


ORIGINAL ARTICLE

Detecting *EGFR* mutations and *ALK/ROS1* rearrangements in non-small cell lung cancer using malignant pleural effusion samples

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Keywords

ALK; *EGFR*; malignant pleural effusion; non-small cell lung cancer; *ROS1*.

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Abstract

Background: The study was conducted to evaluate the feasibility of using malignant pleural effusion (MPE) as a substitute specimen for genetic testing and to determine the significance of genetic profiling of MPE tumor cells to monitor non-small cell lung cancer (NSCLC) progression and therapeutic response.

Methods: We selected 168 NSCLC patients with MPE. We extracted MPE and enriched tumor cells using a custom-designed device. *EGFR* mutations and *ALK/ROS1* fusions were then detected by quantitative real-time PCR, and the results were used to guide targeted therapy. We investigated drug responses through imaging.

Results: MPE tumor cells were detected in all patients. *EGFR* mutations and *ALK/ROS1* rearrangements were detected in biopsy samples, treated MPE, and untreated MPE. We found that treated MPE had higher sensitivity and specificity than biopsy or untreated MPE. Among the 26 *EGFR* inhibitor patients, 13 showed a partial response, 7 had progressive disease, and 6 showed stable disease. Among the 16 patients that received *ALK/ROS1* inhibitors, 8 had a partial response, 4 had progressive disease, and 4 showed stable disease.

Conclusion: Our study provides a new, less invasive, and highly repeatable method of analyzing MPE tumor cells in NSCLC that facilitates precision medicine and genetic testing.

Introduction

Malignant pleural effusion (MPE) is defined as an abnormal amount of fluid between the thin layers of tissue (pleura) that line the outside of the lung and the chest cavity wall in cancer.¹ MPE is frequently observed in multiple malignancies, including lung cancer, breast cancer, and lymphoma, but it is most commonly reported in lung cancer.² The routine diagnostic tests for MPE are chest X-ray, ultrasonic examination, computed tomography (CT), magnetic resonance imaging (MRI), and pathological diagnoses (such as pleural fluid cytology [PFC]). PFC examinations are commonly used for qualitative diagnoses, but they can also be used for subsequent biological analyses. However, the utility of PFC in patients with MPE is controversial.

Moreover, it is difficult to analyze tumor cells within MPE because they coexist with numerous background inflammatory and mesothelial cells. Recently, various technologies have become available that allow mutational analysis of MPE, such as direct PCR sequencing, mutant-enriched PCR, pyrosequencing, and real-time PCR, each with advantages and limitations. However, the reported overall sensitivity varies widely, with the lowest being 10–20%, according to the various technologies. Test sensitivity is closely related to the purity of the tumor sample. The existing enrichment technologies that can achieve high tumor cell purity for analysis include macro-dissection, manual micro-dissection, and laser-capture micro-dissection of smears or cell blocks.³ However, these methods are highly dependent on advanced equipment. Previously, we

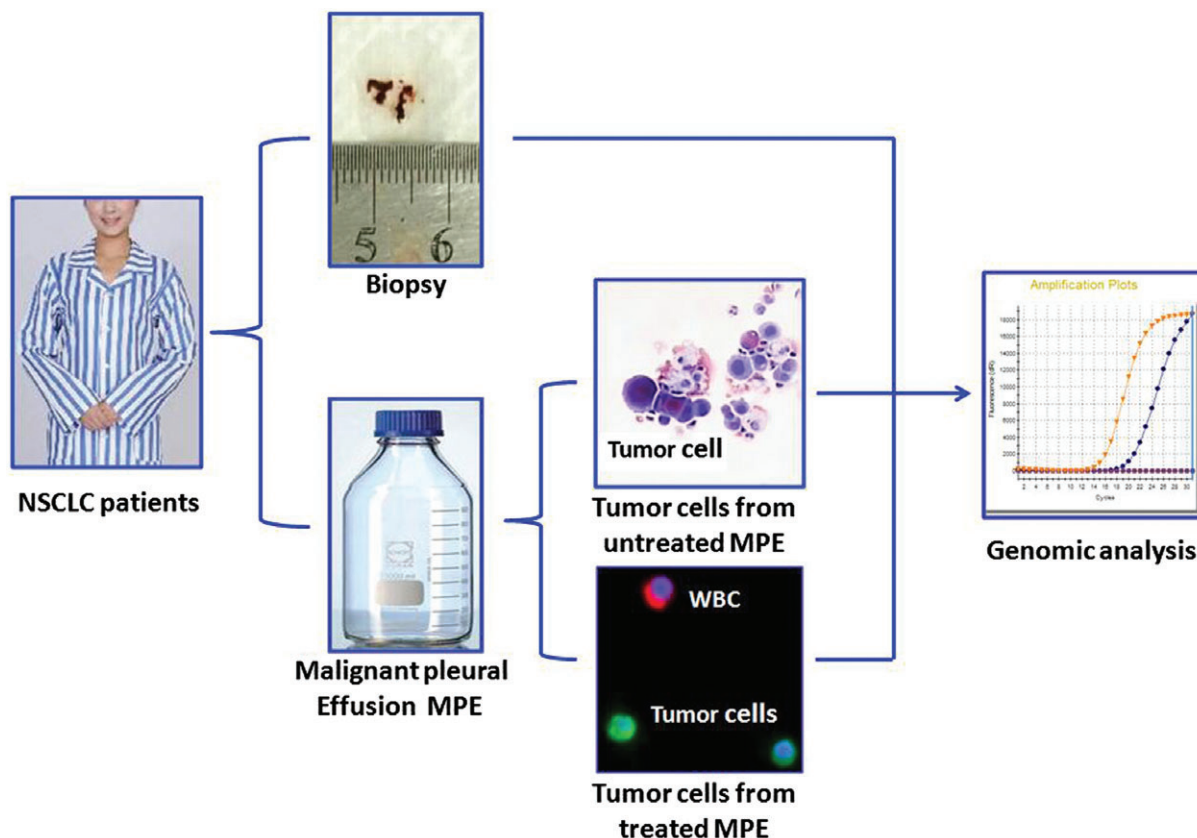


Figure 1 Schematic workflow diagram of tumor cell capture from malignant pleural effusion (MPE) and subsequent mutational analysis in non-small cell lung cancer (NSCLC) patients. WBC, white blood cells.

captured and released circulating tumor cells (CTCs) from the peripheral blood of cancer patients using our custom-designed device.⁴ Herein, we tested whether we could capture and release tumor cells from MPE using this previously established platform to recover high-purity tumor cells for subsequent molecular analyses.

Worldwide, lung cancer has long been the leading cause of cancer-related death in both men and women, and non-small-cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer diagnoses.^{5,6} MPE is one of the most common and sometimes fatal complications, affecting NSCLC patient outcomes and quality of life, especially adenocarcinoma cases, with an incidence rate as high as 60%.⁷ For many NSCLC patients, MPE can be safely and repeatedly obtained and can serve as an alternative specimen for molecular analysis, especially in advanced and unresectable NSCLC patients.⁸ Thus, MPE can act as a substitute sample for the real-time monitoring of disease progression and therapeutic response.

Precision medicine emphasizes using exact molecular diagnosis to guide the appropriate therapeutic regimen, as targeted treatments in oncology rely on inhibiting the validated targets from preclinical research.^{9,10} The detection

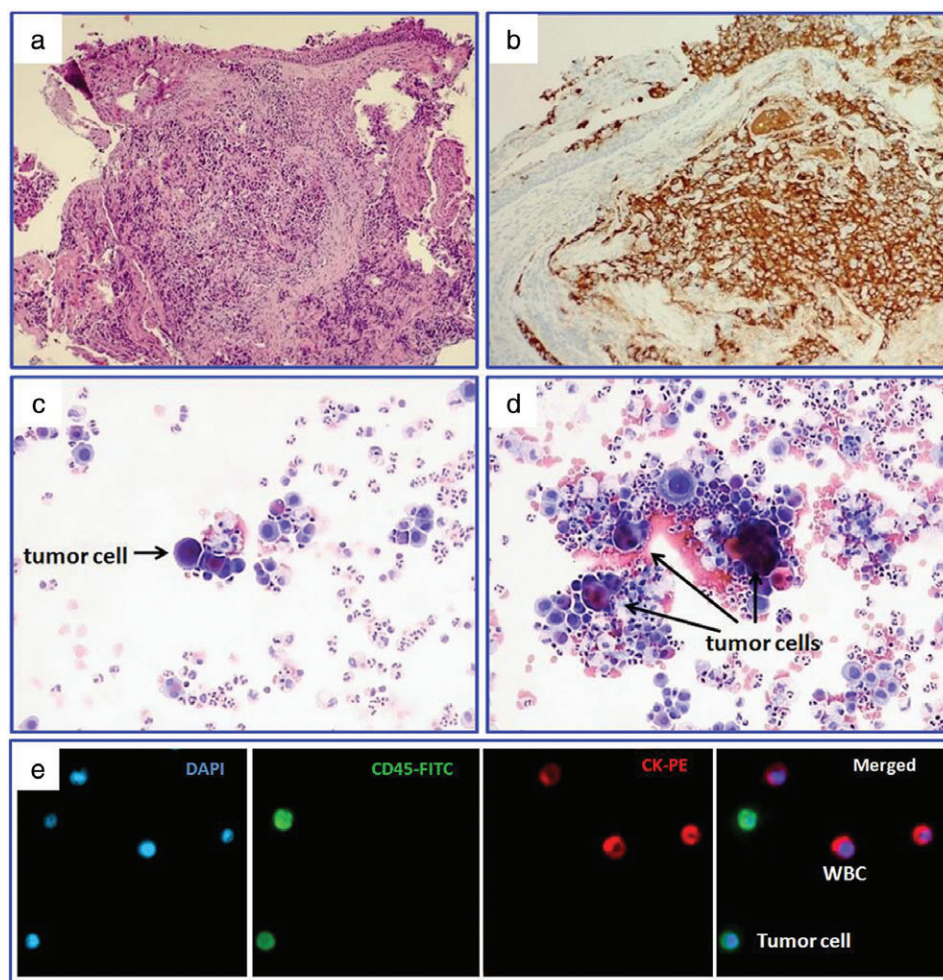
and targeting of driver mutations has brought numerous benefits to cancer therapy. NSCLC is a common tumor with readily targetable genetic alterations.¹¹ These

Table 1 Baseline characteristics of the NSCLC patients

Variables	N = 168
Age	
<60 years	92 (54.8)
≥60 years	76 (45.2)
Gender	
Male	111 (66.1)
Female	57 (33.9)
Smoking status	
Never	74 (44.0)
Occasionally	9 (5.4)
Frequently	85 (50.6)
Histological type	
AC	109 (64.9)
SCC	45 (26.8)
LCC	14 (8.3)
Clinical stage	
III	91 (54.2)
IV	77 (45.8)

AC, adenocarcinoma; LCC, large cell carcinoma; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma.

Figure 2 (a) Hematoxylin and eosin and (b) immunohistochemical staining of biopsied tumor tissue obtained by fiberoptic bronchoscopy. (c) A Wright's-stained single tumor cell and (d) tumor cell cluster in malignant pleural effusion (MPE). (e) Fluorescent micrographs of tumor cells captured from treated MPE samples. WBC, white blood cells.



targetable driver mutations in NSCLC include activating *EGFR* mutations and concurrent *ALK/ROS1* gene rearrangements.¹² Several clinical trials have demonstrated the remarkable efficacy of crizotinib for metastatic NSCLC patients with *ALK/ROS1* rearrangements.¹³ Therefore, we hypothesized that we could analyze *EGFR* mutations and *ALK/ROS1* rearrangements in tumor cells recovered from the MPE of NSCLC patients to monitor relapse/refractory or targeted therapy-responsive disease in real time.

The aim of this study was to provide a less invasive and repeatable method for analyzing MPE tumor cells, and to develop complementary methods for precision cancer medicine based on genetic testing.

Methods

Patient selection and sample collection

This study was approved by the review board of Renmin Hospital of Wuhan University and was conducted according

to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all participants. We enrolled 168 NSCLC patients who were treated at the Cancer Center, Renmin Hospital of Wuhan University (Wuhan, PR China) between March 2014 and March 2016. The inclusion criteria were as follows: (i) NSCLC cases diagnosed by pathological and/or histological examination; (ii) patients aged between 18 and 80 years old; (iii) estimated survival time > 4 months; (iv) patients ineligible or unwilling to undergo surgery and/or radiotherapy; (v) good compliance; (vi) no major organ dysfunction and/or diseases; (vii) Eastern Cooperative Oncology Group performance status score < 3; (viii) clear, objective examination and evaluation with complete disease and physical condition; and (ix) patients who volunteered to join the study and signed informed consent. The exclusion criteria were: (i) patients aged < 18 or > 80 years old; (ii) patients with severe renal dysfunction, cardiovascular or cerebrovascular diseases, hematological or endocrine system diseases, or metabolic diseases; (iii) psychotic patients, pregnant women,

Table 2 Clinical characteristics and therapeutic efficacy in the 26 patients with concurrent EGFR and ALK/ROS1 genetic alterations

Patient	Gender	Age (y)	Smoking history (y)	Histological type	Clinical stage	EGFR type	EGFR-TKI response	ALK/ROS1 type	Crizotinib response
P1	M	46	18	AC	III	19del	PR	EML4-ALK	PR
P2	F	53	0	AC	IV	19del	SD	EML4-ALK	—
P3	M	38	10	SCC	III	L858R	PD	EML4-ALK	—
P4	M	41	0	LCC	IV	19del	PR	EML4-ALK	SD
P5	F	64	0	AC	III	19del	PR	EML4-ALK	—
P6	F	58	0	AC	III	L858R	SD	CD74-ROS1	PD
P7	F	71	0	AC	III	19del	PD	EML4-ALK	—
P8	M	45	23	SCC	IV	L858R	PR	EML4-ALK	SD
P9	F	52	0	AC	IV	19del	SD	EML4-ALK	—
P10	F	66	0	AC	III	G719X	PD	EZR-ROS1	—
P11	M	48	30	SCC	III	L858R	PR	EML4-ALK	PR
P12	M	53	17	AC	III	19del	SD	EML4-ALK	—
P13	F	42	6	AC	III	L858R	PR	CD74-ROS1	SD
P14	F	58	0	AC	IV	19del	PR	EML4-ALK	PR
P15	M	56	0	AC	III	19del	PD	EML4-ALK	PD
P16	M	68	32	AC	III	L858R	PR	EML4-ALK	PR
P17	F	43	0	AC	III	19del	SD	CD74-ROS1	SD
P18	M	49	24	AC	IV	19del	PR	EML4-ALK	PR
P19	F	57	0	AC	III	G719X	SD	EML4-ALK	—
P20	M	69	0	SCC	IV	19del	PD	EML4-ALK	PR
P21	F	63	0	AC	IV	L858R	PR	CD74-ROS1	PR
P22	F	72	0	AC	III	19del	PD	EML4-ALK	—
P23	M	45	0	LCC	III	19del	PR	EML4-ALK	PD
P24	M	61	36	SCC	IV	L858R	PD	EZR-ROS1	—
P25	F	53	0	AC	III	19del	PR	EML4-ALK	PD
P26	M	67	0	AC	III	19del	PR	EML4-ALK	PR

AC, adenocarcinoma; F, female; LCC, large cell carcinoma; M, male; PD, progressive disease; PR, partial response; SCC, squamous cell carcinoma; SD, stable disease; TKI, tyrosine kinase inhibitor.

or lactating women; (iv) poor compliance; (v) severe infection; and (vi) other inappropriate conditions, as considered by the researchers.

Routine diagnostic MPE examinations, including Color Doppler Ultrasound and CT scans, were conducted on all patients. Patients who were surgery candidates underwent curative resection with pathologically confirmed negative margins and regional lymph node dissection. The NSCLC patients who were contraindicated for surgery underwent fiberoptic bronchoscopy to obtain biopsy samples. We obtained approximately 200 mL of MPE by ultrasound-guided thoracentesis, which was stored in clean 500 mL glass bottles for subsequent analysis. Patients that failed platinum-based chemotherapy were then sequentially treated with EGFR-tyrosine kinase inhibitors (TKIs) or ALK/ROS1-TKIs based on the genetic analysis.

Patient identification and cell morphology observations

First, we confirmed every biopsy sample by routine hematoxylin and eosin (H&E) staining and immunohistochemistry

(IHC). Two investigators who were blinded to all clinical data independently scored the staining.¹⁴ To confirm that the MPE contained tumor cells, 10 mL of MPE was used to observe tumor cells. Cell morphologies were observed using an optical microscope (Olympus IX70 Inverted Microscope; Olympus, Tokyo, Japan) following Wright's staining (Sangon Biotech Co., Ltd., Shanghai, PR China).¹⁵

Tumor cell capture and release

Malignant pleural effusion tumor cell capture and release analyses were performed using our previously described well-established method (Aptamer-polymer functionalized silicon nanosubstrates for enhanced recovered CTC viability and in vitro chemosensitivity testing).⁴ Briefly, MPE samples were treated with aptamer-thermoreponsive polymers modified by nanosubstrates to capture and release epithelial cell adhesion molecule-positive tumor cells. Two 100 mL MPE aliquots were separately centrifuged at 6000 rpm for 10 minutes, and the supernatants were removed. One of the cell pellets was collected, resuspended in 2 mL phosphate buffered saline, and stored at -80°C ,

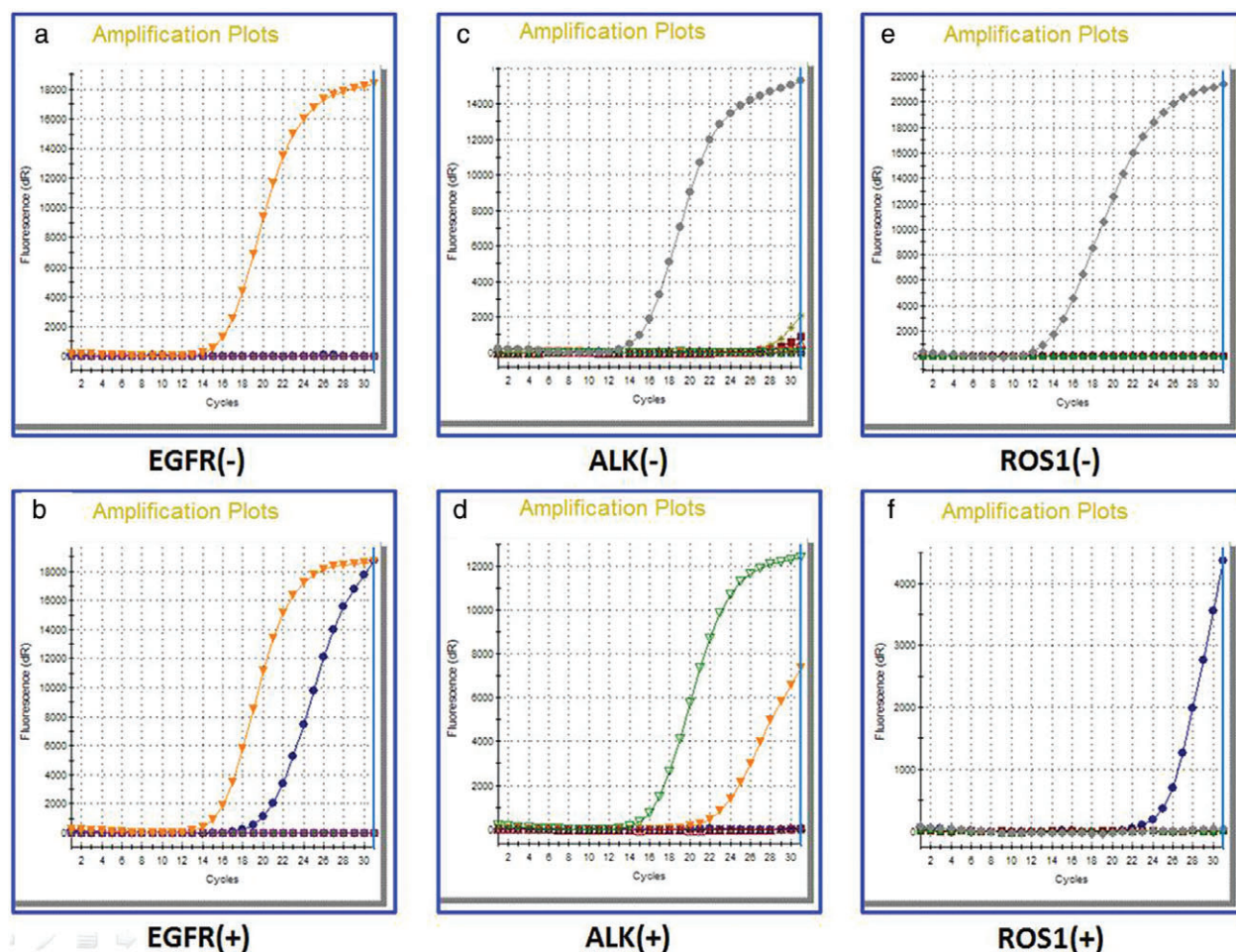


Figure 3 Representative results of patients with co-alterations. Amplification plots demonstrate the typical results of a patient (a) without an *EGFR* mutation, (b) with an *EGFR* mutation, (c) no *ALK* fusion, (d) with *ALK* fusion, (e) no *ROS1* fusion, and (f) with *ROS1* fusion in malignant pleural effusion (MPE) tumor cells from non-small cell lung cancer (NSCLC) patients.

while the other 2 mL cell suspension was put into the device for tumor cell capture and release. High-purity tumor cells were obtained after the heating/cooling cycle and enzyme treatment. Tumor cells were identified with a commonly used three-color immunofluorescence method, as stated in our previous study.⁴ The isolated MPE tumors were stored at -80°C until use.

Analyzing *EGFR* mutations and *ALK/ROS1* rearrangements

Fiberoptic bronchoscopy biopsy samples, tumor cells from MPE treated by our platform, and untreated MPE were analyzed in parallel (Fig 1). Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). To obtain paired samples from each patient, DNA was also extracted using a QIAamp DNA FFPE tissue Kit (Qiagen). DNA concentration was tested using a Quant-iT PicoGreen

dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted from each sample using an RNeasy Mini Kit (Qiagen) and RNA concentration was measured using a spectrophotometer (NanoDrop 2000C; Thermo Fisher Scientific, Waltham, MA, USA). We analyzed *EGFR* mutations and *ALK/ROS1* rearrangement by capillary electrophoresis and quantitative real-time PCR, respectively.

Monitoring *EGFR-TKI* and *ALK/ROS1-TKI* treatment responses in real-time

The NSCLC patients received targeted therapy (*EGFR-TKIs* or *ALK/ROS1-TKIs*) based on genetic detection after failing standard platinum-based doublet chemotherapy. Patients that received oral erlotinib treatment (150 mg daily) were evaluated at 12 weeks for treatment responses and further confirmed four weeks later. The patients were then assessed by imaging every 12 weeks in accordance with Response Evaluation

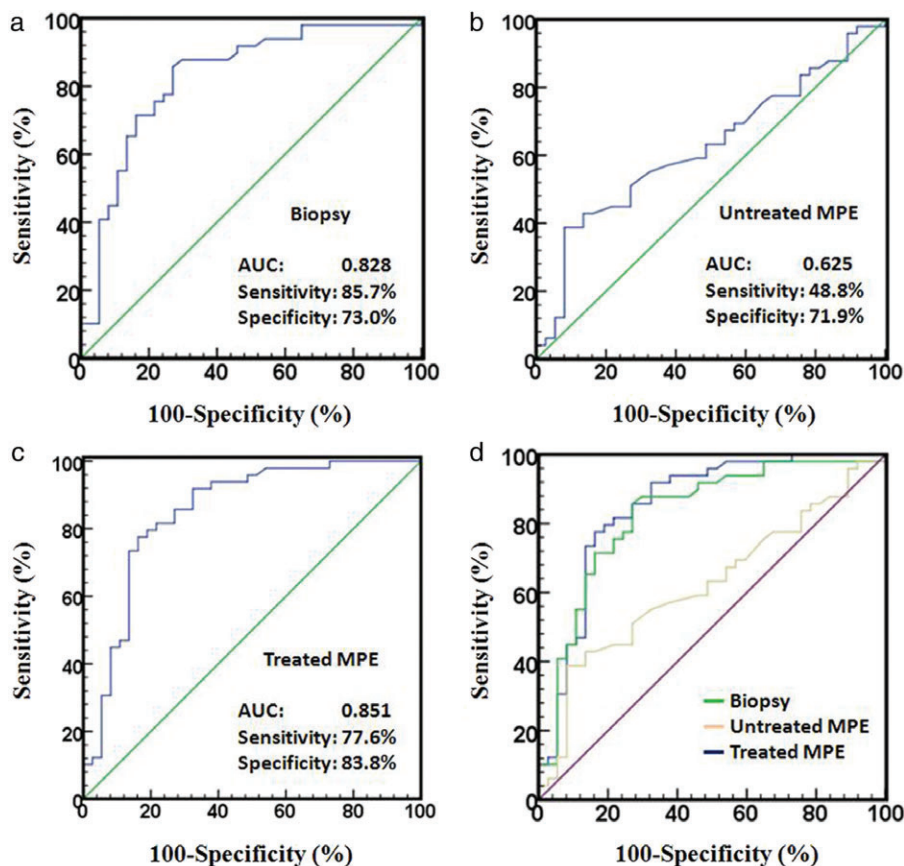


Figure 4 Receiver operating characteristic (ROC) curves to compare the sensitivity and specificity of detecting *EGFR* mutations in non-small cell lung cancer (NSCLC) using the three sample types: (a) biopsy, (b) untreated malignant pleural effusion (MPE), (c) treated MPE, and (d) merged. AUC, area under the curve.

Criteria in Solid Tumors (RECIST) version 1.1.¹⁶ Response measures were defined as follows: complete response (CR) indicates tumor disappearance; partial response (PR) indicates a > 50% reduction in the tumor cross product (the maximum tumor diameter in the axial plane multiplied by the largest diameter in the perpendicular dimension on the same image); and stable disease (SD) indicates a 50% reduction through 25% increase in the tumor cross product. Acquired resistance was defined as an in situ recurrence or new lesion that developed after approximately six months of tumor response. Patients sequentially received oral crizotinib treatment (250 mg twice daily) after being verified for acquired erlotinib resistance according to RECIST. Meanwhile, multiple CT and Color Doppler Ultrasound scans were performed according to requirements for disease evaluation. These scans were monitored for improvements in clinical symptoms, indicating successful erlotinib or crizotinib treatment.

Statistical analysis

Baseline data are presented as frequencies and percentages for categorical variables. The area under the curve (AUC) of a receiver operating characteristic (ROC) curve was

calculated to assess the sensitivity and specificity of the three sample types. All analyses were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA).

Results

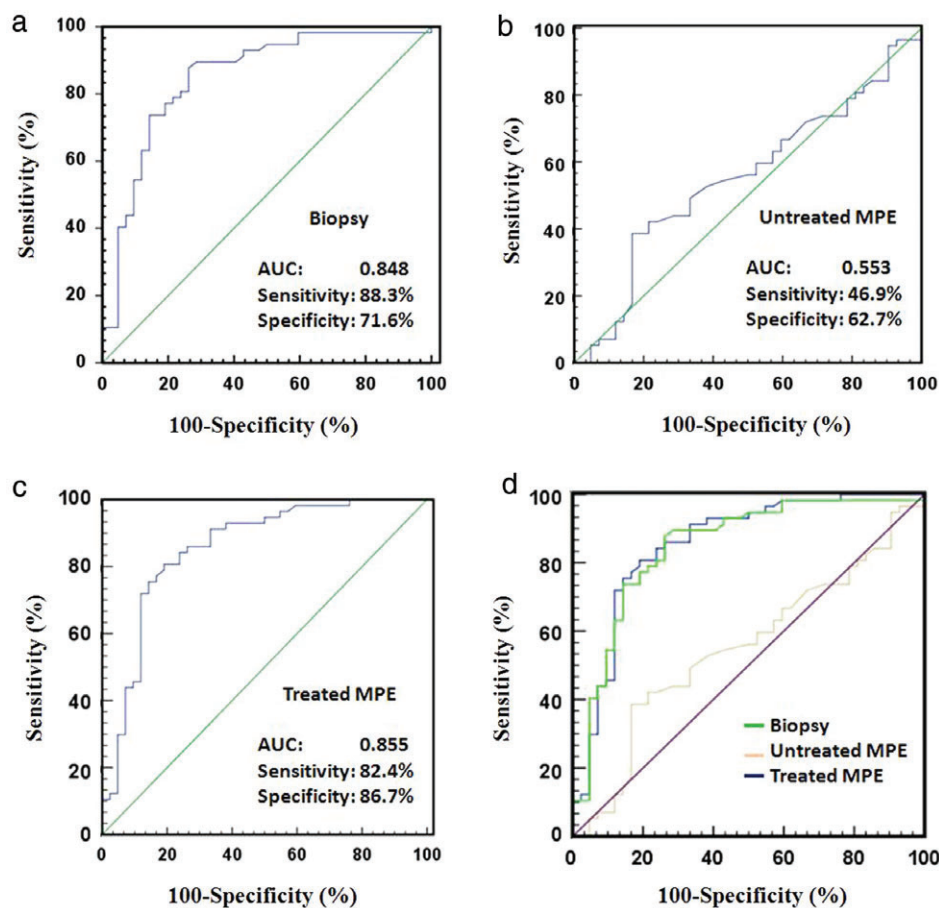
Patient characteristics and tumor classifications

Our study cohort included 168 NSCLC patients; patient characteristics are presented in Table 1. There were 111 men and 57 women, with a median age of 56 years (range: 38–72 years). Thus, 66.1% of the patients were male, 45.2% were aged > 60 years, 50.6% were frequent smokers, 44.0% were never smokers, and 5.4% were occasional smokers. Adenocarcinoma accounted for 64.9% of the cases, and 54.2% were at World Health Organization stage III.

Isolation and identification of tumor cells from malignant pleural effusion

Malignant pleural effusion samples were obtained from the NSCLC patients and paired samples from all patients were

Figure 5 Receiver operating characteristic (ROC) curves to compare the sensitivity and specificity of detecting *ALK* rearrangements in non-small cell lung cancer (NSCLC) using the three sample types: (a) biopsy, (b) untreated malignant pleural effusion (MPE), (c) treated MPE, and (d) merged. AUC, area under the curve.



analyzed. The biopsies obtained by fiberoptic bronchoscopy were estimated by an experienced pathologist blinded to clinical data (Fig 2a,b). Single tumor cells and tumor cell clusters in MPE were identified by Wright stain (Fig 2c,d).

Combined three-color immunofluorescence with cell morphology was used to discriminate tumor cells from white blood cells. Morphologically intact tumor cells that were Cy5+ fluorescein isothiocyanate-/DAP+ were identified as CTCs, while cells that exhibited high fluorescein isothiocyanate and low Cy5 emission were identified as white blood cells (Fig 2e).

Analyzing *EGFR* mutations and *ALK/ROS1* rearrangements

Parallel analysis showed that the rates of detecting *EGFR* mutations were similar in tumor biopsies (39.9%) and cells from treated MPE (41.1%) and both were higher than in cells from untreated MPE (35.1%). Similarly, the rates of *ALK* rearrangements in tumor biopsies (6.5%) were similar to the rates from treated MPE (7.1%), which were both higher than in cells from untreated MPE (5.4%). The *ROS1*

rearrangement rates in tumor biopsies (1.8%) and cells from treated MPE (1.8%) were the same, and both were higher than in cells from untreated MPE (1.2%) (Table 2). Representative results from *EGFR* mutant and *ALK/ROS1* rearrangement cases are shown in Figure 3.

Finally, we compared *EGFR* mutations and *ALK/ROS1* rearrangements in the three sample types. These results showed that treated MPE had a higher sensitivity and specificity than biopsy and untreated MPE, as shown in Figures 4–6.

Evaluating clinical responses to *EGFR* and *ALK/ROS1* tyrosine kinase inhibitors

Among the 168 patients, 26 received targeted therapy based on detected *EGFR* mutations or *ALK/ROS1* rearrangements in captured MPE tumor cells. The clinical responses (according to RECIST version 1.1) to TKI treatment are summarized in Table 2. There were 13 PR cases, 7 PD cases, and 6 SD cases after *EGFR*-TKI therapy. There were 8 PR cases, 4 PD cases, and 4 SD cases after *ALK/ROS1*-TKI therapy. CT images showed that the

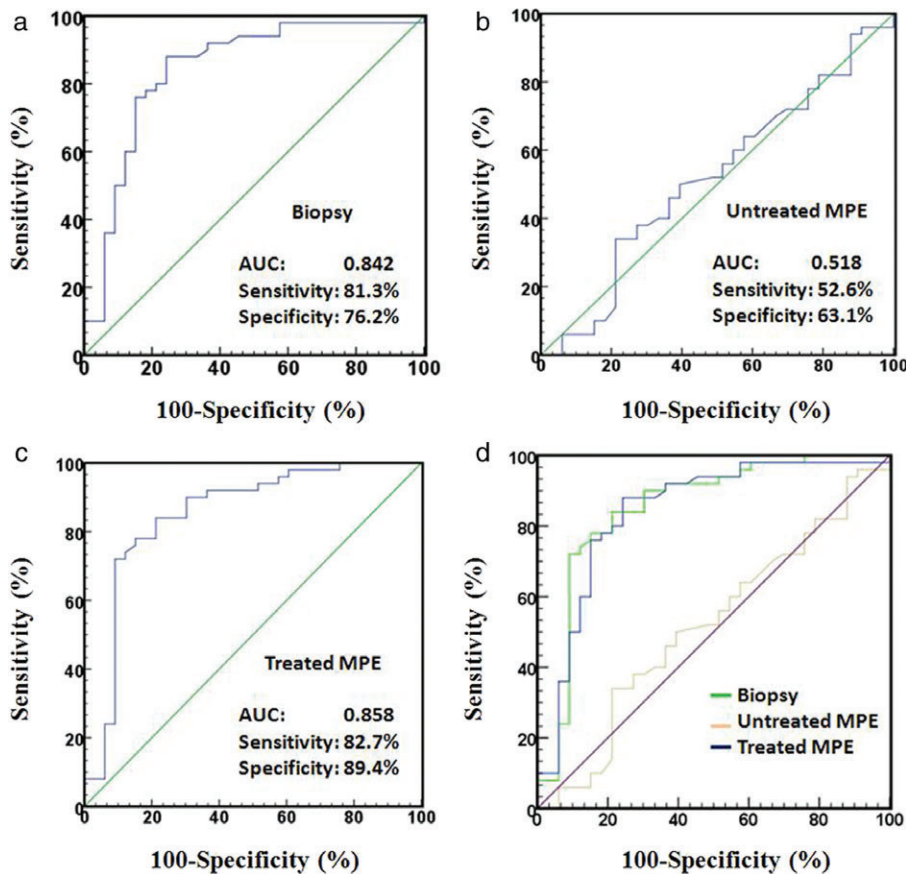


Figure 6 Receiver operating characteristic (ROC) curves to compare the sensitivity and specificity of detecting *ROS1* rearrangements in non-small cell lung cancer (NSCLC) using the three sample types: (a) biopsy, (b) untreated malignant pleural effusion (MPE), (c) treated MPE, and (d) merged. AUC, area under the curve.

primary NSCLC tumors changed at multiple time points during the course of targeted therapy. The red dots on the graph on the right of Figure 7 indicate these five time-points.

Discussion

A vital goal of precision medicine is to apply molecular profiling data to derive accurate diagnoses and individualized therapeutic strategies for cancer patients.^{17,18} We expect to see better patient outcomes from this type of precision medicine based on genomic testing.¹⁹ Technological advancements have greatly promoted the development of precision medicine, especially molecular diagnostics and targeted therapy. However, it can be difficult to obtain adequate tissue from advanced lung cancer patients for molecular profiling. Only 20% of NSCLC cases have adequate tissue accrual to meet the needs of pivotal studies.²⁰ MPE is a potential candidate as cancer cells can be obtained less invasively and repeatedly.¹¹ However, the rarity of tumor cells in MPE among the mixed impurities limits its clinical application. To address this issue, we used our well-established tumor cell isolation platform, and our results

verified that we could extract high-purity tumor cells from MPE.

Many previous studies have considered MPE an alternative tumor cell source for molecular analysis.^{7,8,11,21,22} These reports suggested that the clinical value of MPE was determined by the rate of diagnostic concordance between MPE and biopsy samples. Our results showed that the rates of detecting *EGFR* mutations or *ALK/ROS1* rearrangements were slightly higher in treated MPE than in biopsy samples, and significantly higher than in untreated MPE. Thus, our data suggest that genetic testing using tumor cells isolated from MPE is superior to biopsy and untreated MPE samples. One potential reason for the superiority to biopsy samples may be that the capture and release process improves tumor cell purity, which allows more accurate detection. This conclusion is shared by previous reports.^{11,23} Consequently, if adequate MPE is available, oncologists can attempt genetic analysis, even with negative biopsy samples.

According to the updated 2016 National Comprehensive Cancer Network guidelines, *EGFR*, *ALK*, and *ROS1* status should be detected in NSCLC patients before treatment is administered. Additionally, the “China Graded Lymphoma Kinase (ALK) positive NSCLC diagnosis and treatment

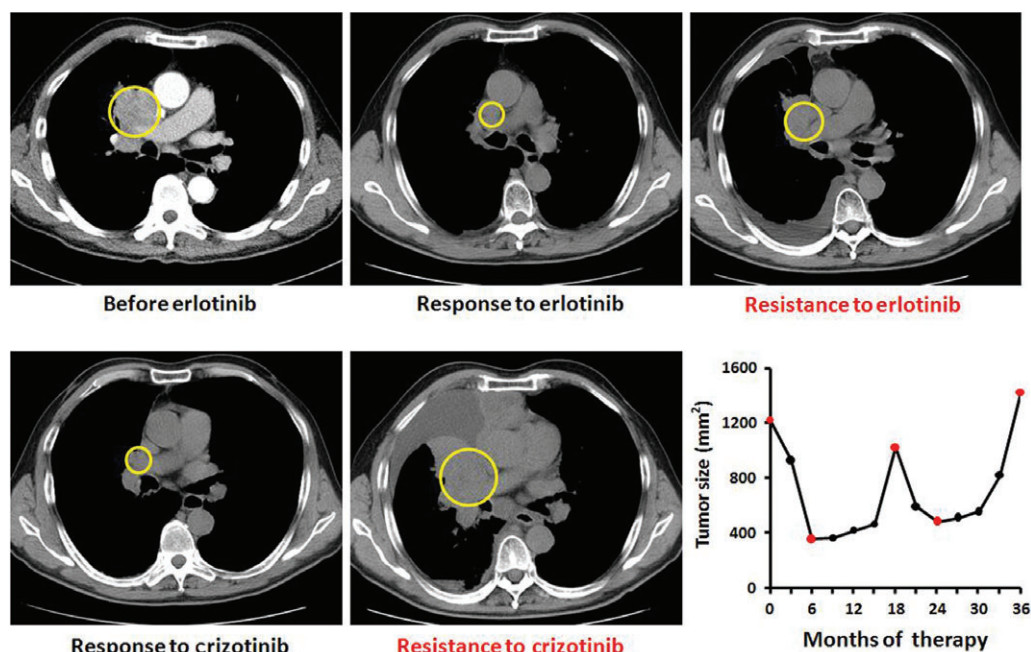


Figure 7 Clinical pattern of acquired resistance to EGFR-tyrosine kinase inhibitor (TKI) and ALK/ROS1-TKI therapy in non-small cell lung cancer (NSCLC) patients. Computed tomography images show an NSCLC primary tumor at baseline, at the time of response to erlotinib, at the time of resistance to erlotinib, at the time of response to crizotinib, and at the time of resistance to crizotinib. Red dots on the right of the graph indicate the five time points.

guidelines” also recommend that NSCLC patients be simultaneously detected for *EGFR*, *ALK* and *ROS1* with patient permission. Clinical diagnosis and treatment for NSCLC has entered a new era given the ability to detect *EGFR* mutations and *ALK/ROS1* fusions.²⁴ Moreover, a monitoring strategy for resistance or re-sensitization to targeted therapies has already been attempted in lung cancer based on the genetic analysis of serial biopsy and imaging data at multiple time points.¹⁶ MPE, which can be safely obtained repeatedly, has obvious advantages over biopsy tissue. The strategy outlined in this study will increase the significance of clinical MPE samples and facilitate observations and molecular profiling during the course of treatment. Our results are consistent with a study that monitored treatment outcomes by observing CTCs.²⁵ Furthermore, these molecular/genetic changes may provide evidence when choosing subsequent therapeutic regimens.

There are several limitations to this study. First, *ALK* and *ROS1* rearrangements are rare, therefore the concordance rates of the small samples are inconclusive. Second, obtaining clean biopsies and high-purity CTCs from MPE are technically challenging procedures that can be highly influenced by the experience of the investigator; deviations may strongly influence the results.

In summary, we applied our well-established platform to isolate tumor cells from MPE and recovered high-purity tumor cells that were suitable for subsequent molecular analysis. Furthermore, we verified the feasibility of MPE as

a less invasive and repeatable substitute specimen for testing for *EGFR* mutations and *ALK/ROS1* rearrangements in NSCLC patients by quantitative real-time PCR. Our study provides a novel method for monitoring treatment that is feasible for guiding targeted therapy based on molecular/genetic testing using tumor cells from MPE.

Acknowledgments

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Disclosure

No authors report any conflict of interest.

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