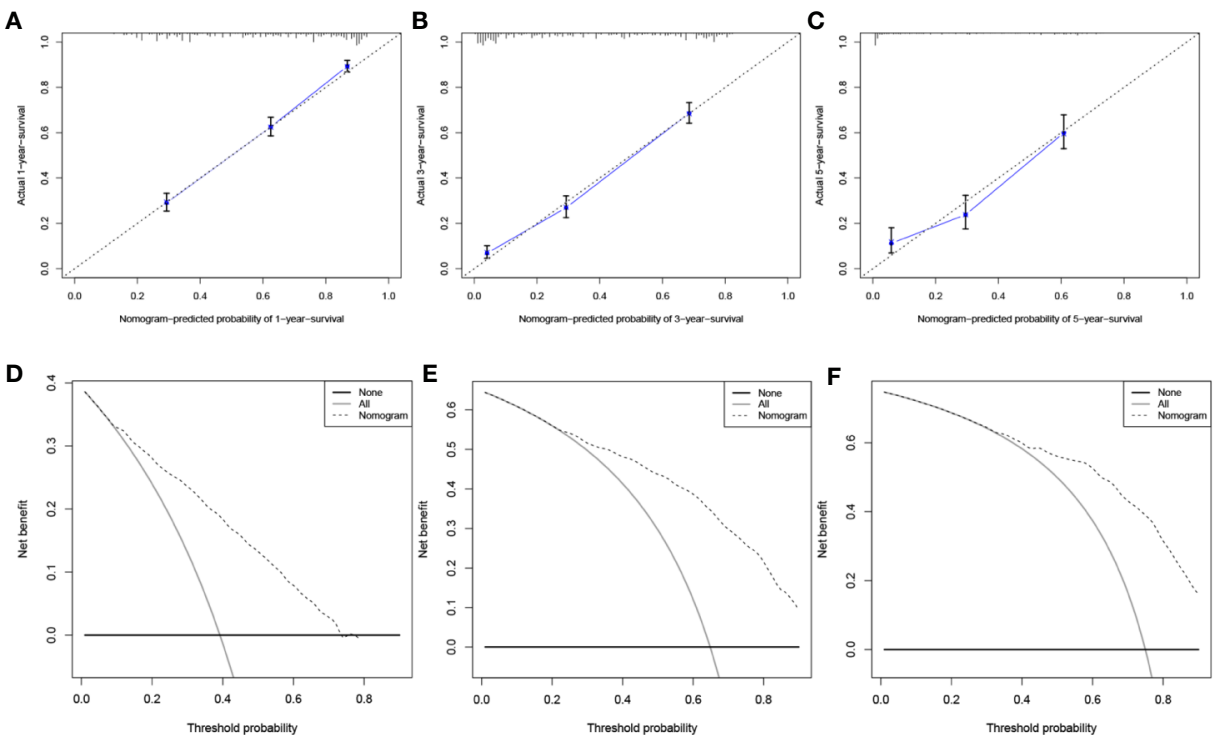

FIGURE 6 | Evaluation of the prognostic nomogram. Calibration curves for 1-year **(A)**, 3-year **(B)**, and 5-year **(C)** OS in the training cohort. DCA curves for 1-year **(D)**, 3-year **(E)**, and 5-year **(F)** OS in the training cohort.

that cancer patients, compared to the general population, had a higher risk to develop new primary tumors. Therefore, we believe that, even if the primary tumor has undergone radical surgery, the cancer patient still needs long-term close follow-up. In addition to paying attention to changes in the organ where the primary tumor is located, changes in other organs should not be ignored.

Good prognosis evaluation is of great significance for the treatment and follow-up of cancer patients. Clinically, due to the lack of a relatively perfect scoring system, clinicians often make empirical judgments based on the patients' age, AJCC stage and pathological results. As an emerging tool widely used in some clinical research (5, 6, 16), a nomogram can integrate the influence of various prognostic factors in the clinic and present the results visually. Compared with traditional methods, it can make predictions more quickly and accurately, and its predictive value has been considered superior to other evaluation systems (17, 18). Thus, a prognosis nomogram was also applied in this study. From the established nomogram, we could intuitively see the influence of each independent prognostic factor on score points. Considering the good prediction performance and clinical utility of this nomogram were fully proven in both internal and external validation sets, this clinical nomogram is expected to be routinely applied to the survival prediction of such patients in the future.

Our study has the following advantages. First, we used the large sample size of the SEER database to determine the common sites of FPC and the median interval between the two primary malignancies in dual primary cancer patients with LCSPM, which was of great significance in improving the effectiveness of follow-up in cancer patients. Second, our study was the first attempt to use a nomogram to predict the survival of dual primary cancer patients with LCSPM, which included 2285 patients from the SEER database, and its data accuracy was up to 95% (19). Third, our preliminary findings can help clinicians understand this disease better and serve as a basis for future, larger multicenter studies.

Admittedly, our study also has some shortcomings. First, the limitations of the SEER database widely discussed in previous studies (20, 21). Second, research on MPC involving lung cancer is still lacking, and thus, the understanding of this special population remains limited. Although this is a multicenter study with a large sample size, this SEER-based study can still not provide important survey information on the risk of multiple primary cancer due to the limitations of the SEER database, including smoking status, genetic conditions (such as gene mutation), family history, exposure history (chemicals), organ transplantation, or chronic immunosuppression to name a few. In the end, this study, as a retrospective analysis, inevitably leads to selective bias. Taking into account the deficiencies of retrospective research, prospective analysis is recommended to proceed further.

CONCLUSION

In summary, dual primary cancer patients with LCSPM have approximately 59.3% of 1-year OS, 34.7% of 3-year OS, and 25.2% of 5-year OS, respectively. Systematic and periodic follow-up is recommended for all cancer patients, and other organs should not be ignored in the follow-up of cancer patients. Early detection for surgical treatment will significantly improve the prognosis of these patients.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://seer.cancer.gov/data/.

ETHICS STATEMENT

The present study was approved by the Institutional Research Committee of Zhongnan Hospital of Wuhan University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CS, WH, and SL: designed the study. CS, SL, and WH: reviewed relevant literature and drafted the manuscript. CS, QW, and YW conducted all statistical analyses. 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.2020.515606/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Evaluation of the prognostic nomogram. Calibration curves for 1-year **(A)**, 3-year **(B)**, and 5-year **(C)** OS in the validation cohort. DCA curves for 1-year **(D)**, 3-year **(E)**, and 5-year **(F)** OS in the validation cohort.

SUPPLEMENTARY TABLE 1 | Univariate and multivariate Cox analysis for NSCLC and SCLC patients in the training cohort.

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Distinct *EGFR* Mutation Pattern in Patients With Non-Small Cell Lung Cancer in Xuanwei Region of China: A Systematic Review and Meta-Analysis

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Background: In the Xuanwei region of China, lung cancer incidence and mortality are among the highest in China, attributed to severe air pollution generated by combustion of smoky coal. No study has yet comprehensively evaluated the prevalence of epidermal growth factor receptor (*EGFR*) mutation characteristics in patients with non-small cell lung cancer (NSCLC) in Xuanwei. This meta-analysis was designed to analyze the *EGFR* mutation pattern in NSCLC patients in Xuanwei region of Yunnan Province in China.

Methods: Electronic databases were comprehensively searched and relevant literatures were retrieved. The odds ratio (OR) for *EGFR* mutations between Xuanwei region and non-Xuanwei region was calculated, and the absolute incidence of *EGFR* mutations in Xuanwei was pooled by mutation subtype.

Results: Seven studies involving 1,355 patients with NSCLC from Yunnan Province (442 in Xuanwei and 913 in other regions) were included. The *EGFR* mutation rate ranged between 30.19% and 55.56%. Higher uncommon *EGFR* mutations (OR: 5.69, 95%CI: 2.23–14.49, P<0.001) and lower common *EGFR* mutations (OR: 0.18, 95%CI: 0.07–0.45, P<0.001) were found in Xuanwei region, compared with non-Xuanwei region. Specifically, the uncommon *EGFR* mutation rate was 59.50% and common *EGFR* mutation rate was 40.50% in Xuanwei. The mutation incidence of exon 18 G719X (OR: 3.21, 95%CI: 1.48–6.97, P=0.003), exon 20 S768I (OR: 6.44; 95%CI: 2.66–15.60; P<0.001), and exon 18 G719X + 20 S768I (OR: 6.55; 95%CI: 1.92–22.33; P=0.003) in Xuanwei were significantly higher, while the frequency of 19 deletion (OR: 0.28, 95%CI: 0.11–0.77, P<0.001) and 21 L858R mutation (OR: 0.51, 95%CI: 0.31–0.84, P=0.007) were lower.

Conclusions: The results highlight the distinct *EGFR* mutation spectrum of NSCLC patients in Xuanwei region compared with other regions, with higher uncommon

mutations but lower common mutations. The distinct Xuanwei featured genetic variations provide a unique model to further study carcinogenesis of lung cancer.

Keywords: lung cancer, *EGFR* mutation, subtype, China, Xuanwei

INTRODUCTION

Lung cancer has the highest morbidity and is the leading cause of cancer-related death worldwide, with 85% of patients having non-small-cell lung cancer (NSCLC) (1, 2). With the advances in molecular oncology, multiple genetic variants have been determined as therapeutic targets for lung cancer, and many onco-targeted drugs had been developed. Epidermal growth factor receptor (EGFR) is a well-accepted carcinogenic variant and driver gene in lung cancer. NSCLC patients with activating *EGFR* mutations are identified in about 40~60% of Asian and 10% of Western populations (3). When EGFR is activated, it can trigger intracellular signaling cascades that affect cellular proliferation, angiogenesis, and apoptosis through transmembrane receptors (4). Studies showed that EGFR tyrosine kinase inhibitors (TKIs) confer better outcome in patients with the *EGFR* common mutations (exon 19 deletion, exon 21 L858R point mutation) (5). A genetic divergence of *EGFR* mutation rates was demonstrated according to ethnicity in previous research (6, 7), and the frequency of *EGFR* was highest among Asians (47%) and lowest among Oceanians (12%) (8).

Xuanwei is a small city located in Yunnan Province of China. This region is one of the major coal-producing regions in Yunnan Province and renowned for distinct lung cancer characteristics (9–11): first, lung cancer incidence in Xuanwei region is regionally specific with a high incidence rate and mortality rate. Second, the mortality rate of females is relatively high and almost all of them are never smokers, 20 times higher than other regions of China, and it is among the highest in the world for female lung cancer mortality. Additionally, the onset of lung cancer happens at a relatively younger age in the Xuanwei region, which occurs in younger than the peak age of onset of lung cancer in other parts of China by more than 10 years (12). Environmental factors are known to play a role in lung cancer development, and indoor air pollution from the use of smoky coal for household purposes has been suggested to be the cause of the high incidence of lung cancer in Xuanwei, especially in female patients with non-smoking history (13). Previously, a small-scale study revealed that non-smoking female NSCLC patients in Xuanwei harbored different *EGFR* mutation patterns when compared with other parts of Asia (14), suggesting that there may exist distinct genetic background in this ethnic group, certain susceptible populations, and unique environments. Therefore, it is of great significance to study the specific *EGFR* mutation patterns in NSCLC patients from the Xuanwei region of China, which may lead to better understanding the carcinogenesis of *EGFR*-mutated NSCLC and more effective targeted therapeutic interventions.

The *EGFR* mutation of NSCLC patients in Xuanwei region has not been fully understood; thus, we conducted this meta-analysis and systematic review to probe further into the *EGFR* mutation pattern of NSCLC patients in the Xuanwei region compared with the non-Xuanwei population in Yunnan Province to further effectively guide clinical treatment.

METHODS

Study design

To obtain a more precise estimate of EGFR mutation pattern in NSCLC patients of Xuanwei region, we pooled the prevalence of *EGFR* mutation (common and uncommon type) in Xuanwei region and control region (non-Xuanwei) in the rest of Yunnan Province where the level of air pollution and lung cancer incidence and mortality were comparable to most parts of China.

Common EGFR mutations (or classic mutations) are deemed as exon 19 deletion and exon 21 L858R substitution, accounting for approximately 90% of EGFR mutations in NSCLC (15, 16). Uncommon EGFR mutations are deemed as mutations other than 19 deletion and 21 L858R, and they account for 10% to 20% of all EGFR mutations; the substitution mutations of G719X in exon 18, L861Q in exon 21, S768I in exon 20, exon 20 insertions, and complex mutations are the most frequent among the uncommon mutations (17–19).

Search Strategy

A comprehensive literature search and systematic review of online databases PubMed, Embase, Web of Science, Medline, Cochrane Library, and Chinese Biomedical Literature Database was performed to identify relevant studies published before 6 November 2019 that examined EGFR mutation frequency in non-small cell lung cancer in southwest China's Yunnan Province. The search key words including "EGFR" AND "mutation" AND ("NSCLC" OR "lung cancer") AND ("Yunnan" OR "Xuanwei") were used. No search limitations were set. The relevant abstracts and presentations from conferences were also manually searched. Then, we examined the publication of the list of references and searched for additional research.

Inclusion and Exclusion Criteria

Eligible studies should meet the following criteria: (i) Publications describing the mutation subtype in *EGFR*-mutated NSCLC of Yunnan in southwestern China were retained. (ii) Mutation detection in paraffin-embedded tumor tissues or cytological specimens or blood samples can be included.

Studies were excluded if (i) data were insufficient to calculate the pooled incidence for this meta-analysis or (ii) they were review

articles, case reports, editorials, expert opinions, non-comparative studies, unrelated to research topics, or duplicate reports.

Data Extraction and Quality Assessment

This meta-analysis was conducted according to the Cochrane and Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (20). Two researchers (Li Lv and Zhichao Liu) independently performed all of the screening of studies and data extraction. The third researcher (Yang Liu) resolved the disagreements. For eligible research, we extract all available information: the first name of the author, year of publication, region, research type, number of patients, gender, age, smoking status, *EGFR* mutations, detection methods, tumor histology, disease stage, and metastases type. The quality of the included studies was assessed using the Agency for Healthcare Research and Quality Tool. Any disagreements were resolved through discussion and consensus.

Statistical Analysis

Pooled odds ratio (OR) of common and uncommon *EGFR* mutation rate between Xuanwei region and non-Xuanwei region in Yunnan province of China was calculated, and the pooled frequency of *EGFR* mutations in Xuanwei was also calculated. Subgroup pooled OR and incidence were generated according to *EGFR* mutation subtypes. Cochran's Q test and I^2 were used to estimate the heterogeneity effect among the studies. I^2 statistics more than 50% was suggestive of statistical heterogeneity between studies, and random effects model was used if heterogeneity existed. All tests were two-sided and a P value less than 0.05 was considered statistically significant. All statistical analyses were conducted with STATA 12.0 software (Stata Corporation, College Station, TX, USA) and R 3.4.1 software (R foundation for Statistical Computing, Vienna, Austria).

RESULTS

The Selection and Characteristics of Study

A total of 1,473 publications were retrieved through the initial literature search, of which 658 papers were excluded due to duplication. With title and abstract review, 10 potentially relevant articles were identified for detailed review (13, 14, 21–28). These articles were further assessed for eligibility by reviewing the full texts, and 3 articles (23–25) were removed due to insufficient data. Finally, a total of 7 articles (13, 14, 21, 22, 26–28) were identified as eligible to be included in the meta-analysis (**Figure 1**).

Table 1 summarized the characteristics of all included studies. Seven studies involving 1,355 NSCLC patients from different regions of Yunnan Province in southwestern China (442 from Xuanwei region, 913 from other region in the rest of Yunnan Province) were included. The majority of patients were adenocarcinoma and non-smoker. Four studies provided a comparison of *EGFR* mutations between Xuanwei and non-Xuanwei regions, while the other 3 studies (14, 26, 27) only

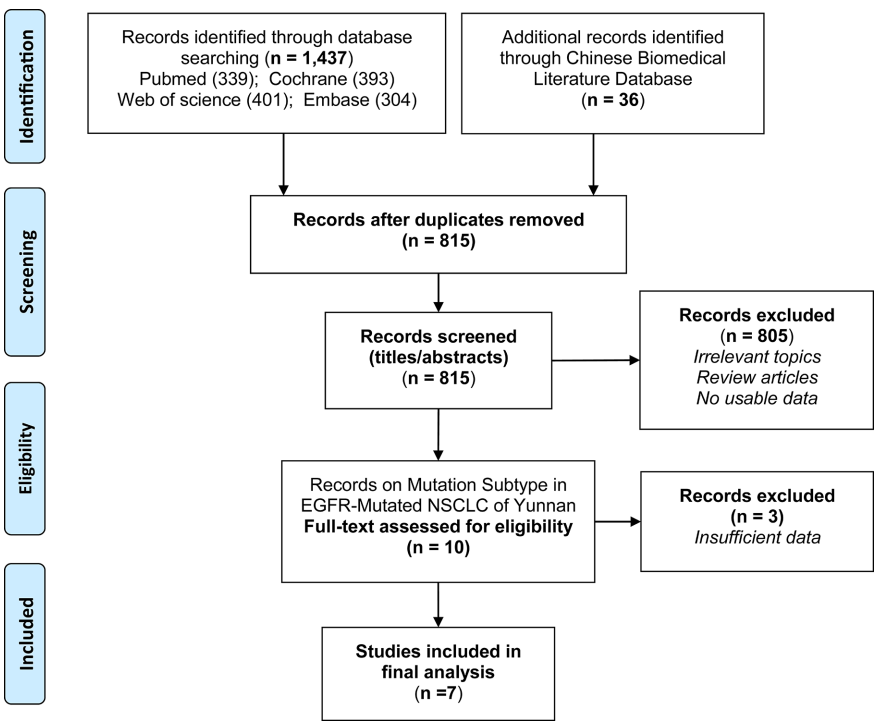

FIGURE 1 | Flow chart detailing the search strategy and identification of studies.

TABLE 1 | Characteristics of the included studies.

First author	Publish year	Region	Study type	Number of patients			*EGFR* mutation			Female (%)	Age(years)	Smoker (%)	Adenocarcinoma (%)	Stage	Lymph node metastasis (%)	Brain metastasis (%)	*EGFR* mutation detection methods	Scores Of AHRQ
				Xuanwei	Non-Xuanwei	Total	Yes	No	Rate									
Guo et al. (27)	2019	China, Xuanwei	Retro	85	NA	85	39	46	45.88%	41.18%	54 (36-78)	NG	80.00%	NG	NG	NG	Targeted NGS	10
Zhou et al. (28)	2018	China, Yunnan	Retro	20	192	212	64	148	30.19%	38.21%	57.1 (31-86)	44.81%	81.60%	I, II, III, IV	NG	18.87%	Super ARMS-PCR and dd PCR	11
Zhou et al. (21)	2017	China, Yunnan	Retro	63	384	447	156	291	34.90%	44.97%	<65 (69.4%); 65-75 (23.5%); >75 (7.2%)	47.43%	86.58%	I, II, III, IV	NG	14.32%	ARMS-PCR	11
Chen et al. (13)	2016	China, Yunnan	Retro	90	168	258	124	134	48.06%	52.33%	<=60 (68.6%); >60 (31.4%)	27.13%	87.60%	I, II, III, IV	29.07%	NG	ARMS-PCR	10
Yang et al. (22)	2016	China, Yunnan	Retro	81	169	250	131	119	52.40%	NG	NG	NG	NG	NG	NG	NG	ARMS-Taqman	10
Yang et al. (26)	2016	China, Xuanwei	Retro	63	NA	63	35	28	55.56%	42.86%	<50 (54.0%); >=50 (46.0%)	39.68%	84.13%	NG	17.46%	NG	ARMS-Taqman	10
Hosgood et al. (14)	2013	China, Xuanwei	Retro	40	NA	40	14	26	35.00%	NG	46.5 ± 10	0	80.00%	NG	NG	NG	SNaPshot and separate PCR-based sizing technique	10

Retro, Retrospective study; EGFR, Epidermal growth factor receptor; NA, Not Available; NG, Not given; AHRQ, Agency for Healthcare Research and Quality; NGS, Next generation sequencing; ARMS, super amplification refractory mutation system; PCR, polymerase chain reaction; dd PCR, Droplet Digital polymerase chain reaction; GS, gene sequencing.

evaluated *EGFR* mutations in Xuanwei region. In all included studies (13, 14, 21, 22, 26–28), the *EGFR* mutation status was mainly (5 out of 7) detected by Amplification Refractory Mutation System (ARMS), while next generation sequencing (NGS) or SNaPshot were used in the other 2 studies. Overall, the *EGFR* mutation positive rate was 30.19% to 55.56% across studies. All studies gained 10 to 11 scores in study quality assessment on a scale of 0 to 11 with the Agency for Healthcare Research and Quality Tool.

Frequency and Odds Ratio of EGFR Mutation in Xuanwei and Non-Xuanwei Regions

Four studies (13, 21, 22, 28) that simultaneously reported the *EGFR* mutation rate in Xuanwei and non-Xuanwei region were included in the comparative analysis. The difference in *EGFR* mutation rates is summarized according to regions (**Table 2**). One hundred and twenty-nine patients from Xuanwei region harbored *EGFR* mutations, with 64.34% (83/129) uncommon mutation and 35.66% (46/129) common mutation. In non-Xuanwei region, the incidence of uncommon and common *EGFR* mutation was 25.43% (88/346) and 74.57% (258/346) respectively. The frequency of uncommon mutations was higher in Xuanwei region than that in non-Xuanwei region (4 studies (13, 21, 22, 28) OR: 5.69, 95%CI: 2.23–14.49, P<0.001) (**Figure 2B**). By contrast, patients in Xuanwei regions were less likely to have common *EGFR* mutations (19 deletion or 21 L858R) compared with non-Xuanwei region (OR: 0.18, 95%CI: 0.07–0.45, P<0.001) (**Figure 2A**).

Overall Incidence of Common and Uncommon EGFR Mutation in Xuanwei Region

To further illustrate the distribution of common and uncommon *EGFR* mutations in Xuanwei region, 7 studies (13, 14, 21, 22, 26–28) were included to calculate the pooled incidence (**Table 3**). A total of 217 patients had *EGFR* mutation in Xuanwei region. The pooled incidence of uncommon *EGFR* mutation was 59.5% (95% CI: 53.2%–65.9%), while the pooled incidence of common *EGFR* mutation was 40.5% (95%CI: 34.1%–46.8%).

Subgroup Analysis of Different EGFR Mutation Subtypes in Xuanwei and Non-Xuanwei Regions

Patients from Xuanwei and non-Xuanwei regions with specific *EGFR* mutation subtypes were further analyzed (**Table 4**). Results showed that mutation of 18 G719X, 20 S768I, and 18 G719X + 20 S768I were more likely to appear in Xuanwei patients compared with non-Xuanwei patients (OR: 3.21, 95% CI: 1.48–6.97, P=0.003; OR: 6.44, 95%CI: 2.66–15.60, P<0.001, OR: 6.55, 95%CI: 1.92–22.33, P<0.05, respectively). In contrast, NSCLC patients of Xuanwei regions harbored lower frequency of 19 deletion (OR: 0.28, 95%CI: 0.11–0.77, P<0.001) and 21 L858R mutation (OR: 0.51, 95%CI: 0.31–0.84, P=0.007).

TABLE 2 | Comparison of the incidence of *EGFR* mutation of NSCLC patients between Xuanwei and non-Xuanwei regions.

			Xuanwei				Non-Xuanwei				META		
			Yes	No	Total	Rate	Yes	No	Total	Rate	OR	95%CI	*P*-value
Common mutation	Zhou et al. (28)	2018	3	5	8	37.50%	28	28	56	50.00%	0.600	(0.131-2.755)	
	Zhou et al. (21)	2017	8	19	27	29.63%	101	28	129	78.29%	0.117	(0.046-0.295)	
	Chen et al. (13)	2016	14	37	51	27.45%	63	10	73	86.30%	0.060	(0.024-0.149)	
	Yang et al. (22)	2016	21	22	43	48.84%	66	22	88	75.00%	0.318	(0.148-0.686)	
	Overall		**46**	**83**	**129**		**258**	**88**	**346**		**0.176**	**(0.069-0.448)**	**<0.001**
											(**Heterogeneity**: I^2 = 72.4%, p=0.0012)		
Uncommon mutation	Zhou et al. (28)	2018	5	3	8	62.50%	28	28	56	50.00%	1.667	(0.363-7.652)	
	Zhou et al. (21)	2017	19	8	27	70.37%	28	101	129	21.71%	8.567	(3.393-21.628)	
	Chen et al. (13)	2016	37	14	51	72.55%	10	63	73	13.70%	16.650	(6.720-41.256)	
	Yang et al. (22)	2016	22	21	43	51.16%	22	66	88	25.00%	3.143	(1.458-6.777)	
	Overall		**83**	**46**	**129**		**88**	**258**	**346**		**5.685**	**(2.231-14.486)**	**<0.001**
											(**Heterogeneity**: I^2 = 72.4%, p=0.0012)		

OR, odds ratio; CI, confidence interval.

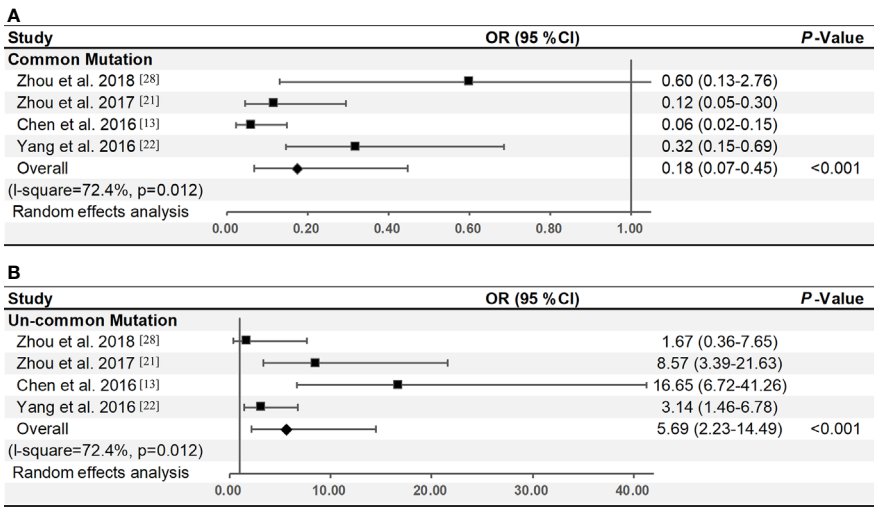

FIGURE 2 | Odds of common **(A)** or uncommon **(B)** EGFR mutation of Xuanwei compared to non-Xuanwei regions (four studies). The center of each square is the odds ratio (OR) for individual trials and corresponding horizontal line is the 95% CI. The broken line and center of the blue diamond is overall pooled OR and the horizontal tip of the diamond is the 95% confidence interval (CI).

DISCUSSION

Due to the low incidence of NSCLC with the so-called uncommon *EGFR* mutations in the general population, information on their significance of carcinogenesis and treatment is still incomplete and deserves further investigation. We conducted a systematic review and meta-analysis of current researches (13, 14, 21, 22, 26–28) to evaluate the mutation pattern of *EGFR*-mutated NSCLC of Yunnan province in southwestern China. The pooled analysis confirmed a distinct *EGFR* mutation spectrum in Xuanwei region. The NSCLC patients in Xuanwei region are present with significantly higher incidence of uncommon *EGFR* mutations, especially 18 G719X mutation, 20 S768I mutation, and their combined mutation, but lower incidence of the two common mutations (19 deletion and 21 L858R substitution), providing a unique model for uncommon-*EGFR*-mutation-related lung cancer.

EGFR mutations in NSCLC is one of the most common genetic variations, especially in East Asians, females, and non-smokers (29). Studies suggested that NSCLC patients with *EGFR* mutation were significantly related to adenocarcinoma and light smoking, rather than gender (30). It was suggested that the dominant mutation rate of *EGFR* in women is a reflection of a higher frequency of adenocarcinoma (31). The results of our analysis revealed that the overall *EGFR* mutation rate of NSCLC patients varied from 30.2% to 55.6% in Yunnan province (Xuanwei region: 40.0%–56.7%; non-Xuanwei region: 29.2%–52.1%), which was in the range of other reports in East Asian areas (31%–56%) (8, 31–34) and similar to other studies performed in other regions of China (ranging from 33.64% to 53.69%)(**Table S1**). Most patients covered by our survey were carriers of adenocarcinomas (ranging from 80.0% to 87.6%) and female (ranging from 38.2% to 52.3%), which was similar to

TABLE 3 | Incidence of common and uncommon *EGFR* mutation in Xuanwei region.

			Xuanwei				META	
			Yes	No	Total	Rate	Rate	95%CI
Common mutation	Guo et al. (27)	2019	22	17	39	56.41%	0.564	(0.408-0.720)
	Zhou et al. (28)	2018	3	5	8	37.50%	0.375	(0.040-0.710)
	Zhou et al. (21)	2017	8	19	27	29.63%	0.296	(0.124-0.469)
	Chen et al. (13)	2016	14	37	51	27.45%	0.275	(0.152-0.397)
	Yang et al. (22)	2016	21	22	43	48.84%	0.488	(0.339-0.638)
	Yang et al. (26)	2016	16	19	35	45.71%	0.457	(0.292-0.622)
	Hosgood et al. (14)	2013	6	8	14	42.86%	0.429	(0.169-0.688)
	Overall		**90**	**127**	**217**		**0.405**	**(0.341-0.468)**
								(**Heterogeneity**: I^2 = 48.0%, p=0.073)
Uncommon mutation	Guo et al. (27)	2019	17	22	39	43.59%	0.436	(0.280-0.592)
	Zhou et al. (28)	2018	5	3	8	62.50%	0.625	(0.290-0.960)
	Zhou et al. (21)	2017	19	8	27	70.37%	0.704	(0.531-0.876)
	Chen et al. (13)	2016	37	14	51	72.55%	0.725	(0.603-0.848)
	Yang et al. (22)	2016	22	21	43	51.16%	0.512	(0.362-0.661)
	Yang et al. (26)	2016	19	16	35	54.29%	0.543	(0.378-0.708)
	Hosgood et al. (14)	2013	8	6	14	57.14%	0.571	(0.312-0.831)
	Overall		**127**	**90**	**217**		**0.595**	**(0.532-0.659)**
								(**Heterogeneity**: I^2 = 48.0%, p=0.073)

other studies performed in East Asian countries (8, 33, 34). Notably, despite similar incidence of overall *EGFR* mutations, the NSCLC patients in Xuanwei region present a distinctive characteristic with higher uncommon *EGFR* mutations and lower common *EGFR* mutations (**Table 2**, **Table S2**).

Generally, exon 19 deletions and 21 L858R substitutions, accounting for approximately 90% of *EGFR* mutations in NSCLC, are termed common or classic mutations and lead to high sensitivity to *EGFR* TKIs (35). Other *EGFR* mutations are termed uncommon mutations, accounting for 10%–20% of all *EGFR* mutations, and patients with uncommon *EGFR* mutation are a heterogeneous group exhibiting different responses to *EGFR* TKIs (36, 37). There are significant regional differences in *EGFR* gene mutation in China. It has been reported that exon 21 of *EGFR* gene mutation is dominant in Taiwan (38), while exon 19 is dominant in Yunnan (21) and exon 20 is dominant in Guangdong (36). Our results show that *EGFR* gene mutation mainly occurred in exon 21 L858R mutation and in exon 19 deletion NSCLC patients in most regions of Yunnan, which was consistent with other reports. Another study showed that the uncommon *EGFR* mutations were present in 11.9% of all patients with documented *EGFR* mutation (34%) in a Chinese NSCLC cohort (36). However, according to our study, the detection rate of uncommon EGFR mutations varied from 43.6% to 72.6% in NSCLC patients in Xuanwei, which was significantly higher than that of the general Chinese population and other regions of the world (ranging from 3.4% to 15.5%) (**Table 2**, **Table S2**). This result further emphasizes the uniqueness of the Xuanwei population in lung cancer tumorigenesis.

The majority of clinical trials evaluating the efficacy of *EGFR* TKIs have included only patients with common *EGFR* mutations due to the low incidence of uncommon *EGFR* mutations in the general population; thus, the efficacy of *EGFR* TKIs in patients with uncommon *EGFR* mutations remains elusive. A post-hoc study showed that NSCLC patients with uncommon *EGFR* mutation (L861Q or G719X) was significantly associated with shorter overall survival (11.9 vs. 29.3 months) compared with patients harboring common *EGFR* mutation (L858R or 19 deletion) in the EGFR TKI treatment group (39). The patients with uncommon *EGFR* mutation (41% vs. 62%–83%) had a significantly lower overall response rate (ORR) of *EGFR* TKIs and shorter progression-free survival (PFS) (2.2 vs. 11.4 months) than that in those with common *EGFR* mutation (39). Additionally, in other studies, a poor ORR and shorter PFS were also found in uncommon *EGFR* mutations compared with the classic *EGFR* mutations. However, a similarly poor response to *EGFR* TKIs was observed in other uncommon mutation rather than G719X and L861Q (31, 40). It has also been documented that patients with NSCLC *EGFR* mutation in Xuanwei have poor prognosis after treatment with *EGFR* TKIs (21), possibly owing to the high incidence of uncommon *EGFR* mutations in this area.

Recent studies have suggested that the lung cancer in Xuanwei region showed distinct mutational signatures and signaling pathways, and two single nucleotide variants located in exon 20 and exon 18 of the *EGFR* gene were known to be related to lung adenocarcinoma in Xuanwei (41). In line with this, our results also suggested that patients with exon 20 S768I and exon 18 G719X mutations presented higher mutation rates than those from other regions. The specific genotypes of complex *EGFR* mutations (double or multiple concomitant *EGFR* mutations) are diverse, which can be common single mutation combined with common single mutation, common single mutation combined with rare single mutation, rare

TABLE 4 | Subgroup analysis of different *EGFR* mutation subtypes between Xuanwei and non-Xuanwei regions.

Mutation type		Guo et al. (27)	Zhou et al. (28)		Zhou et al. (21)		Chen et al. (13)		Yang et al. (22)		Yang et al. (26)	Hosgood et al. (14)	META(Xuanwei vs. Non-Xuanwei)			META(Xuanwei)	
		Xuanwei	Xuanwei	non-Xunwei	Xuanwei	non-Xunwei	Xuanwei	non-Xunwei	Xuanwei	non-Xunwei	Xuanwei	Xuanwei	OR[a]	95% CI	*P*-value	Rate[b]	95% CI
Common	19 deletion	56.41%	37.50%	26.79%	18.50%	45.00%	7.80%	49.30%	13.95%	39.77%	14.30%	28.57%	0.284	(0.105-0.766)	<0.001	0.13	(0.081-0.178)
	21 L858R		0	23.21%	11.10%	33.30%	19.60%	37.00%	34.88%	35.23%	31.40%	14.29%	0.511	(0.313-0.836)	0.007	0.197	(0.109-0.302)
Uncommon	18 G719X	7.69%	12.50%	1.79%	22.20%	3.90%	7.80%	1.40%	9.30%	7.95%	14.30%	50.00%	3.212	(1.481-6.965)	0.003	0.11	(0.070-0.151)
	20 T790M	0	12.50%	14.29%	0	2.30%	0	0	4.65%	4.55%	2.90%	0	0.927	(0.286-2.999)	0.899	0.0076	(0-0.034)
	20 S768I	0	25.00%	3.57%	11.10%	3.10%	3.90%	2.70%	23.26%	1.14%	17.10%	0	6.437	(2.657-15.598)	<0.001	0.109	(0.036-0.207)
	20 insertion	0	0	1.79%	0	3.90%	2.00%	0	0	0	0	7.14%	1.335	(0.316-5.634)	0.694	0.0014	(0-0.021)
	21 L816Q	0	0	0	3.70%	2.30%	2.00%	2.70%	0	0	0	0	1.395	(0.339-5.741)	0.644	0.0022	(0-0.023)
	18 G719X + 20 S768I	15.38%	12.50%	10.71%	18.40%	1.60%	45.10%	4.10%	9.30%	3.41%	17.10%	0	6.549	(1.921-22.332)	0.003	0.213	(0.048-0.439)
	18 G719X + 20 G779C	5.13%	0	0	0	0	2.00%	0	0	0	0	0	3.969	(0.603-26.132)	0.152	0.0035	(0-0.023)
	18 G719X + 21 L816Q	2.56%	0	0	0	0.80%	2.00%	1.40%	0	0	0	0	1.959	(0.366-10.489)	0.432	0.0017	(0-0.019)
	18 G719X + 21 L858R	0	0	0	3.70%	0	0	0	0	0	0	0	4.421	(0.736-26.554)	0.104	0	(0-0.013)
	18 G719X + 21 L858R + S768I	0	0	0	3.70%	0	0	0	0	0	0	0	4.421	(0.736-26.554)	0.104	0	(0-0.013)
	19 deletion + 21 L858R	0	0	1.79%	0	0.80%	2.00%	0	0	4.55%	0	0	0.95	(0.242-3.731)	0.941	0.0001	(0-0.016)
	19 deletion + 20 T790M	0	0	10.71%	0	2.30%	0	0	0	0	0	0	0.776	(0.156-3.852)	0.756	0	0
	20 S768I + 20 T790M	0	0	0	7.40%	0.00%	0	0	0	0	0	0	6.046	(1.119-32.677)	0.037	0.0002	(0-0.017)
	20 S768I +20 insertion	0	0	0	0	0	3.90%	1.40%	0	0	0	0	3.284	(0.607-17.760)	0.167	0.0013	(0-0.021)
	20 S768I + 21 L858R	0	0	0	0	0.80%	2.00%	0	0	0	0	0	2.963	(0.534-16.429)	0.214	0.0001	(0-0.016)
	20 T790M + 21 L858R	0	0	5.36%	0	0	0	0	4.65%	3.41%	2.90%	0	1.431	(0.374-5.472)	0.601	0.0048	(0-0.029)

OR, odds ratio; CI, confidence interval.

[a]*the pooled OR of EGFR mutation for Xuanwei region vs. non-Xuanwei region.*

[b]*the pooled incidence of EGFR mutation in Xuanwei region.*

single mutation combined with rare single mutation, or compound mutation combined with known resistance genes. In this study, in patients with complex mutations, there was only one case with the common mutation combination (19deletion + L858R), while the others were the rare mutation combination, mainly including G719X, S768I, T790M, 20 insertions, and L861Q. Specifically, the 20 S768I mutation, 18 G719X mutation, co-mutation in 20 S768I + 18 G719X, and co-mutation in 20 S768I + 20 T790M were significantly more likely to present in Xuanwei patients compared with non-Xuanwei patients (all $P<0.05$). These mutations were thought to promote the development and progression of lung cancer (42). For other uncommon *EGFR* mutation types, there was no statistically significant difference between Xuanwei and non-Xuanwei regions. However, it should be noted that some co-mutations with uncommon *EGFR* mutation did present an un-negligible high OR value in Xuanwei region, such as 18 G719X+21 L858R (OR: 4.42), 18 G719X + 20 G779C (OR: 3.97), and 20 S768I +20 insertion (OR: 3.28). In other ways, the 20 S768I + 20 T790M was significantly more frequently present in Xuanwei region (OR, 6.05) whereas 19 deletion + 20 T790M tends to be present less in Xuanwei region (OR, 0.77), which may reflect an acquired resistance after *EGFR*-TKI therapy in these NSCLC patients with prevalent uncommon *EGFR* mutation in Xuanwei region. Overall, these results revealed a relatively high mutation frequency of so-called uncommon mutations (e.g., 18 G719X, 20 S768I, or 18 G719X/20 S768I in conjunction with other mutation) in the unique Xuanwei population, strikingly divergent from those in other populations from Asia. Given that our subjects (Xuanwei population) live in an area where coal is typically burned indoors, our analysis implies that the tumorigenesis and progression of lung cancer in Xuanwei region is different from that in other geographic areas, which may due to its distinctive etiology and the different environmental exposures.

The main difference between environmental exposure may be related to indoor solid fuel use related to indoor air pollution. In Xuanwei, indoor air pollution from bituminous coal burning in unvented fire pits was suggested to be the main cause of high lung cancer mortality (11). Polycyclic aromatic hydrocarbons (PAHs)-DNA adducts have been observed in the bronchoalveolar lavage fluid of coal-burning residents in Xuanwei region (11). Previous study showed that the mutations in *EGFR* exons 21 and 18 were associated with emissions from coal combustion (14). In addition, PAHs have been found to increase intracellular calcium in human cells (43), which may result in EGFR-dependent cell proliferation (4), suggesting that PAHs may lead to a unique mutation pattern. Liu et al. (44) evaluated the relationship among indoor air pollution, tobacco use, and lung cancer risk, showing that the risk association between smoking and lung cancer increased with the decrease of bituminous coal consumption. In other words, the relationship between smoking and lung cancer is relatively weak when there is a strong correlation between lung cancer and bituminous coal. Xuanwei is located in one of China's largest tobacco producing provinces (45). In Xuanwei, the smoking rates of males in high, medium, and low incidence areas were 75%, 78%, and 63% respectively, and the exposure rates of second-hand smoke were 85%, 88%, and 58%, which were much higher than the national

average level of male smoking rate (52.1%) and second-hand smoke exposure level (72.4%) (46). The discrepancy of mutation subtypes may provide clues for the mechanism of the occurrence of EGFR mutation. And this comparative analysis provides reference data, allowing a better understanding of a possible mechanism of *EGFR* mutation in Xuanwei, paving the way toward better exploration of uncommon EGFR mutation in lung cancer pathogenesis.

There are several limitations to the study. First, all included publications were retrospective studies with inherent biases. Second, the sample size of the study was not big enough. Third, only one study used NGS method to detect EGFR mutation; most of the included studies applied ARMS method to detect EGFR mutation, which is limited to the detection of known mutations and therefore might miss some rare EGFR mutations. Nevertheless, it should be noted that the detection methods in this analysis were already sufficient for the detection of classic (19 deletions and 21 L858R) and those known non-classic *EGFR* mutations in exons 18 to 21. A high concordance was found between ARMS and NGS, but more detailed information (unknown genetic mutations and deep sequencing) could be revealed by NGS (30, 47, 48). Therefore, a larger amount of sample and adoption of NGS is needed to validate the results of this study.

In conclusion, our analysis suggested the prevalence of *EGFR* mutation in Xuanwei region that is differentiating it from the general population. The frequency of S768I, G719X, and G719X +S768I were higher, but the 19 deletions and L858R mutations were lower in Xuanwei region. The difference of EGFR mutation between patients in Xuanwei region and in other areas may indicate the difference of lung cancer pathogenesis, dietary habits, coal-burning factors, or genetic backgrounds, which are worthy of more detailed studies.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JL and WL designed and financed the study. LL performed the literature search and review, data extraction, and drafted the manuscript. ZL performed all of the screening of studies and data extraction. Any disagreements were resolved by YL. WZ, LJ, and TL were responsible for the analysis of pooled data. 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.2020.519073/full#supplementary-material

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CASE REPORT
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Osimertinib Resistance With a Novel *EGFR* L858R/A859S/Y891D Triple Mutation in a Patient With Non-Small Cell Lung Cancer: A Case Report

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Targeted drug therapy based on the types of epidermal growth factor receptor (*EGFR*) gene mutations has been widely used in the diagnosis and treatment of patients with non-small cell lung cancer (NSCLC). With the development of next-generation sequencing (NGS) technology, more and more *EGFR*-tyrosine kinase inhibitor (TKI) resistance mutation sites have been revealed. Here, we report a novel *EGFR* L858R/A859S/Y891D triple mutation in plasma-derived circulating tumor DNA (ctDNA) was identified in a 53-year-old male patient with NSCLC resistant to osimertinib treatment, using an ultra-deep (20,000×) 160-gene panel through the NGS platform. Our case confirms that dynamic monitoring of liquid biopsy based on ctDNA is conducive to the selection of targeted therapy and the realization of the patient's full course management.

Keywords: case report, osimertinib, epidermal growth factor receptor, circulating tumor DNA, non-small cell lung cancer

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INTRODUCTION

Accurate identification of oncogenic driver mutations has revolutionized the clinical management of non-small cell lung cancer (NSCLC). Targeted drug therapy based appropriate epidermal growth factor receptor (*EGFR*) gene mutations has been widely used in the treatment of NSCLC patients (1, 2). Based on the kinase domain of *EGFR*, several *EGFR*-tyrosine kinase inhibitor (TKI) drugs have been developed and applied effectively, including the first-generation inhibitors erlotinib, gefitinib and icotinib, the second-generation inhibitors afatinib and dacomitinib, and the third-generation inhibitor osimertinib (3–8). Although targeted therapy has improved the prognosis of NSCLC patients, inevitable drug resistance remains widespread (9).

With the rapid development of next-generation sequencing (NGS) technology, more and more novel *EGFR* mutation sites have been revealed gradually, indicating the sensitivity and resistance to drugs (10, 11). The biological simulation of protein structure suggested that the first-generation TKI resistance of a patient with NSCLC harboring *EGFR* L858R mutation treated with erlotinib was related to the secondary *EGFR* Y891D mutation (12).

Here, we report a novel *EGFR* L858R/A859S/Y891D triple mutation in plasma-derived circulating tumor DNA (ctDNA) was identified in a patient with NSCLC resistant to osimertinib

treatment, using an ultra-deep (20,000×) 160-gene panel through the NGS platform. We present the following case in accordance with the CARE Guideline (13).

CASE PRESENTATION

A 53-year-old male with a history of smoking for approximately 30 years was presented to hospital in July 2017. The patient was previously in good health and had no history of other diseases or medication. Computed tomography (CT) scans showed a lung mass of the upper left lobe along with nodules involvement (**Figure 1B**). Brain magnetic resonance imaging (MRI) revealed brain metastases (**Figure 1B**). Broncho-alveolar lavage fluid (BALF) confirmed squamous cell carcinoma. He was diagnosed with stage IVb lung squamous cell carcinoma (T4N2M1c) with a *EGFR* L858R/A859S double mutation, detected using ctDNA through the NGS platform. Variant allele frequencies (VAFs) of the detected *EGFR* L858R and A859S mutations were 8.7 and 8.41%, respectively (**Figures 1A**, **2**). Icotinib (125 mg, three times per day) was then administered from July 2017.

After seven months of icotinib treatment, the patient presented obvious nausea and vomiting, accompanied by lack of consciousness and limited movement of legs. Chest CT scans showed original lung mass was significantly reduced, but the brain MRI showed the brain metastases was enlarged according to the Response Evaluation Criteria in Solid Tumors (RECIST v1.1) (**Figure 1C**). These phenomena can be inferred that the patient had developed resistance to icotinib. Plasma ctDNA detection results showed two secondary *EGFR* T790M and Y891D mutations, and VAFs were 1 and 1.89% respectively, accompanied with decreased VAFs of *EGFR* L858R and A859S mutations to 6.25 and 5.61%, respectively (**Figures 1A**, **2**). Based on ctDNA testing results, the patient received osimertinib (80 mg per day) and experienced significant improvements in nausea, vomiting, consciousness and legs movement within one week. In our subsequent follow-up, the symptoms of the patient were

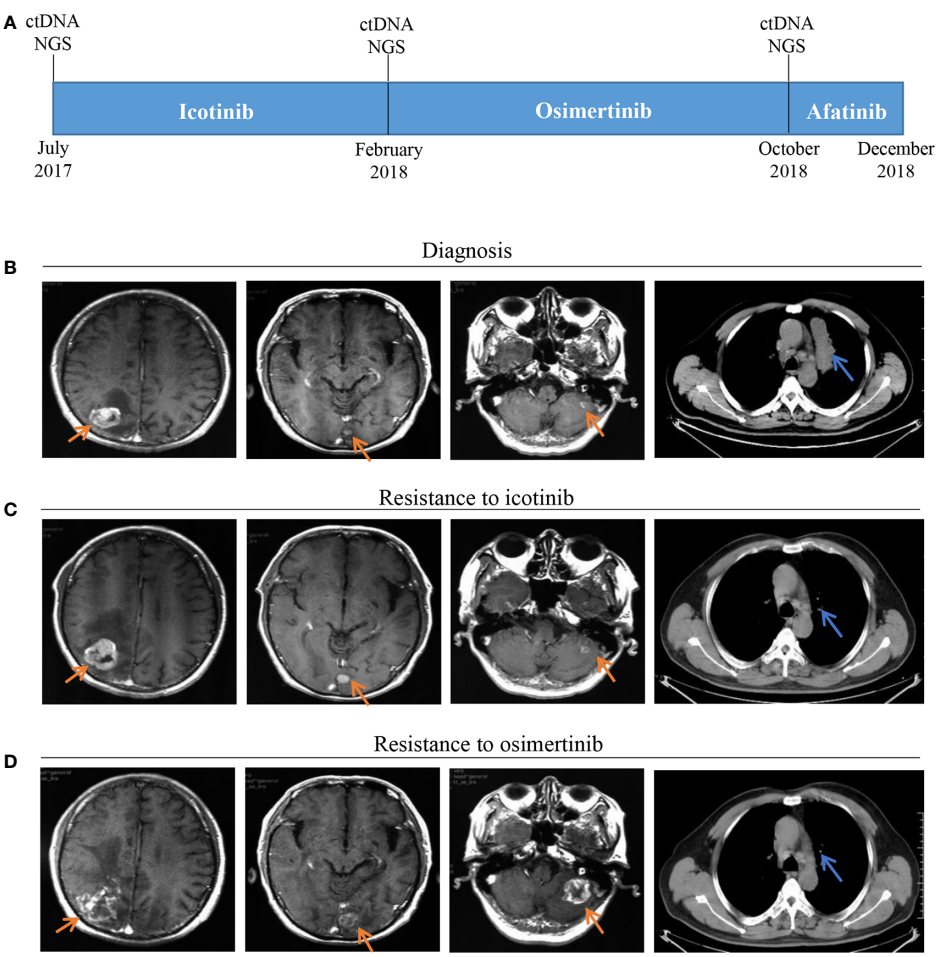

FIGURE 1 | Timeline and effect of *EGFR*-TKI treatments. **(A)** Treatment timeline; **(B)** Baseline images of CT and MRI at diagnosis; **(C)** Brain metastases progressed and original lung mass reduced after seven months of icotinib treatment; **(D)** Brain metastases progressed and thoracic lesions controlled after eight months of osimertinib treatment; EGFR, epithelial growth factor receptor; TKI, tyrosine kinase inhibitor; CT, computed tomography; MRI, magnetic resonance imaging.

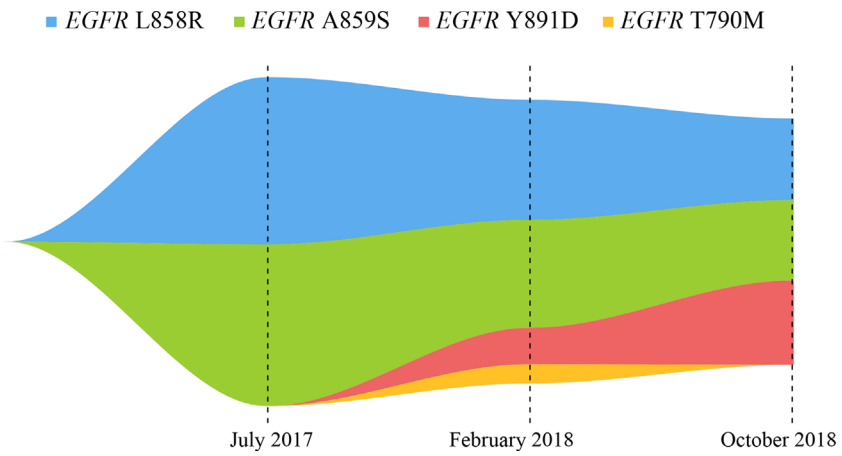

FIGURE 2 | Map of clonal evolution. The different colors represent different mutation sites in *EGFR* gene, as measured by the NGS platform; NGS, next-generation sequencing.

gradually alleviated and the patient was able to take care of himself normally. This good performance status lasted for eight months, suggesting that the symptoms of brain metastasis were well controlled.

However, the disease progressed again after eight months, and the patient presented the neurological symptoms again. Chest CT scans showed thoracic lesions were still well controlled, but brain MRI revealed the brain metastases were larger and the edema was more obvious than before (**Figure 1D**). Compared with the VAFs of *EGFR* mutation sites in the second ctDNA testing, the third gene detection results revealed *EGFR* T790M disappeared, L858R and A859S decreased to 4.25 and 4.18% respectively, while Y891D increased to 4.38% (**Figures 1A**, **2**). The patient received afatinib (40 mg per day) and experienced improvements in nausea, vomiting, consciousness and legs movement within two weeks.

The symptom was well controlled for nearly two months. Eventually, the patient's condition deteriorated dramatically, resulting in loss of consciousness and paralysis of both legs. His death was certified at home in December 2018, most likely due to the stroke caused by the brain metastases.

DISCUSSION

Patients with NSCLC harboring *EGFR* mutations are usually treated with *EGFR*-TKIs for targeted therapy, but some patients' progress was due to acquired drug resistance. *EGFR* mutations are rare in patients with the squamous cell carcinoma, whose outcomes are usually inferior to *EGFR*-positive adenocarcinoma. To date, more and more reports have been published on the mechanisms of third-generation *EGFR*-TKI acquired resistance, such as gatekeeper *EGFR* C797S mutation, human epidermal growth factor receptor 2 (HER2) or hepatocyte growth factor receptor (MET) amplification, and histological transformation (9, 14). Therefore, considering the importance of gene-guided therapy and the difficulty of repeated biopsy or insufficient tissues during the progression, dynamic monitoring of gene mutation variations through liquid biopsy is of great value for the management of NSCLC.

Here, we report a case of whole-course management in a patient with NSCLC carrying *EGFR* gene mutations in ctDNA through the NGS platform, to guide drug selections for *EGFR*-TKI treatments. The NGS detection platform included the detection of gene amplifications, but no gene amplification was found in our study. Previous studies have reported that a patient with *EGFR* L858R/A859S responded well to the first-generation *EGFR*-TKI, which was consistent with our findings (15). Qin et al. inferred that *EGFR* L858R/Y891D was resistant to erlotinib through the energy simulation of protein structure biology and the clinical manifestations of the patient, and found that osimertinib treatment could control the disease status (12). Our present study found that the *EGFR* L858R/A859S/Y891D triple mutation showed drug resistance to osimertinib, so it was concluded that the triple mutation might be the drug resistance mechanism of the patient. In our report, this patient developed the common *EGFR* T790M drug-resistant mutation and the rare *EGFR* Y891D mutation after seven months of treatment with icotinib. Although VAFs of *EGFR* T790M, L858R and A859S were all expected to decrease, VAF of *EGFR* Y891D was significantly increased after osimertinib treatment for eight months (**Figure 2**). The clinical significance of single *EGFR* A859S somatic mutation was not clearly determined, this site was only detected in multiple myeloma, but not reported in lung cancer (16). Combined with these data, we concluded that the secondary *EGFR* Y891D mutation may be the main cause of drug resistance to osimertinib. This phenomenon may be caused by the selection pressure of different *EGFR*-TKI drugs, and the resistance to treatments may be caused by the expansion of the pre-existing subclonal population (17).

It has been reported that patients with rare *EGFR* mutations may be sensitive to targeted treatment with afatinib (18). In this case study, since the *EGFR* A859S and Y891D were considered to be rare *EGFR* mutations, the patient was treated with afatinib for the third-line therapy. Although the symptoms were relieved for

nearly two months, the patient died from a stroke caused by brain metastases.

There are some limitations in our study. First of all, the osimertinib resistance mechanism of the novel *EGFR* L858R/A859S/Y891D triple mutation needs to be further verified from the in vitro cell line experiments and protein structural biology energy calculation. Secondly, whether afatinib can be used for the treatment of such patients with this triple mutation remains to be further studied.

CONCLUSION

In summary, our report indicates that a novel *EGFR* L858R/A859S/Y891D formed by secondary *EGFR* Y891D may be the potential cause of the drug resistance mechanism of the first- and third-generation *EGFR*-TKIs, which may be a new target for the treatment of NSCLC. In addition, it is confirmed that dynamic monitoring of liquid biopsy based on ctDNA is conducive to the selection of targeted therapy and the realization of the patient's full course management.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. YY, XZ, and XS carried out the studies, participated in collecting data, and drafted the manuscript. RW, JQ, JW, and ZL performed the NGS platform and statistical analysis.

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Conflict of Interest: Authors RW, JQ, JW and ZL were employed by the company Annoroad Gene Technology Co., Ltd.

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

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Clinical Features and Outcomes Analysis of Surgical Resected Pulmonary Large-Cell Neuroendocrine Carcinoma With Adjuvant Chemotherapy

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Objective: Large-cell neuroendocrine carcinoma (LCNEC) is a rare subtype of pulmonary cancer with poor survival. Optimal adjuvant chemotherapy for resected LCNEC is controversial till now; clinical features together with the prognostic factors in LCNEC should be clarified better.

Methods: Clinicopathological characteristics, driven genes' status (EGFR, ALK, and ROS1), adjuvant chemotherapy strategy for 94 surgical resected LCNECs were extracted from digital database, tumor relapse or progression, and survival were analyzed with clinical profiles.

Results: Driven gene mutants were scarce in LCNEC, 8.3% (4/48) samples harbored EGFR mutations, 5.8% (3/52) with ALK positive, and none of ROS1 positive. A total of 44 patients suffered tumor relapse or progression during follow-up. Tumor/lymph node (N) stage, serum carcinoembryonic antigen (CEA) level before surgery, different adjuvant chemotherapies were associated with tumor relapse ($P < 0.05$); poorer disease-free survival (DFS) appeared in N2/stage III, serum CEA positive and pemetrexed based chemotherapy ($P < 0.05$); for overall survival (OS) analysis, the T/tumor stage, serum positive CEA/neuron-specific enolase (NSE) at baseline were associated with worse OS ($P < 0.05$). Moreover, in the multivariate analysis, N stage still acted as prognostic for DFS ($P = 0.019$); OS differed significantly in different T stages, chemotherapy selection and serum CEA levels after adjustment ($P < 0.05$).

Conclusion: Classical driven gene mutations were rare in LCNEC. Tumor N stage appeared as prognostic for DFS, while serum positive CEA, different adjuvant chemotherapy strategies, and T stage were independent prognostic factors for OS. Etoposide–platinum regime seemed to be a better choice which should be confirmed by further prospective investigations.

Keywords: large-cell neuroendocrine carcinoma, adjuvant chemotherapy, prognosis analysis, driven genes, serum tumor markers

INTRODUCTION

Large-cell neuroendocrine carcinoma (LCNEC) is a rare type of lung cancer, which accounts lower than 3.5% of all (1, 2), while according to the Surveillance, Epidemiology, and End Results (SEER) (2001–2007) database, LCNEC incidence seemed to increase (3). Since this subtype is a high-grade malignancy and presents as neuroendocrine features (4), LCNEC is used to be a subcluster of large-cell carcinoma (LCC) and part of neuroendocrine tumors (NETs) of the lung before 2015, and the World Health Organization (WHO) lung tumor classification revised the criteria (2015) which moved LCNEC from LCC to NET chapter (5). Previous reports indicated LCNEC appeared aggressive and the prognosis was poor (6, 7) and shared some similarities with small-cell lung cancer (SCLC) (8) or non-small cell lung cancer at the same time (9).

The rarity of LCNEC impeded large-scale randomized clinical trials in seeking the optimal therapy; majority of the present were data derived from retrospective studies, and the sample size was also small. Similar to NSCLC, early stage (stages I–II) LCNEC usually received surgical resection, while for local advanced or metastatic tumors, the treatment selection is still controversial, either for adjuvant chemotherapy or first-line therapy. Reported data evaluated platinum–etoposide combination, which was widely used in treating SCLC, as a better choice for prolonging survival (1, 10, 11); however, most of the results focused on IIIB/IV stage tumors, and treatment for patients with operation should be clarified further.

As targeted therapy provided a promising prognosis for specific patients in NSCLC, driven gene detection is necessary before clinical decision, while gene mutant data related to LCNEC at present was rare. Recently, Zhuo et al. reported genetic subtyping was associated with tumor prognosis (12), which indicated treatment selection might rely on genomic status. Considering the gloomy outcomes in LCNEC, clinical characteristics, genomic information, and survival should be investigated with deeper insight. Herein we conducted this retrospective study to provide an overview of LCNEC in Chinese population, especially for resected tumors; the adjuvant chemotherapy effects, driven gene spectrum and survival will be concentrated in order to help understand LCNEC better.

MATERIALS AND METHODS

Study Population

During August 2011 to October 2019, a total of 105 LCNEC underwent surgical resection in Shanghai Chest Hospital, and all samples were confirmed as LCNEC or combined LCNEC (N = 11) following the 2015 WHO lung tumor classification criteria (13), and only LCNECs were collected (N = 94). Informed consent was obtained from all patients, and the present study was approved by the Institutional Review Board (IRB) in ShangHai Chest Hospital [No. KS(Y)1982].

Data Extraction

An independent database was established based on hospital digital medical records; details of these individuals were extracted such as patients' age, gender, smoking status, primary tumor size, tumor location, tumor-nodal-metastasis (TNM) staging information, peripheral blood tumor marker carcinoembryonic antigen (CEA), squamous cell carcinoma antigen (SCC), cytokeratin-19 fragment (CYFRA21-1), neuron-specific enolase (NSE), cancer antigen-125 (CA125), and gene detection results. Blood tumor markers were evaluated before surgery, epidermal growth factor receptor (EGFR) mutants were detected with amplification refractory mutation system (ARMS), anaplastic lymphoma kinase (ALK) rearrangement was detected by immunohistochemistry (IHC), and ROS1 fusion was determined with fluorescence *in situ* hybridization (FISH). All tumor stage was performed according to the Eighth edition of American Joint Committee on Cancer (AJCC) staging system (14).

Patient Follow-Up and Definition of End-Point

Serial clinical physical examination and image evaluation (included chest computed tomography, brain magnetic resonance imaging, abdomen ultrasound or whole-body ^{18}F-Fluorodeoxyglucose positron emission tomography/computed tomography if necessary) were recommended to all patients in a sequential follow-up. Disease-free survival (DFS) was defined as the time from surgery to the first confirmed relapse or alive at final follow-up; overall survival (OS) was defined as time to death by any cause or last follow-up from diagnosis. Survival information was collected mainly by phone communication and outpatient visit. Last follow-up date was set at November 2019.

Statistical Analysis

The Chi-square ($\chi 2$) test or Fisher's exact test was used for clinicopathological characteristics comparison analysis in LCNEC. Survival differences were analyzed by Kaplan–Meier survival function with the method of log-rank test. Moreover, variants including age, gender, tumor location and size, tumor staging, chemotherapy or radiotherapy status, and peripheral blood tumor markers were evaluated by fitting logistic multivariable regression with Cox proportional hazard models. All statistical analyses were performed by the SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 7 (GraphPad Software, Inc.); significance level was set at two-sides $P < 0.05$.

RESULTS

Characteristics of Resected LCNEC Patients

Among the 94 LCNEC patients, 84 (89.4%) were males, and 10 (10.6%) were females; median age was 60 (range 35–80 years) and 35 (37.2%) were current or former smokers. More than half (60, 63.8%) of the patients had a tumor with diameter larger than 3 cm, and 64 (68.1%) patients had tumors located in the left lobe.

Stage I, II, and III tumors accounted for 33% (31/94), 23.4% (22/94), and 43.6% (41/94), respectively. Of the 94 patients, three received neoadjuvant chemotherapy, and all these three patients refused the adjuvant chemotherapy after surgery. 75 (79.8%) patients received adjuvant chemotherapy, of which pemetrexed/cisplatin (PEM) or carboplatin contained 26 (34.7%), and etoposide–platinum (PE) based regime contained 21 (28%), 28 (37.3%) were gemcitabine/vinorelbine/paclitaxel–platinum (GVTP). A total of 38 patients received radiotherapy of which 16 (42.1%) were followed by chemotherapy, and 22 (57.9%) received radiotherapy for tumor recurrence. Detailed clinicopathological characteristics of the study patients were presented in **Table 1**.

Serum Tumor Biomarkers Level and Genetic Alternations Profiles of LCNEC

Five kinds of peripheral blood tumor biomarkers were selected for evaluation, which included CEA, SCC, CYFRA21-1, NSE, and CA125. Positive rates of these biomarkers were 27.4% (23/84) for CEA, 10.7% (9/84) for SCC, 14.3% (12/84) for CYFRA21-1, 15.5% (13/84) for NSE, and 13.1 (11/84) for CA125. Furthermore, common driven genes such as EGFR, ALK, and ROS1 mutations were confirmed in this cohort; mutant status was available in 51.1% (48/94), 55.3% (52/94) and 26.6% (25/94), respectively. Four (8.33%) patients harbored EGFR mutations, of which two were L858R and two with 19 deletions. 5.77% (3/52) of patients appeared ALK positive, and all ROS1 status was negative in the present study.

TABLE 1 | Clinicopathological characteristics of 94 resected LCNEC patients.

Characteristics	Number (%)
Gender	
Male	84 (89.4)
Female	10 (10.6)
Age	
≥60	55 (58.5)
<60	39 (41.5)
Smoking history	
Yes	35 (37.2)
No	52 (55.3)
Missing	7 (7.5)
Primary tumor location	
Left upper	30 (31.9)
Left lower	11 (11.7)
Right upper	27 (28.7)
Right lower/middle	26 (27.7)
Tumor size (cm)	
>3	60 (63.8)
≤3	34 (36.2)
Tumor stage	
I	31 (33.0)
II	22 (23.4)
III	41 (43.6)
Adjuvant chemotherapy strategy	
PEM	26 (34.7)
PE	21 (28.0)
GVTP	28 (37.3)
CEA (ng/ml)	
Positive (>5)	23 (27.4)
Negative (≤5)	61 (72.6)
SCC (ng/ml)	
Positive (>1.5)	9 (10.7)
Negative (≤1.5)	75 (89.3)
CYFRA21-1(ng/ml)	
Positive (>5)	12 (14.3)
Negative (≤5)	72 (85.7)
NSE (ng/ml)	
Positive (>25)	13 (15.5)
Negative (≤25)	71 (84.5)
CA125 (U/ml)	
Positive (>35)	11 (13.1)
Negative (≤35)	73 (86.9)

Outcomes Predictors With Univariate Analysis in LCNEC

Until the final follow-up, we obtained information of tumor relapse in 84.04% (79/94) of patients, and 55.7% (44/79) suffered relapse or tumor progression during the follow-up, of which nine (20.5%) had intrapulmonary tumor recurrence, eight (18.2%) with brain and five (11.4%) with bone metastasis, eight (18.2%) suffered lymph node metastasis, and nine (20.5%) already died (**Table 2**). The tumor/nodal (N) stage was significantly associated with recurrence, with $P = 0.021$ and 0.022. Positive serum CEA level (>5 ng/ml) appeared to be more likely with relapse (75% *vs* 49%, $P = 0.047$); moreover, different chemotherapies were also associated with tumor recurrence ($P < 0.005$). As for DFS evaluation, N2 tumor indicated poorer DFS (median 54-N0 *vs* 23-N1 *vs* 12-N2 months, $P = 0.004$), and tumor stage (median 12 months in stage III), pemetrexed–platinum based chemotherapy (median 21 months) and serum CEA positive were also significantly with worse DFS (median 48-positive *vs* 13-negtive months), with $P = 0.002$, $P = 0.025$, $P = 0.014$, respectively (**Figure 1**). Furthermore, median DFS was longer with PE than with others (not reached), and PEM indicated the worst survival (21 months). Over-all survival was analyzed in 77.66% (73/94) of patients. T (tumor) stage ($P < 0.0001$), tumor stage ($P = 0.014$) and serum positive CEA ($P = 0.003$)/NSE ($P = 0.04$) at baseline were all significantly associated with shorter OS (**Figure 2**); furthermore, different chemotherapy regimes also appeared a significant trend ($P = 0.059$) (**Supplement Figure 1**).

Multivariate Analysis of Outcomes Predictors in LCNEC

In the multivariate analysis, T and N stages, peripheral blood CEA/NSE level, tumor stage and chemotherapy in relation to patients' DFS and OS were selected. The N stage still acted as an

TABLE 2 | Tumor relapse/progression patterns in 44 surgical resection LCNEC patients.

Relapse/progression patterns	Number (%)
Intrapulmonary	9 (20.5)
Brain metastasis	8 (18.2)
Bone metastasis	5 (11.4)
Lymph node metastasis	8 (18.2)
Death	9 (20.5)
Abdomen metastasis	
Liver	2 (4.5)
Pancreas	1 (2.2)
Adrenal gland	1 (2.2)
Abdominal cavity	1 (2.2)

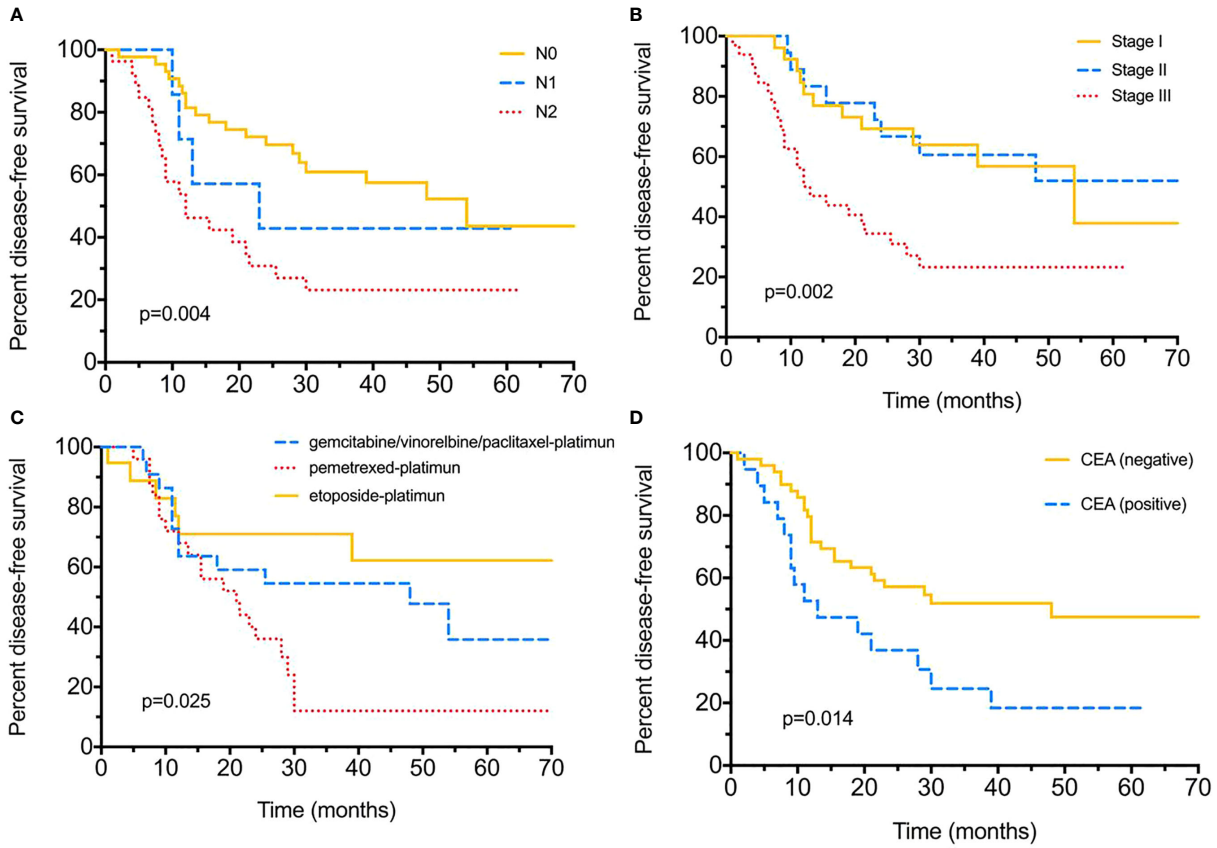

FIGURE 1 | Disease-free survival (DFS) for surgical resected LCNEC. **(A)** Disease-free survival by different nodal (N) stages. **(B)** Disease-free survival by different tumor stages. **(C)** Disease-free survival with different adjuvant chemotherapy strategies. **(D)** Disease-free survival between positive/negative serum CEA levels.

independent prognostic factor for DFS (P = 0.019), and OS differed significantly in different chemotherapies (P = 0.027), T stage (P = 0.01), and serum CEA levels (P = 0.032) after adjustment. No other associations were discovered in survival analysis (**Table 3**).

DISCUSSION

Since LCNEC was a rare type of lung cancer, the reported results were scarce and mostly derived from small sample size studies. Moreover, comprehensive analysis was limited, and clinical management for LCNEC remains controversial in some respects (10, 12, 15, 16). Hence we conducted this retrospective study in order to give an overview the clinical characteristics and prognostic variants of LCNEC. Diagnosis and treatment of lung carcinomas thrived dramatically, while few data was related to uncommon cancer types. We provide the landscape of tumor relapse and adjuvant therapy for resected LCNEC and confirmed PE was a priority for these patients; furthermore, normal serum tumor markers such as CEA and NSE could be utilized for prognosis evaluation, which was convenient and non-invasive for clinical practice.

Like most reported studies, LCNEC more likely occurred in males, with 89.4% in our study and 62.5–90.6% in others (2, 10, 12, 15, 16). Driven genes such as EGFR and ALK forecasted targeted therapy in NSCLC and detected as routine in clinical management. As for LCNEC, we found EGFR and ALK mutants were both rare in this subtype; the mutation rates were 8.33 and 5.77% respectively. Naidoo et al. also evaluated these genes in 49 LCNECs, they discovered none EGFR mutation or ALK rearrangement in 17 patients (15); however, 24% (4/17) harbored KRAS mutants. Considering all recruited patients were stage IV, the genomic alternations might differ between different tumor stages, which should be investigated in the future, and whether targeted therapy could be used was also controversial.

CEA is a widely used serum tumor marker and if positive before surgery seemed to be a risk in tumor relapse; moreover, positive CEA is also significantly associated with poorer DFS and OS in LCNEC. Zhang et al. also evaluated CEA in LCNEC prognosis, and no significance was mentioned (10), while 30.7% (117/381) in the study were stage IV patients. The mixed groups induced different proportions of positive CEA in the whole cohort, for 27.4% in our study and 42.2% in theirs (n = 301). Metastasis always obtained heavy tumor burden, which influenced the CEA level in the peripheral blood. Kim et al.

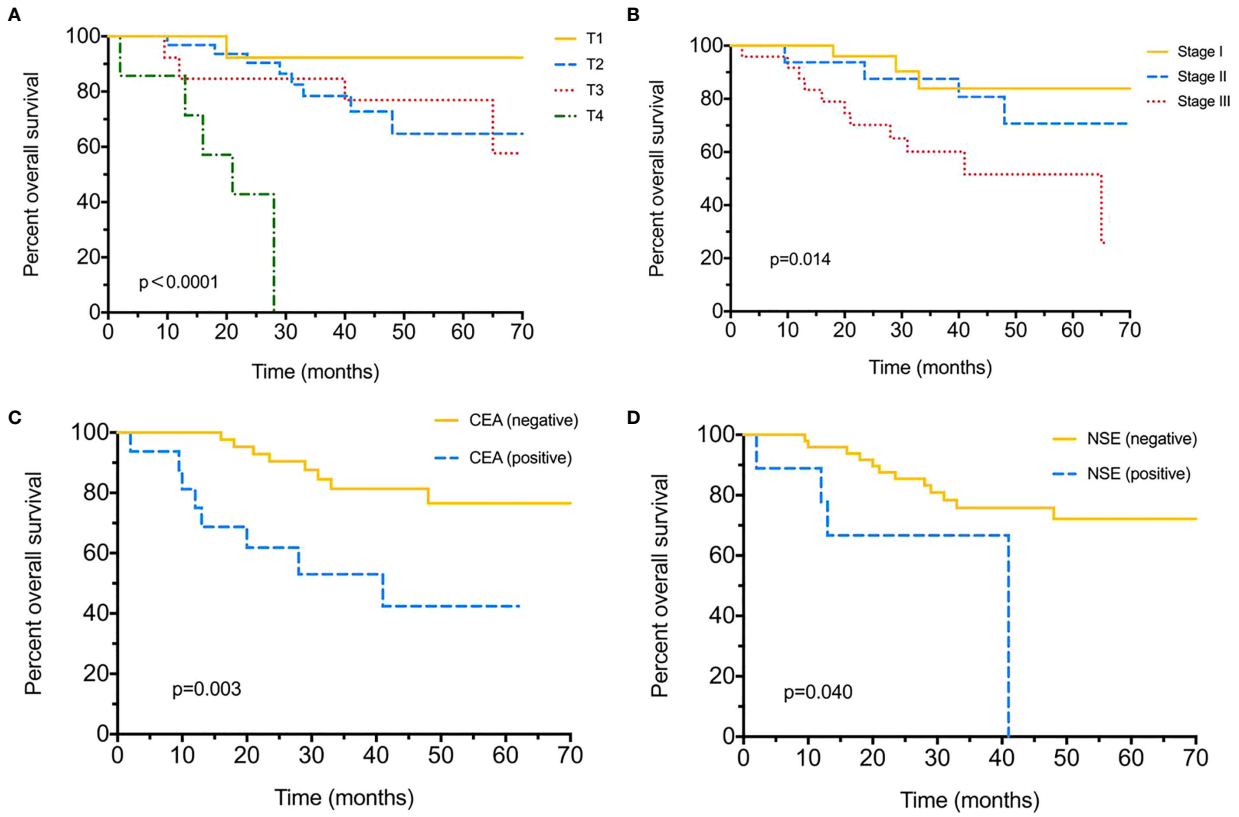

FIGURE 2 | Over-all survival (OS) for surgical resected LCNEC. **(A)** Over-all survival by different T (tumor) stages. **(B)** Over-all survival by different tumor stages. **(C)** Over-all survival between positive/negative serum CEA levels. **(D)** Over-all survival between positive/negative serum NSE levels.

TABLE 3 | Multivariate analysis of outcomes predictors in LCNEC patients.

Prognostic characteristics	P value	DFS HR	95% CI	P value	OS HR	95% CI
T stage	0.15	1.51	0.86–2.66	0.01	2.39	1.23–4.63
N stage	0.019	1.53	1.07–2.19	0.11	3.26	0.76–13.96
TNM stage	0.26	0.53	0.17–1.61	0.12	0.22	0.035–1.45
Chemotherapy	0.43	0.81	0.47–1.38	0.027	0.30	0.10–0.87
CEA	0.28	1.58	0.69–3.61	0.032	4.20	1.14–15.49
NSE	0.77	1.19	0.37–3.78	0.60	1.61	0.27–9.62

collected 139 LCNEC patients who received operation; however, no tumor marker information was involved (16). Positive NSE at baseline was significantly associated with shorter OS in the univariate analysis although only 15.5% was positive in the present study, and 50.6% (n = 241) in Zhang et al.'s (10). The result was also consistent with theirs. However, 36.7% of the samples lacked the NSE information, and tumors involved in the final analysis would be different between studies since NSE was specific for NETs. Maybe further investigation could notice this.

Due to lack of randomized clinical trials in adjuvant treatment for LCNECs, the optimal therapy in these patients was still in debate. In a large scale investigation whether adjuvant treatment could benefit LCNECs, patients with stage II or higher seemed to obtain better DFS and OS (16); however, the chemotherapy information was not provided. Although some previous researches evaluated different treatments in LCNECs, Treut et al. demonstrated that cisplatin–etoposide doublet may induce poor survival with advanced LCNEC (11). Another study chose platinum–etoposide in metastatic LCNEC, with 37% objective response rate (ORR; complete response + partial response) (15), and no response to other regimens. Metro G et al. investigated the survival outcomes and incidence of brain recurrence in advanced high-grade neuroendocrine carcinoma (HGNEC) which included 53 LCNECs and 108 SCLCs (17); the LCNECs shared a worse overall response and survival outcomes (both PFS and OS) compared with SCLCs based on the PE regime. Besides, LCNECs are at high risk of brain recurrence when prophylactic cranial irradiation (PCI) is lacking. Most of

the studies focused on advanced LCNEC. A recent study involved 56 patients for adjuvant chemotherapy, and SCLC-based regimens might be more effective than NSCLC-like therapy ($P > 0.05$), while no details such as tumor stage distribution and drugs usage were provided (10). We presented that in resected LCNEC, the PE regime might be a better choice for these patients and even acted as an independent prognostic factor for OS. While stage I patients were distributed more in the PE group (57.1%), the conclusion should be confirmed in the future. As genetic classification was implemented in clinical decision, some researches also explored genomic profiling in LCNEC. Zhuo et al. used next generation sequencing (NGS) to classify LCNEC as SCLC-like and NSCLC-like LCNEC (12), and treatment could be recommended based on genomic subtyping. Zhou et al. also provided the genomic landscape for LCNEC and indicated a group of gene alternations contained RUNX1, ERBB4, BRCA1, and EPHA3 (18), which may distinguish LCNEC from SCLC. Since the sample size was small (14 LCNECs and 10 SCLCs), more work should be undertaken in future investigations. Gene-based subtyping and further treatment options might emerge.

There are several limitations of the present study. First, the sample size was relatively small, and some data bias/missing may exist in a retrospective study; for instance, serum tumor markers were not detected in some patients for some unknown reasons, and we could not provide the missing part in present study, and the single-center samples with only Chinese ethnicity population involved in the present study may impede the capacity of obtaining robust conclusions to general populations; multicenter investigation in the future could be performed. Second, only resected patients were collected, and no advanced tumors were involved, then overview of LCNEC with different stages was difficult. Third, genomic and immune biomarkers such as PD-L1 information were insufficient. Since immunotherapy might be a choice in the future for LCNEC (19, 20), related immune markers should be investigated better.

In conclusion, LCNEC was a rare type of lung cancer with a high relapse rate. Our results demonstrated common driven gene mutants were scarce in LCNEC. Nodal (N) stage was associated with tumor recurrence and proved to be an independent prognostic factor for DFS, while OS significantly differed from different T stages. Serum positive CEA before surgery could be used for survival evaluation; besides, different adjuvant chemotherapies influenced the outcomes, with PE seemed a better choice. Perspective clinical trials were essential to provide a more confirmed conclusion, and deeper investigations of genomic/immune biomarker in LCNEC were also important.

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 Review Board (IRB) in ShangHai Chest Hospital [No. KS(Y)1982]. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization of the study was achieved by YS and BH. The research methodology was designed by YS, JX, and CL. Formal analysis of the data was conducted by YS, FH, and TC. Project administration was carried out by BH and XZ. The study resources were obtained by YS, JX and RZ. Software analysis of data and figures was conducted by YS and FH. In addition, supervision of the research was conducted by TC, XZ and BH. Writing the manuscript was carried out by YS and BH. Review and editing of the manuscript were carried out by TC and XZ. 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.2020.556194/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Over-all survival (OS) for surgical resected LCNEC with different adjuvant chemotherapy strategies.

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Treatment Outcomes of 9,994 Patients With Extensive-Disease Small-Cell Lung Cancer From a Retrospective Nationwide Population-Based Cohort in the Korean HIRA Database

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To investigate the efficacy of irinotecan-based (IP) and etoposide-based (EP) platinum combinations, and of single-agent chemotherapy, for treatment of extensive-disease small cell lung cancer (ED-SCLC), we performed a large-scale, retrospective, nationwide, cohort study. The population data were extracted from the Health Insurance Review and Assessment Service of Korea database from January 1, 2008, to November 30, 2016. A total of 9,994 patients were allocated to ED-SCLC and analyzed in this study. The primary objectives were to evaluate the survival outcomes of systemic first-line treatments for ED-SCLC. For first-line treatment, patients who received IP showed a better time to first subsequent therapy (TFST) of 8.9 months (95% confidence interval [CI], 8.50–9.40) than those who received EP, who had a TFST of 6.8 months (95% CI, 6.77–6.97, $P < 0.0001$). In terms of overall survival (OS), IP was superior to EP (median OS, 10.8 months; 95% CI, 10.13–11.33 vs. 9.5 months; 95% CI, 9.33–9.73; $P < 0.0001$). Taken together, in the Korean population, first-line IP combination chemotherapy had significantly favorable effects on OS and TFST.

Keywords: efficacy, systemic chemotherapy, population-based cohort study, prognosis, extensive-disease small cell lung cancer

INTRODUCTION

Lung cancer is the main cause of cancer-related death worldwide, and the small cell lung cancer (SCLC) subtype includes only 11%–14% of total lung cancer diagnoses (1–3). Biologically, SCLC is aggressive lung cancer subtype, with a high frequency of metastasis and early dissemination. At diagnosis, more than two-thirds of patients have extensive-disease (ED) SCLC. The majority of

patients with ED-SCLC die within 1 year of initial diagnosis due to relapse, despite the initial sensitivity of platinum-based chemotherapy (2).

Platinum-based chemotherapy including etoposide or irinotecan can produce a 60–80% response rate (RR) and 7–12 months of median survival in patients with ED-SCLC (4). However, despite good response, improvement during the past decade has been limited; the 2-year survival rate increased only from 3.4% to 5.6% (5). Etoposide with platinum (EP) is currently the standard first-line treatment used to obtain longer overall survival (OS) and progression-free survival (PFS) in Western populations; however, it results in a only 2% 5-year survival rate (6). In contrast, subsequent Eastern Asian studies have yielded contradictory results. A Japanese phase III study, comparing the efficacy of irinotecan with cisplatin (IP) versus EP as first-line chemotherapy, showed improved survival for IP compared to EP (7). However, the following trials did not support the superiority of IP over EP (8, 9). In a recent phase III study, first-line IP also did not significantly improve survival compared to EP (2). Also, there is no established consensus regarding the most effective second-line regimen. Especially in Korea, there is a tendency to use a less toxic single agent rather than a platinum-based combination because of patients' poor performance and organ dysfunction (10–12). Therefore, determining the clinical efficacy of first- and second-line systemic therapies in a larger population would enable treatment strategies for ED-SCLC to be refined.

To date, no large-scale studies have assessed the efficacy of each systemic regimen in ED-SCLC patients in an East Asian population. Korean health insurance covers the entire population of Korea, and the Health Insurance Review and Assessment Service of Korea (HIRA) provides information on healthcare services provided to the Korean population. Thus, using the HIRA database, we could approach the entire Korean population and analyze the efficacy of systemic chemotherapy in a large population of patients with ED-SCLC who received palliative systemic treatment.

MATERIALS AND METHODS

Study Design

This large-scale, retrospective, nationwide cohort study was approved by the institutional review board of the Uijeongbu St. Mary Hospital and HIRA (No. UC18ZESI0145). The requirement for written informed consent was waived because this study retrospectively analyzed national insurance cohort data. The data-mining scheme used in this study is shown in **Figure 1**. The HIRA data include all ICD-10 diagnostic codes and billing codes for all medical services, such as diagnostic procedures and treatment modalities (such as drug prescriptions, radiotherapy, or surgery), provided to the entire population of Korea. We performed data mining using a query program to classify the appropriate SCLC patient cohort.

Study Population

A total of 252,656 patients were identified as having C34 ICD-10 diagnostic code in the HIRA database from January 1, 2008, to March 31, 2018. 238,166 were excluded, as they had received chemotherapy regimens only used for NSCLC such as pemetrexed, gemcitabine, docetaxel, vinorelbine, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), or anaplastic lymphoma kinase (ALK) TKI, or had other types of cancer. In the first query step, billing codes were used to identify SCLC; these consisted of chest radiotherapy and drugs such as belotecan, irinotecan, etoposide, vincristine, ifosfamide, cyclophosphamide, topotecan, cisplatin, carboplatin, doxorubicin, and paclitaxel, which are covered for ED-SCLC by the Korean national health insurance service. SCLC patients were defined and classified according to the types and orders of use of chemotherapeutic regimens for each stage of SCLC defined by the national health insurance service regulations of Korea. The remaining 14,490 patients were selected as having undergone chemotherapy appropriate for SCLC. The operational definition of patients with limited disease (LD) SCLC was those who received definitive concurrent chemoradiotherapy (CCRT); otherwise, the patient was considered to have ED-SCLC. A total of 4,496 patients with LD-SCLC were defined using the operational criteria, while the remaining 9,994 patients were allocated to ED-SCLC and analyzed in this study. To verify the reliability of the operational criteria for SCLC staging, we used single-institution data from 357 SCLC patients with known

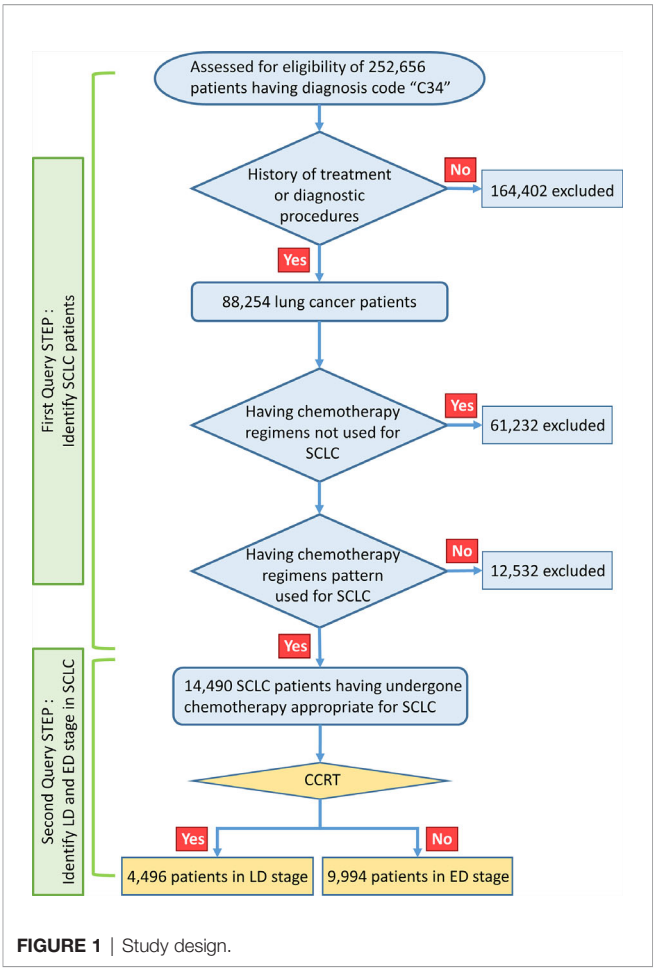

FIGURE 1 | Study design.

disease status. Using these operational criteria, patients with ED-SCLC were predicted with a sensitivity of 100%, specificity of 64.6%, and accuracy of 88.5% (**Supplementary Table 1**).

In patients with LD-SCLC defined by our operational criteria, the median survival duration was 21.8 months (95% confidence interval [CI], 20.86–22.96); in patients with ED-SCLC, 9.6 months (95% CI, 9.43–9.83, **Supplementary Figure 1**). Five-year survival rates were 24.73 ± 0.75% and 8.13 ± 0.30%, respectively. These findings are comparable with the survival outcomes of LD and ED-SCLC patients in recent studies (13–15). Thus, our operational criteria were considered acceptable.

Definition of Survival Outcomes

The time to first subsequent therapy (TFST) duration was defined as the time from the date of first-line chemotherapy until subsequent chemotherapy or death due to any cause, whichever was observed first. The overall survival (OS) duration was calculated from the date of diagnosis to the date of death or the last follow-up visit. The date of diagnosis was defined as the date when first chemotherapy or surgery or radiotherapy was started after the first application of the C34 diagnostic code.

Statistical Analysis

The primary objectives were to evaluate the survival outcomes of systemic first-line treatments for ED-SCLC. The secondary objectives were to evaluate the survival outcomes of the regimens as second-line treatments. Baseline characteristics are presented as means (± standard error) and medians (ranges) for continuous variables and frequencies (%) for categorical variables. A *t*-test was performed for comparisons of continuous variables, and Pearson's chi-squared test or a two-sample proportion z-test for comparisons of categorical variables. We performed a Cox proportional hazards regression to identify the risk factors for overall mortality, because the Cox proportional hazards assumption was satisfied for the variables analyzed in this study. The survival curves were estimated using the Kaplan–Meier method and compared using the log-rank test. SAS Enterprise Guide version 6.1 (SAS Inc., Cary, NC, USA), Visual Basic for Applications 7.0 (Microsoft Inc., Redmond, WA, USA), and Excel 2010 (Microsoft Inc., Redmond, WA, USA) were used to perform all data mining and statistical analyses.

RESULTS

Baseline Characteristics

A total of 9,994 patients were analyzed as having ED-SCLC. Their demographic features are shown in **Table 1**. The mean age was 68 years. As first-line treatment, 9,618 patients received combination chemotherapy (combination chemotherapy group [CG]), and the remaining 376 received a single agent (single agent group [SG]). The most common first-line regimen was an etoposide with platinum combination. For the second-line regimen, combination chemotherapy was used in 2,213 patients and single-agent chemotherapy in 2,085. Irinotecan combined with platinum in CG and belotecan in SG were the most frequently used second-line treatment regimens.

TABLE 1 | Demographic characteristics of 9,994 patients with ED-SCLC with systemic chemotherapy.

	Total (n = 9,994)
Age	68 (SD 8.4)
Gender (Male/Female)	8,634 (86.4%)/1,360 (13.6%)
Comorbidities	
HBP	5,677 (56.8%)
DM	2,719 (27.2%)
Dyslipidemia	4,623 (46.3%)
COPD	2,015 (20.2%)
First-line chemotherapy	
Combination chemotherapy	9,618 (96.2%)
Etoposide/platinum	8,142 (81.4%)
Irinotecan/platinum	1,476 (14.8%)
Single-agent chemotherapy	376 (3.8%)
Etoposide	213 (2.1%)
Irinotecan	71 0.7%)
Belotecan	92 (0.9%)
Second-line chemotherapy	
Combination chemotherapy	2,123 (21.2%)
Etoposide/platinum	598 (6.0%)
Irinotecan/platinum	1,525 (15.3%)
Single-agent chemotherapy	2,085 (20.8%)
Etoposide	31 (0.3%)
Irinotecan	561 (5.6%)
Belotecan	920 (9.2%)
Topotecan	573 (5.7%)

ED-SCLC, extensive-disease small-cell lung cancer; SD, standard deviation; HBP, hypertension; DM, diabetes mellitus; COPD, chronic obstructive pulmonary disease.

Survival Outcomes With the First-Line Treatment

Within a 9.6-month median follow-up period, analysis of the survival data revealed 8,907 death (89.1%) events in the ED group of 9,994. In CG, of note, the IP combination showed significantly better TFST of 8.9 months (95% CI, 8.50–9.40) than the EP combination at 6.8 months (95% CI, 6.77–6.97) (P < 0.0001, **Figure 2A**). In terms of OS, significantly improved survival benefit was also found in patients with the IP combination at 10.8 months (95% CI, 10.13–11.33) compared with the EP combination at 9.5 months (95% CI, 9.33–9.73) (P < 0.0001, **Figure 2B**). The median TFST time was 7.1 months (95% CI, 6.70–7.23) in the CG group and 6.1 months (95% CI, 5.37–6.77) in the SG group (P < 0.0001, **Supplementary Figure 2A**). The median OS time was 9.7 months (95% CI, 9.50–9.90) in the CG group and 7.3 months (95% CI, 6.23–8.53, P < 0.0001) in the SG group (**Supplementary Figure 2B**). In SG, there were no significant differences among the three monotherapies for TFST (P = 0.4101). Belotecan showed better OS than etoposide or irinotecan monotherapy (14.7 months, 95% CI, 12.83–17.00 vs. 4.16 months, 95% CI, 3.06–5.56 vs. 6.66 months, 95% CI, 5.26–8.53, respectively, P < 0.0001, **Supplementary Figure 2C**).

Survival Outcomes With the Second-Line Treatment

Following failure of first-line chemotherapy, the combination chemotherapy in the second line demonstrated significantly

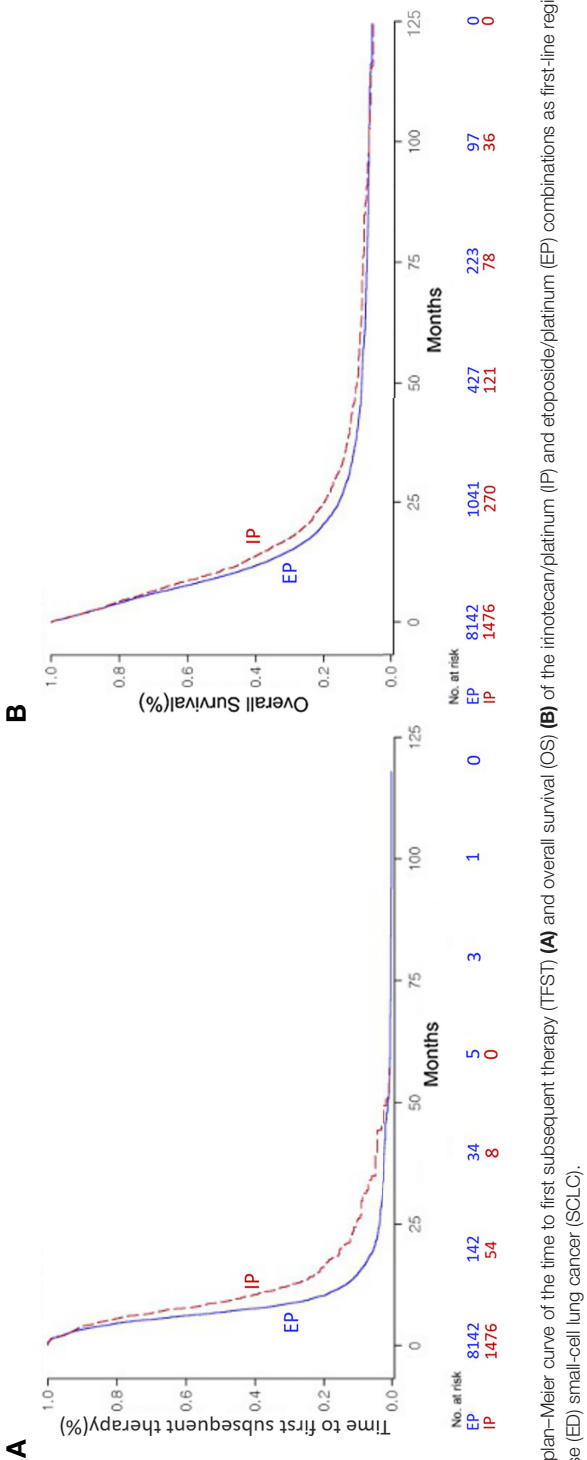

FIGURE 2 | Kaplan–Meier curve of the time to first subsequent therapy (TFST) **(A)** and overall survival (OS) **(B)** of the irinotecan/platinum (IP) and etoposide/platinum (EP) combinations as first-line regimens for extensive-disease (ED) small-cell lung cancer (SCLC).

improved OS of 6.6 months (95% CI, 6.36–6.96) compared with the single regimens at 5.1 months (95% CI, 4.93–5.36) (P < 0.0001, **Supplementary Figure 3A**). In patients with SCLC who failed or relapsed after first-line EP chemotherapy, a similar finding was also observed (SG: 5.1 months, 95% CI, 4.93–5.36 vs. CG: 6.5 months, 95% CI, 6.23–6.86, P < 0.0001, **Supplementary Figure 3B**). Unlike with first-line treatment, the EP combination showed significantly better OS of 6.9 months (95% CI, 6.13–7.53) than the IP combination at 6.6 months (95% CI, 6.30–6.90) (P = 0.0009, **Figure 3A**). However, OS did not differ significantly among single-agent regimens as second-line treatment (P = 0.5856, **Figure 3B**).

Factors Associated With Survival Outcomes in Patients With Extensive-Disease Small Cell Lung Cancer

The univariate analyses demonstrated that elder age, male gender, hypertension, chronic obstructive pulmonary disease (COPD), absence of hypercholesterolemia and diabetes mellitus, EP combination compared with the IP combination were significantly associated with shorter OS (**Table 2**). In the multivariate Cox proportional hazards regression analysis, all factors retained their independence toward OS. Also, the EP combination was significantly associated with poorer OS (adjusted odds ratio [OR], 1.18, 95% CI, 1.10–1.27; P < 0.0001) compared to IP as first-line treatment.

DISCUSSION

In this study, the survival outcomes of patients with ED-SCLC were better among those who received the IP regimen than those who received the EP regimen in the first-line setting. If a single agent was required, despite inferior tumor control to platinum-based combination chemotherapy, the OS of patients who received belotecan as the first-line treatment was better than that of those administered irinotecan or etoposide alone. In the second-line setting, EP had a better OS than IP, unlike the first-line setting. The single-agent chemotherapies as the second-line treatments did not significantly differ in terms of OS. This study provides evidence that the irinotecan and platinum combination as the first-line therapy may be the gold standard first-line regimen for Korean patients with ED-SCLC. To the best of our knowledge, this analysis includes the largest study population to date.

Nowadays, based on IMpower 133 and Caspian trial, atezolizumab or durvalumab combined platinum-based doublet chemotherapy have been updated as the standard of care in the first-line regimen of extensive disease of SCLC (16, 17). However, over the past 20 years, standard therapy for most patients with ED-SCLC has been a platinum-based etoposide combination regimen. In 2002, in the Japanese Clinical Oncology Group (JCOG)-9511 phase III study, which compared EP to IP, the tumor response and patient survival outcomes were significantly better in the IP group at the interim analysis, prompting early termination of further accrual (7). Because of the small number of patients (n = 174), the study involved a

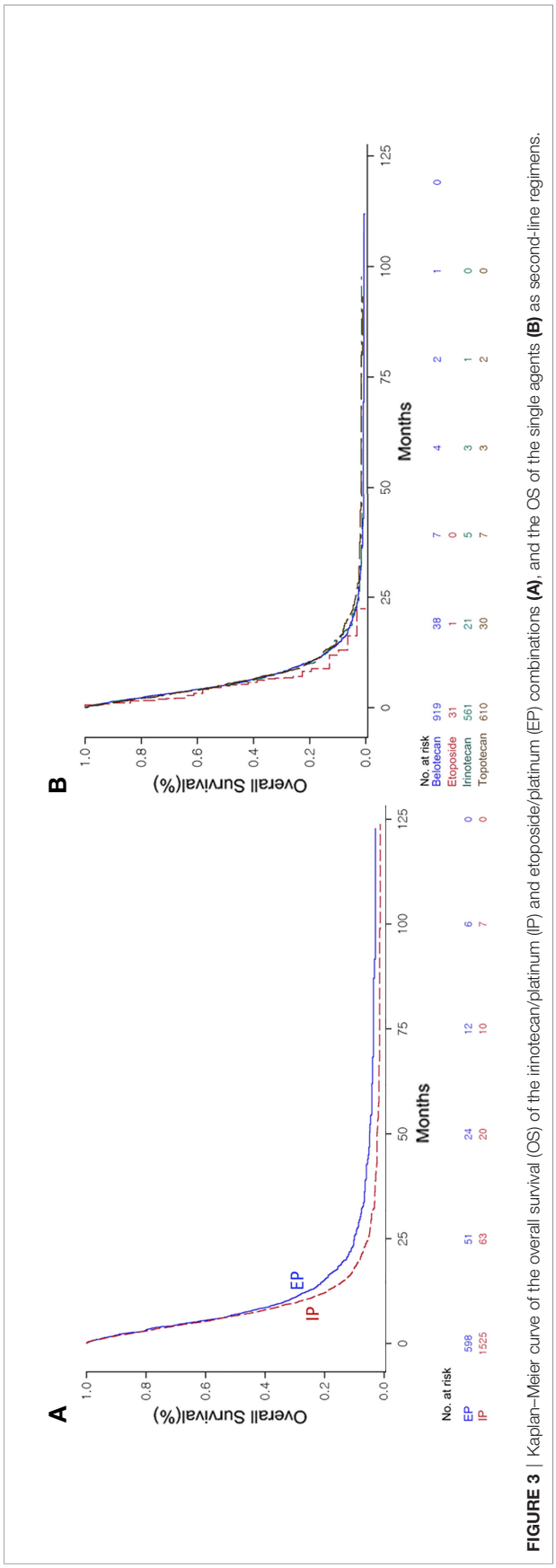

FIGURE 3 | Kaplan–Meier curve of the overall survival (OS) of the irinotecan/platinum (IP) and etoposide/platinum (EP) combinations **(A)**, and the OS of the single agents **(B)** as second-line regimens.

solely Japanese population. Subsequently, a phase III trial by the Southwest Oncology Group (SWOG)-0124 was conducted to confirm the results of JCOG-9511 in 651 people from North America, with similar eligibility criteria to those in the Japanese trial (8). SWOG-0124 found no significant differences between IP and EP in terms of tumor response, PFS, and OS. Thus, EP remains the standard of care for patients with ED-SCLC, at least for non-Japanese populations. In a comparison of two trials, there was no difference in the PFS of the EP group (9.4 months in JCOG-9511 vs. 9.1 months in SWOG-0124). On the contrary, for the IP group, there was a definite difference between the two studies: a median PFS of 12.8 months for JCOG-9511 and 9.9 months for SWOG-0124 ($P < 0.001$) (18). However, patients of male sex and with a poor performance status, who are generally regarded as having a poor prognosis, were present in larger numbers in the JCOG-9511 IP group than in the SWOG-0124 IP group. Thus, differences according to ethnicity are possible.

The most reasonable explanation for differences of irinotecan efficacy across ethnicities could be pharmacogenomic differences in the metabolism of irinotecan between Asian and Western populations. There has been no direct comparison of irinotecan metabolism-related genes and the efficacy of irinotecan in SCLC patients across geographic regions. According to Gandara et al. differences in genes involved in paclitaxel disposition or DNA repair were observed between Japanese and American patients with lung cancer (19). Also, Lampe et al. reported that the allele and genotype frequencies of *UGT1A1*, which is related to glucuronidation of a metabolite of irinotecan, varied between Asians and Caucasians (20). A specific single nucleotide polymorphism in the adenosine triphosphate-binding cassette (ABC) gene is correlated with the efficacy of irinotecan-based chemotherapy. Han et al. reported that the ABCC2-24TT and 3972TT genotypes were associated with a higher RR and longer PFS in Korean patients with advanced lung cancer (21). We infer a possible association between gene polymorphism, such as the ABC gene, and efficacy of irinotecan in SCLC. However, there have not been any reports of differences of ABC gene polymorphism according to ethnicity. Moreover, to date, UGT1A1 has not been reported as significantly correlated with irinotecan efficacy in SCLC (22, 23). Therefore, analysis of differences in genes related to the metabolism of irinotecan-based chemotherapeutics and of their direct correlation with efficacy is warranted.

In a recent phase III trial in Korean patients with ED-SCLC, although OS and PFS were not significantly different between the EP and IP arms, there was a favorable trend toward the IP regimen (OS, 10.9 months vs. 10.3 months, $P = 0.120$; PFS, 6.5 months vs. 5.8 months, $P = 0.115$). A higher RR was observed in IP (62.4% vs. 48.2%, $P = 0.006$) (2). Of note, the authors concluded that IP chemotherapy might be beneficial for these particular subgroups: male gender, < 65 years old, and ECOG PS 0/1 patients. In 62 Chinese patients, a randomized, prospective phase II study showed the efficacy of IP was similar to that of EP for untreated ED-SCLC; median OS was 18.1 months in IP vs.15.8 in EP (23). In a meta-analysis by Jiang et al., six randomized controlled trials involving 1,476 patients, without considering ethnicity, showed that irinotecan/platinum

TABLE 2 | Relative risk for overall survival of 9,994 patients with ED-SCLC.

	Unadjusted OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
Age	1.52 (1.42–1.62)	<0.0001	1.47 (1.37–1.57)	<0.0001
Gender (**male** vs. female)	1.19 (1.09–1.30)	<0.0001	1.19 (1.09–1.30)	<0.0001
HBP (**HBP** vs. normal)	1.08 (1.01–1.14)	0.0103	1.07 (1.00–1.14)	0.03
DM (**normal** vs. DM)	1.13 (1.06–1.21)	0.0002	1.12 (1.04–1.20)	0.001
Hypercholesterolemia (**normal** vs. hypercholesterolemia)	1.08 (1.02–1.15)	0.005	1.11 (1.04–1.18)	0.001
COPD (**COPD** vs. normal)	1.25 (1.16–1.34)	<0.0001	1.17 (1.09–1.26)	<0.0001
1st line chemotherapy regimen (reference, irinotecan/platinum combination)				
Belotecan	1.05 (0.81–1.35)	0.7114	1.04 (0.80–1.34)	0.75
Etoposide	2.37 (1.98–2.84)	<0.0001	2.25 (1.88–2.69)	<0.0001
Irinotecan	1.34 (1.02–1.75)	0.0337	1.38 (1.05–1.81)	0.02
Etoposide/platinum	1.18 (1.09–1.26)	<0.0001	1.18 (1.10–1.27)	<0.0001

ED-SCLC, extensive-disease small-cell lung cancer; OR, odds ratio; 95% CI, 95% confidence interval; HBP, hypertension; DM, diabetes mellitus; COPD, chronic obstructive pulmonary disease. Variables with odds ratio are shown in bold type.

significantly improved the risk ratio (RR) and OS compared with etoposide/platinum with less hematological toxicity (4). In addition, in a recent meta-analysis of 12 randomized controlled trials involving 2,030 patients, including more Asian populations, the irinotecan/platinum regimen also significantly improved the 1- and 2-year survival rates of patients with previously untreated ED-SCLC (RR 1.16, 95% CI, 1.03–1.31, P = 0.02 vs RR 1.79, 95% CI, 1.22–2.61, P = 0.003, respectively) (24). Taken together, the IP regimen should be strongly considered as first-line therapy in Asian populations.

Interestingly, belotecan, a new camptothecin analog, was superior as a single agent in the first-line regimen compared to irinotecan or etoposide alone. In a preclinical study, belotecan was a more potent topoisomerase I inhibitor and had superior antitumor activity to camptothecin and topotecan (25). In a phase II study, belotecan showed a 42.9% RR and a modest OS of 11.4 months as the first-line treatment for ED-SCLC, comparable to irinotecan alone (26). Neutropenia occurred in 74% of the patients but was reversible, generally manageable, and not cumulative. There has been no confirmatory trial of the efficacy of belotecan in the first-line setting for patients medically unfit for combination chemotherapy. However, our findings will enable a confirmatory trial of the efficacy of belotecan alone compared with irinotecan, etoposide, topotecan, and paclitaxel alone.

Regarding strategies to use cytotoxic chemotherapy in the second-line setting, a consensus has not been reached on the best and most effective regimen. However, based on our results, in patients receiving combination treatment as their second-line treatment, a statistically significant increase in OS was observed compared to those receiving single agents, even in the population with the EP regimen as first-line treatment. OS did not significantly differ between single-agent regimens such as topotecan, irinotecan, belotecan, or etoposide at the second-line treatment. In a phase III study comparing topotecan alone and cyclophosphamide/adriamycin/vincristine combination therapy as second-line therapy, both groups showed similar response and survival rates, but the group receiving topotecan had less toxicity than the combination therapy group (27). Although there are limited data on similar effects between irinotecan and topotecan, this has not been evaluated in randomized studies. Meanwhile, in the Western population, there was a new selective oncogenic transcription inhibitor, lurbinectedin, which showed anti-tumor activity in 105 patients with small-cell lung cancer who had received prior platinum-based chemotherapy and had no brain metastases (28). The objective response rate of lurbinectidine was 35.2%, median progression-free survival was 3.5 months, and median overall survival 9.3 months. When it comes to our results showing no superior single agent in 2nd line setting and lubinectidine studied only in Europe and USA population, the interpretation and application of results of lurbinectedin should be with caution. There might be other metabolic and genetic differences of drugs according to ethnicity as we mentioned above.

Poor prognostic factors for patients with ED-SCLC were elderly age, male gender, COPD, normal lipids, and EP chemotherapy. COPD could be a driving factor in lung cancer, but there have been conflicting results from previous studies about whether COPD affects the survival of lung cancer patients on chemotherapy or a tyrosine kinase inhibitor (28). Recent analysis showed that COPD is an independent prognostic risk factor for lung cancer (29). The prognostic role of lipidemia in cancer patients is controversial. Hypocholesterolemia in malignancy might come from an increased demand for cholesterol from neoplastic cells, resulting in increased LDL removal (30).

Several limitations are apparent in this study and must be considered when interpreting the results. First, there may be bias in identifying LD or ED-SCLC patients using the operational definition. However, to overcome such bias we used a strict multistep approach. The operational definition showed high accuracy to differentiate LD and ED patients when validated among 357 SCLC patients at a single institution. Moreover, the survival rates of the subgroups defined were comparable to those reported previously. Second, we analyzed the HIRA data retrospectively; they do not include information on the frequency of adverse drug reactions, dose intensity, or cause of

death. However, this is, to our knowledge, the largest comparative analysis in Asian patients with ED-SCLC.

CONCLUSIONS

Authors found that IP as the first-line regimen had a significantly favorable effect on the OS and TFST of patients with ED-SCLC compared to EP. Among the single agents, belotecan showed a superior OS to irinotecan or etoposide alone. As the second-line therapy, combination chemotherapy had clinical benefits over single agents; there were no significant differences among the single agents.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are not publicly available due to Data Protection Laws and Regulations in Korea, but final analyzing results are available from the corresponding authors on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Uijeongbu St. Mary Hospital of the Catholic University of Korea and HIRA. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

YKo, JL, and HL conceptualized the work. JL, YKi, JH, YKo, HL, SK, and S-YS acquired, analyzed, and interpreted the data. YKo, JL, and JH have drafted the work. JL, JH, and YKo substantively revised it. JH and YKo were co-corresponding authors in this work. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.546672/full#supplementary-material

Supplementary Table 1 | Sensitivity, specificity, and accuracy for prediction of LD- or ED-SCLC using the operational definition. LD, limited-stage disease; ED, extensive-stage disease; SCLC, small cell lung cancer; PPV, positive predictive value; NPV, negative predictive value.

Supplementary Figure 1 | Kaplan–Meier curve for overall survival (OS) between extensive and limited stage disease small cell lung cancer (ED-SCLC and LD-SCLC, respectively) patients who underwent systemic treatment.

Supplementary Figure 2 | Kaplan–Meier curve for time to first subsequent therapy (TFST) **(A)** and overall survival (OS) **(B)** of the combination chemotherapy (combination-chemotherapy group [CG]) and the single-agent group [SG]); **(C)** OS of patients who received belotecan, etoposide, and irinotecan as a single agent as first-line treatment of ED-SCLC.

Supplementary Figure 3 | Kaplan–Meier curve for overall survival (OS) of the combination chemotherapy (combination-chemotherapy group [CG]) and the single-agent group [SG]) as the second-line regimen in the total population **(A)** and post-EP chemotherapy population **(B)**.

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