Application of pleural effusion cell blocks for immunohistochemistry and EGFR gene mutation testing for advanced lung cancer

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Abstract. In the present study, the diagnostic capability of pleural effusion cell blocks for immunohistochemistry and epidermal growth factor receptor (EGFR) mutation detection in advanced lung cancer was explored. Samples of pleural effusion were collected from 231 patients with advanced lung cancer, treated at Weifang No. 2 People's Hospital (Weifang, China) from July 2018 to December 2022, and were transformed into cellular blocks by embedding them in paraffin. Lung cancer subtypes were determined using immunohistochemical staining, and EGFR gene mutations were identified through the use of the amplification refractory mutation system technology (ADx-ARMS). Of the 231 specimens analyzed by immunohistochemistry, 222 were adenocarcinoma, seven were small cell carcinoma and two were squamous cell carcinoma. EGFR testing was performed on 161 lung adenocarcinoma cases, revealing an EGFR mutation rate of 52.8% (85/161). The mutations discovered in the EGFR gene encompassed L858R in exon 21 (49 instances), deletion of exon 19 (31 instances), L861Q in exon 21 (2 instances), G719X in exon 18 (1 instance), a co-mutation of G719X/L861Q (1 instance) and a co-mutation of S768I/G719X (1 instance). The EGFR mutation rate was significantly higher in female patients with lung adenocarcinoma (32.30%) compared with that in male patients (20.50%) ($P<0.05$). Furthermore, patients with EGFR-mutant lung adenocarcinoma undergoing treatment with EGFR tyrosine kinase inhibitors exhibited a significantly extended survival rate compared with those with wild-type EGFR receiving chemotherapy. In conclusion, the present study demonstrated that immunohistochemistry with pleural effusion cell blocks can aid in clarifying the histological subtype of lung cancer, and enable EGFR mutation detection, which can effectively guide molecular targeted therapy.

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Introduction

Globally, lung cancer is listed as one of the most common and lethal types of cancer (1,2). In total, ~85% of lung cancer cases are non-small cell lung carcinoma (NSCLC) (3), with lung adenocarcinoma being the most common subtype of NSCLC. Early-stage lung cancer often presents with minimal or mild clinical symptoms; therefore, ~75% of patients are already at an advanced stage at initial diagnosis (4). When diagnosed, pleural effusion is present in 20% of patients with lung cancer (5) and the survival of patients with lung cancer with malignant pleural effusion (MPE) is 5.5 months from diagnosis (6). Furthermore, most patients with pleural effusion are not eligible for surgical intervention due to the advanced stage of the disease.

Mutations in the EGFR gene have been recognized as key markers for individualized therapy and evaluating prognosis in advanced lung cancer, given their close association with the appropriateness of specific treatments, such as targeted therapy (7). However, for numerous patients with advanced lung cancer, sufficient tumor tissue for molecular diagnosis cannot be obtained due to the inherent invasiveness and sampling limitations of puncture biopsy or surgical procedures. MPE cell blocks, often containing tumor cells from patients, are recognized by pathologists as a valuable resource for genetic testing, offering a unique opportunity to directly analyze and understand the molecular characteristics of malignancies (8). Pleural effusion may be an alternative specimen to tumor tissue, as it can be safely and repeatedly collected via thoracentesis (9). A previous study showed that the use of pleural effusion cell blocks combined with immunohistochemistry and molecular testing markedly improved the accuracy and specificity of advanced lung cancer diagnosis compared with standalone pleural effusion cytology (10).

The objective of the present study was to provide clinical proof to confirm the diagnostic and predictive significance of pleural effusion cell block technology in diagnosing lung

cancer and guiding decisions for patient-specific clinical treatments. Immunohistochemical analysis was performed on pleural effusion cell blocks from patients with advanced lung cancer for histological classification, and an amplification refractory mutation system (ARMS) was used to detect the EGFR target gene mutation status in cell blocks from patients with confirmed lung adenocarcinoma.

Materials and methods

Patients. The present study encompassed 231 patients with lung cancer with pleural effusion complications (MPE) who presented at Weifang No. 2 People's Hospital (Weifang, China) between July 2018 and December 2022. The population comprised of 129 male patients and 102 female patients, aged 30-91 years old (mean age, 68.61±10.53 years). Samples of pleural effusion were collected from each patient through the hospital medical system. No patient had undergone targeted therapy. Data on clinicopathological features were collected from the Weifang No. 2 People's Hospital health record system. Patient overall survival (OS) information was acquired via telephone follow-ups. The monitoring phase commenced in July 2018 and concluded in February 2022.

Criteria for inclusion were as follows: i) Patients diagnosed with lung cancer after admission on the basis of clinical symptoms, bronchoscopy and pathological examination of cell blocks using hematoxylin and eosin (H&E) staining and immunohistochemistry; ii) patients with lung cancer who had not received targeted therapy prior to pleural effusion collection; iii) patients with sufficient pleural effusion material for the preparation of cell blocks and subsequent experimental procedures (at least 20 tumor cells in one H&E-stained section of the cell block); and iv) comprehensive clinical data for patients provided by the medical record system, encompassing the patient's age, sex, clinical stage and smoking history.

The exclusion criteria were: i) Individuals suffering from acute respiratory, heart or brain vessel disorders; and ii) individuals with a background of or simultaneous presence of other cancerous diseases, such as breast, colon or cervical cancer.

The Ethics Committee of Weifang No. 2 People's Hospital approved the present study (approval no. KY2023-015-01) and all participants provided written informed consent for the use of their tissues/data for scientific research purposes.

Reagents and instruments. The primary antibodies used in immunohistochemistry were purchased from Fuzhou Maixin Biotechnology Development Co., Ltd. The secondary antibody and colorimetric reagents were obtained from Roche Diagnostics (Shanghai) Co., Ltd.

A low-speed centrifuge (TDL-40B) was obtained from Shandong Bio-Medical Technology Co., Ltd. The BenchMark GX automated staining system was purchased from Roche Diagnostics (Shanghai) Co., Ltd. The SLAN-96S fully automated medical polymerase chain reaction (PCR) analysis system was purchased from Shanghai Hongshi Medical Technology Co., Ltd.

Preparation of cell blocks. After collection of pleural fluid specimens, the samples were promptly delivered to the Department of Pathology of Weifang No. 2 People's Hospital. The sample was maintained at ambient temperature for a duration of 15-30 min, following which 50-100 ml liquid was collected from the bottom of the container. Samples underwent centrifugation at 1,000 x g for 5 min at room temperature, followed by the extraction of the pellet for cytological smear analysis by H&E staining. Following removal of the supernatant, a solution of 4% neutral buffered formalin was introduced to fix the pellet samples for 15 min at room temperature. Subsequently, samples underwent centrifugation at 1,000 x g for 5 min at room temperature, after which the pellet was moved to qualitative filter paper (Nanchang Yulu Experimental Equipment Co., Ltd.) and then routinely dehydrated through a graded ethanol series for 1-2 h at each concentration to remove water, followed by clearing with xylene to achieve transparency for a duration of 1-3 h. The slides were embedded in paraffin and subsequently stained with H&E, with hematoxylin applied for 5 min and eosin applied for 30 sec, both at room temperature. An optical microscope was used to observe the results.

Immunohistochemistry. The cell blocks were sectioned into 4-μm slices and stained using the Roche BenchMark GX fully automated immunohistochemistry system. Positive (antigen-expressing tissue) and negative (replacement of primary antibody with negative reagent) controls were routinely set up. The primary antibodies used included antibodies against pan-cytokeratin (pCK), CK7, CK5/6, napsin A, thyroid transcription factor-1 (TTF-1), P40, Wilms tumor 1 (WT-1), synaptophysin, chromogranin A (CgA), homeobox protein CDX2, villin, CD56, Ki-67, desmin and calretinin (Table SI; Data SI). All cell blocks were independently evaluated by two pathologists to ascertain whether the cytomorphology matched the immunophenotyping results and to assess whether the tumor cell content met the requirements for PCR testing (a cell block H&E-stained section must contain at least 20 tumor cells). Tumor cells of lung adenocarcinoma exhibit glandular/acinar patterns, while tumor cells of small cell carcinoma are tightly arranged in a 'stacked or regimented' formation and tumor cells of squamous cell carcinoma are often dispersed as single cells or in small clusters.

Genomic DNA extraction from paraffin-embedded samples. After the tumor cell content was evaluated by immunohistochemistry (number of tumor cells and percentage of tumor cells relative to the total tissue area in the H&E-stained section), 4-12 cell block sections were cut to a 5-μm thickness using a disposable microtome blade and transferred into clean 1.5-ml centrifuge tubes. DNA extraction was performed using a nucleic acid extraction kit (FFPE DNA; cat. no. 8.02.0017; Amoy Diagnostics Co., Ltd.) following the manufacturer's instructions. DNA purity and concentration were quantified using an Eppendorf UV spectrophotometer (Eppendorf SE).

Detection of EGFR gene mutations. EGFR gene mutation status was evaluated by ADx-ARMS using the human EGFR gene mutation detection kit (cat. no. 8.01.0131; Amoy Diagnostics Co., Ltd.), including primers and fluorophore, following the manufacturer's guidelines. Using this kit, the ARMS technique was used to identify 21 prevalent forms

Table I. TKI treatments (patients received only one oral medication listed).

EGFR mutation type	Specific TKI	Generation	Dosage	Treatment duration
L858R 19-del	Gefitinib	1st Generation	250 mg once daily	Until disease progression or unacceptable toxicity
	Erlotinib	1st Generation	150 mg once daily	
	Icotinib	1st Generation	125 mg three times daily	
	Afatinib	2nd Generation	40 mg once daily	
	Osimertinib	3rd Generation	80 mg once daily	
G719X,L861Q	Afatinib	2nd Generation	40 mg once daily	

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

of mutations in EGFR exons 18, 19, 20 and 21. The thermocycling conditions used were as follows: 1 cycle at 95˚C for 5 min; 15 cycles at 95˚C for 25 sec, 64˚C for 20 sec and 72˚C for 20 sec; and 31 cycles at 93˚C for 25 sec, 60˚C for 35 sec and 72˚C for 20 sec. Upon completion of the reaction, the SLAN-96S fluorescence quantitative PCR instrument provided the amplification curve, and the mutations were determined following the interpretation principles outlined in the kit instructions. Each experiment included a positive control from the kit and nuclease-free purified water as a negative control.

Patient treatment. Patients with single EGFR mutations received EGFR tyrosine kinase inhibitors as first-line treatment, as detailed in Table I. Patients without EGFR gene mutations were treated with chemotherapy, specifically cisplatin (a platinum-based drug) at 75 mg/m^2 combined with pemetrexed (an antimetabolite agent) at 500 mg/m^2 for 4-6 cycles, followed by pemetrexed maintenance at 500 mg/m^2. Treatment continued until disease progression or the occurrence of intolerable adverse reactions. Based on the completeness of follow-up data, prognostic analysis was performed only on 36 cases with EGFR gene mutations and 65 cases without EGFR gene mutations.

Statistical and prognostic analyses. Statistical evaluation was performed using SPSS 25.0 software (IBM Corp.). The χ^2 test was employed to explore the associations between mutations in the EGFR gene and clinicopathological features. Data are presented as n (%). The analysis of prognosis was performed with GraphPad Prism 9.4.0 software (Dotmatics). Survival curves were generated using Kaplan-Meier analysis. Statistical evaluation of the survival rates for patients with EGFR mutations and those with wild-type EGFR was performed using the log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Histological types of lung tumors in the study group. H&E analysis and immunohistochemistry with different antibodies was conducted on the cell blocks from pleural effusions of 231 patients with advanced lung cancer for histological classification. TTF-1, P40, CDX-2, Ki-67 and WT-1 exhibited nuclear staining, pCK, CK7, CK5/6, napsin A, villin, desmin, CgA and synaptophysin showed cytoplasmic staining, CD56 displayed membrane staining, and calretinin showed either nuclear or cytoplasmic staining. Among the 231 cell blocks analyzed, 222 showed cancer cells arranged in glandular and rosette-like patterns (Fig. 1A), with positive staining for the lung adenocarcinoma markers TTF-1, napsin A and CK7 (Figs. 1B and C and S1A). Two cases were positive for the squamous cell carcinoma markers P40 and CK5/6 (Figs. 1D and S2D). Seven cases showed positive staining for the neuroendocrine markers synaptophysin, CD56 and CgA (Figs. 1E and F and S2B), along with focal perinuclear dot-like expression of cytokeratin (Fig. S2A), supporting a diagnosis of small cell carcinoma. Additionally, the Ki-67 proliferation marker was highly expressed in the small cell carcinoma cases (Fig. S2C). The remaining proteins (calretinin, WT-1, villin, CDX2 and desmin) showed negative staining in the tumor cells (Fig. S1B-F).

EGFR gene mutations in the study group. Among the 231 pleural effusion cell blocks from patients with lung cancer, 161 underwent EGFR gene mutation testing, all of which were obtained from patients with lung adenocarcinoma. The remaining 70 patients did not receive the testing for various reasons, such as financial constraints. Among the 161 cases, 85 were positive for EGFR gene mutations (positive rate, 52.8%). The detected genetic alterations encompassed 49 instances of the L858R mutation in exon 21, 31 instances of deletions in exon 19, 2 instances of the L861Q mutation in exon 21 and a single case of the G719X mutation in exon 18. Another 2 patients showed complex mutations; 1 patient had the G719X mutation in exon 18 alongside the L861Q mutation in exon 21, whereas the other patient had the S768I mutation in exon 20 coupled with the G719X mutation in exon 18 (Figs. 2 and S3).

Association between EGFR gene mutations and clinicopathological characteristics in patients with lung adenocarcinoma. Subsequently, the present study focused on the link between the clinicopathological traits of 161 patients with lung adenocarcinoma and EGFR mutations detected through pleural effusion cell block analysis. There was a significantly greater

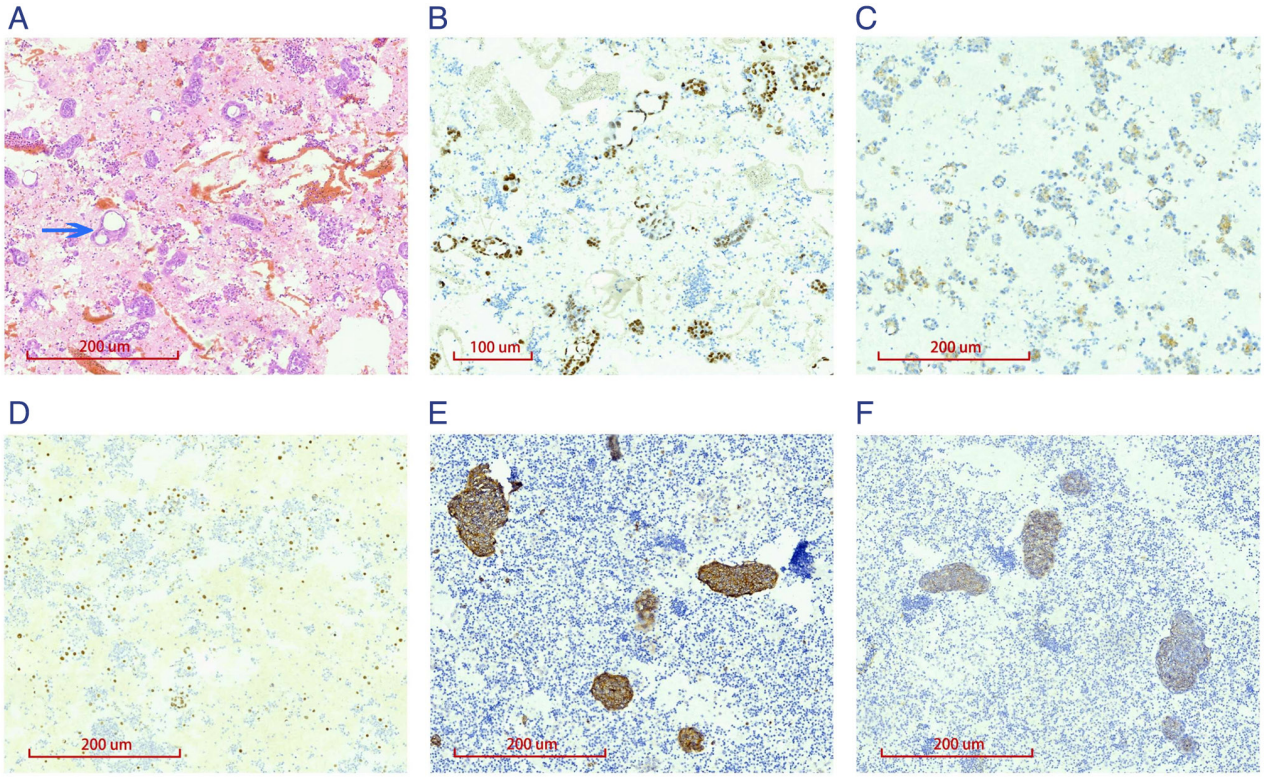

Figure 1. Histological classification of cell blocks from pleural effusion in patients with lung cancer. (A) Cancer cells were arranged in glandular patterns (blue arrow) in the cell block of pleural effusion from a patient with lung adenocarcinoma (hematoxylin and eosin staining). Immunohistochemical staining showing (B) thyroid transcription factor-1 and (C) napsin A positivity in lung adenocarcinoma. (D) Immunohistochemical staining showing P40 positivity in lung squamous cell carcinoma. Immunohistochemical staining showing (E) synaptophysin and (F) CD56 positivity in lung small cell carcinoma.

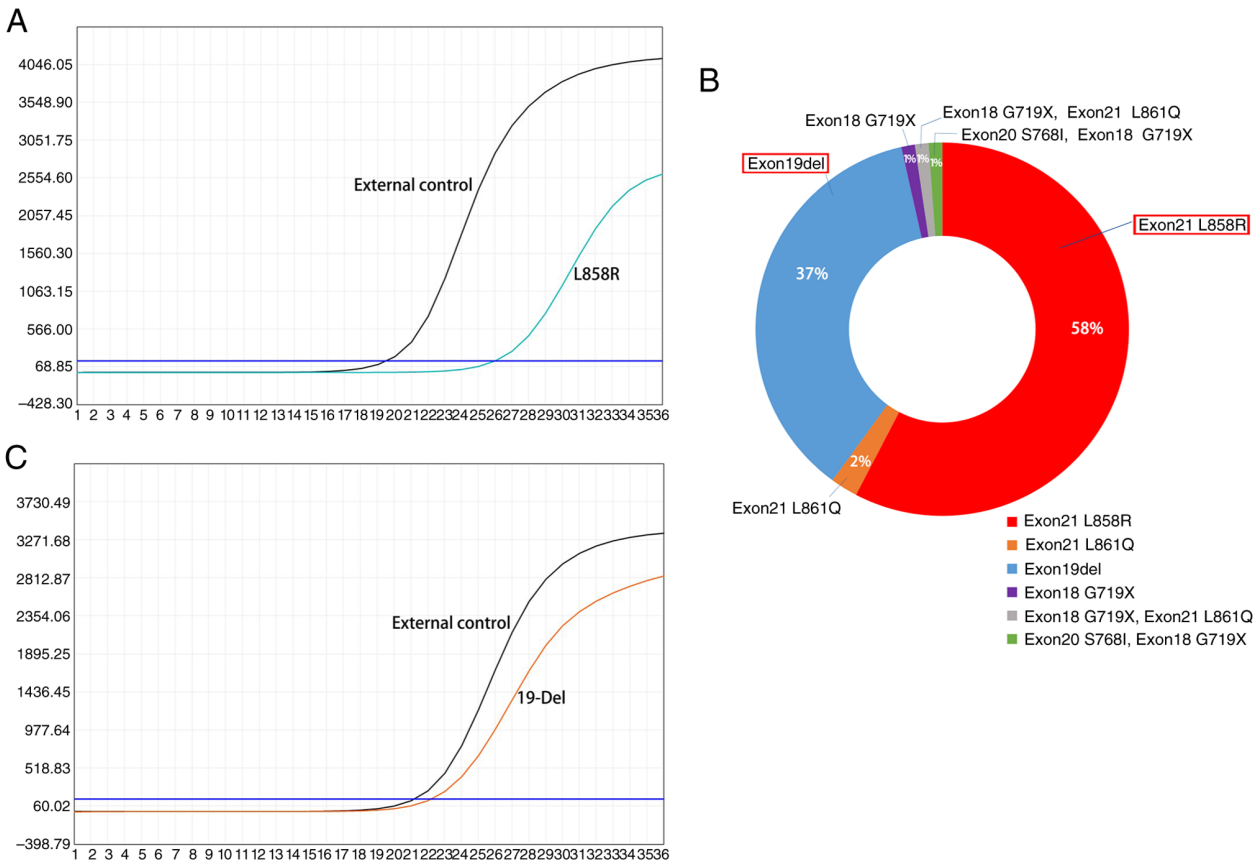

Figure 2. EGFR gene mutation analysis. (A) EGFR exon 21 L858R mutation. (B) EGFR exon 19del mutation. (C) Results of EGFR gene mutation analysis. del, deletion; EGFR, epidermal growth factor receptor.

Table II. Association of EGFR gene mutations with patient clinical parameters.

Clinical parameter	Patient number	EGFR gene mutations			χ^2-value	P-value
		Mutation	Wild-type	Mutation rate, %		
Sex					11.682	0.001
Male	83	33	50	20.50		
Female	78	52	26	32.30		
Age, years					0.328	0.608
<65	48	27	21	16.77		
≥65	113	58	55	36.03		
Smoking history					3.520	0.086
Smoker	66	29	37	18.02		
Non-smoker	95	56	39	34.78		

EGFR, epidermal growth factor receptor.

occurrence of EGFR gene mutations in female patients (32.3%) than in male patients (20.5%) (P=0.001) (Table II). Whereas EGFR gene mutations tended to show differences in patients stratified by age and smoking history, the results did not reach statistical significance (Table II).

Prognostic comparison. An observational analysis was performed on 36 patients exhibiting single EGFR mutations determined from pleural effusion cell blocks and receiving EGFR tyrosine kinase inhibitor (TKI) as their primary therapeutic intervention and 65 patients without EGFR gene mutations determined from pleural effusion cell blocks and treated with chemotherapy as first-line treatment. The monitoring phase concluded in February 2022. Patients carrying EGFR mutations exhibited a significantly extended OS rate compared with patients with wild-type EGFR (hazard ratio=0.4155, P=0.0133; Fig. 3).

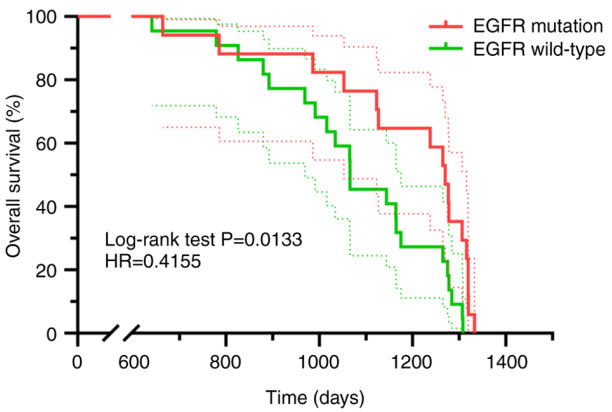

Figure 3. Evaluation of overall survival rate in patients with EGFR mutations, detected in cell block samples of pleural effusion. Solid lines represent cumulative survival rate and dashed lines indicate the confidence interbal. EGFR mutation (n=36) and EGFR wild-type (n=65). EGFR, epidermal growth factor receptor.

Discussion

Over the last 20 years, studies have shown that focusing on specific driver genes in therapy can extend the duration of progression-free survival and OS in patients with lung cancer. A study by Ramalingam *et al* (11) demonstrated that patients with EGFR mutations (exon 19 deletion or L858R) who received osimertinib treatment showed significantly extended OS. Alterations in the EGFR gene frequently emerge as the primary driver of genetic changes in lung cancer (12). Multiple studies (13-15) have demonstrated the superior efficacy and reduced toxicity of targeted drugs compared with traditional chemotherapy, indicating that targeted drug treatments provide sustained clinical benefits and markedly improve prognostic outcomes for patients with lung cancer. For patients with advanced NSCLC and sensitizing EGFR mutations, EGFR TKIs serve as the primary treatment option (16); however, sufficient tumor tissue for genetic testing is not available for all patients with advanced NSCLC.

The present study demonstrates that detecting genetic mutations through MPE cell blocks is of value for predicting patient response to targeted therapy. The ARMS-PCR technique was employed to detect genetic mutations that drive tumor progression in MPE cell blocks from patients with NSCLC lacking tumor tissue samples. The findings showed a 52.8% detection rate of EGFR mutations in MPE specimens, a rate similar to that observed with tumor tissue samples (8,17). The detected mutations included the L858R mutation in exon 21 and the deletion mutation in exon 19 of EGFR, detected in 49 and 31 patients, respectively, constituting 95% of all detected EGFR mutations. These proportions align with previous results in studies of tumor tissues with reported rates of 85-90% (18-20). The results of the present study also revealed the presence of three less common mutation types (L861Q, G719X and S768I), with no other rare EGFR mutations identified. These findings indicated that the frequency of EGFR oncogenic driver gene mutations identified in MPE may mirror that found in tumor tissues.

While a previous study has verified the diagnostic precision of MPE in detecting oncogenic driver genes compared with tumor tissue (21), to the best of our knowledge there is almost no research on the efficacy of TKI targeted therapy administered solely on the basis of the detection of mutations through MPE analysis. The current study revealed that patients with single EGFR mutations identified via MPE analysis and treated initially with EGFR-TKIs experienced a significantly longer OS rate compared with patients without EGFR mutations who received chemotherapy as first-line treatment. This aligns with a previous study on patients with EGFR sensitizing mutations detected on tumor tissue who were administered EGFR-TKIs as an initial treatment (11). Additionally, the findings of the present study demonstrated variations in EGFR mutation rates in MPE based on sex, with a notably higher incidence in female patients than in male patients, consistent with the outcomes of previous studies based on tumor tissue analysis, where the detection rate of EGFR gene mutation was 52.8% in women and 31.6% in men (22-24).

The present study has certain limitations. First, only ARMS-PCR was used for detection of EGFR gene mutations, and this technique has limitations in terms of detection sensitivity and coverage (25,26), particularly in identifying low-frequency mutations and rare mutations related to EGFR-TKI resistance. This limitation affects the completeness of the mutation spectrum in MPE samples. Upcoming initiatives ought to focus on employing diverse mutation detection platforms, and amalgamating advanced detection methods, such as next-generation sequencing and droplet digital PCR, for a more detailed gene mutation profile. Secondly, since this study only collected data from malignant pleural effusion cell blocks of patients with lung cancer, and the data analysis was based on comparisons with other literature rather than a direct comparison with the patients' own tumor tissue specimens, there are inherent differences in the research subjects and tumor heterogeneity, which impose relative limitations on the results. In future research, the study design will be refined further by conducting advance planning and patient recruitment to ensure standardized and consistent data collection.

In conclusion, the findings of the present study indicated that the OS rate of EGFR-TKI-treated patients, with EGFR gene mutations identified solely via MPE cell block tests, may be comparable with that of patients with similar mutations identified on tumor tissues in previous studies. Furthermore, the frequency of EGFR gene alterations identified in MPE paralleled that reported in cancerous tissues in previous studies. Therefore, when sufficient tumor tissue cannot be obtained, MPE is recommended as an alternative specimen for the detection of EGFR mutations.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SZ, YuL and XC designed the present study. SZ, YuL and TW conducted the experiments. ZC and YiL performed statistical analysis, and SZ wrote the manuscript. XC and YiL conducted the systematic literature search and supervised the writing of the manuscript. SZ, YuL and XC confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with ethical standards and was approved by the Ethics Committee of Weifang No. 2 People's Hospital (approval no. KY2023-015-01; Weifang, China). Written informed consent was obtained from all patients for the use of their data and specimens in scientific research.

Patient consent for publication

Written informed consent was obtained for publication of the patient data and images.

Competing interests

The authors declare that they have no competing interests.

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