



# Profiling Cell-Free DNA from Malignant Pleural Effusion for Oncogenic Driver Mutations in Patients with Treatment-Naive Stage IV Adenocarcinoma: A Multicenter Prospective Study

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## Abstract

**Introduction** Comprehensive next-generation sequencing (NGS) of non-small-cell lung cancer specimens can identify oncogenic driver mutations and their corresponding targeted therapies. Plasma cell-free DNA (cfDNA) genotyping is easy to perform; however, false negatives cannot be overlooked. We explored malignant pleural effusion (MPE), a rich source of cfDNA, as a non-inferior alternative to tumor tissues for genotyping.

**Methods** We conducted a prospective trial including 39 patients with newly diagnosed stage IV lung adenocarcinoma who presented with MPE. Tissue tests matching hotspot variants, including *EGFR*, *ALK*, and *ROS1*, were compared with the AlphaLiquid100 of PE-cfDNA.

**Results** Among the 39 PE-cfDNA samples successfully sequenced, 32 (82.1%) had a PE cell-block tumor content of < 10%. Standard tissue or cell-block testing for *EGFR*, *ALK*, and *ROS1* identified 20 mutations (51.3%), whereas PE cfDNA identified 25 mutations (64.1%). Five *EGFR* mutations were observed in PE cfDNA but not in Cobas *EGFR* owing to coverage or insufficient tumor content issues. The overall rate of oncogenic mutations identified in the PE cfDNA was 92.3%, and the mutation distribution was as follows: even with a very low cfDNA input, high detection rates could be achieved. Otherwise, most patients harbored co-mutations. Comparison of pleural fluid NGS with traditional testing revealed differences in accuracy. We also followed up with patients with *EGFR*-sensitizing mutations who had a treatment response rate of 97.2% after 3 months.

**Conclusions** Genotyping of MPE supernatant cfDNA is feasible in clinical practice, in addition to plasma and tumor testing, to improve diagnostic yield and extend patients' benefit from targeted therapies.

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## Key Points

This study was one of the first in Taiwan to explore cfDNA-based next-generation genetic testing for malignant pleural effusion (MPE).

The success rate for identifying *EGFR* (Cobas), *ALK* (IHC), and *ROS1* (IHC) using standard cell-block testing of pleural effusion sediments was approximately 50%, while the AlphaLiquid® 100 target capture panel identified over 90% of oncogenic mutations in cfDNA.

cfDNA analysis from pleural effusion supernatant is a practical method that complements plasma and tumor testing, improving diagnostic accuracy and patient access to targeted therapies.

## 1 Introduction

Recent advancements in pathological diagnostic methods have led to a more precise classification of lung cancers based on pathology and genetics, thus improving treatment options [1]. Emphasizing the importance of obtaining an accurate diagnosis for small cytology and biopsy samples highlights the need to identify specific histological types and molecular characteristics of non-small-cell lung cancer (NSCLC). Tissue genotyping is considered the gold standard method for identifying human genomic changes in cancer diagnosis; however, this technique has many limitations. It is highly invasive, expensive, and time-consuming, making it impractical for small amounts of tumor tissues or follow-up in cancer treatment regimens. Hence, liquid biopsy is considered a promising alternative to invasive diagnostic methods [2]. Liquid biopsy candidates, including circulating tumor cells (CTCs), circulating cell-free tumor DNA (cfDNA), tumor-educated platelets (TEP), and extracellular vesicles (EVs), contain cancer-derived biomolecules. They allow the identification of driver mutations that initiate cancer, acquired resistance due to the generations of therapeutic agents, refractory disease, prognosis, and surveillance [3]. In patients with cancer, the majority of cfDNA is released through apoptosis or necrosis of tumor cells. The clinical potential of noninvasive diagnostic tests using cfDNA is significant. The fragmentation pattern of cfDNA can indicate the tissue origin in patients with cancer. Although next-generation sequencing (NGS) is extensively used in tumor tissue profiling, its analytical sensitivity for cfDNA applications is limited. This limitation typically arises from the efficiency of capturing or enriching genetic regions of interest from cfDNA and the higher error rate in sequencing reactions

[4]. Plasma cfDNA is frequently used as a liquid biopsy for genetic testing across various cancers, but tumor-derived cfDNA within the total plasma cfDNA can be low [5].

Currently, studies are investigating the use of tumor-derived cfDNA isolated from different body fluids, including plasma, pleural effusion (PE), cerebrospinal fluid, saliva, and urine, for cancer genomic profiling [6, 7]. Patients with advanced lung cancer develop malignant pleural effusion (MPE) owing to the impaired drainage of pleural fluid and increased leakage of plasma into the pleural space due to the tumor cell invasion of the pleural cavity [8]. PE samples can be collected simultaneously to avoid additional invasive sampling, which contain floating malignant cells and tumor-derived cfDNA in the supernatant [9]. Previous studies have analyzed *EGFR* mutations by using cfDNA extracted from MPE supernatant samples, revealing that the supernatant from MPE exhibited a notably higher detection rate and sensitivity for tumor-specific mutations than the sediment-containing tumor cells [10]. Diagnosing MPE can be challenging, with positive cytology findings in only 60–80% of adenocarcinoma cases. Furthermore, the potential for PE in patients with negative pleural aspiration cytology is largely unexplored in clinical diagnostic settings [11].

Molecular tests recommended for advanced NSCLC according to the NCCN guidelines are not routinely performed because of insufficient samples. In Taiwan, under the National Health Insurance system, the standard method for testing PE is the isolation of the sediment for cell-block staining or polymerase chain reaction (PCR), including the identification of *EGFR* (Cobas), *ALK* (IHC), and *ROS1* (IHC). However, owing to the limited number of tumor cells in the PE sediment, sensitivity and accuracy are often questioned. To compare the genotyping of cfDNA extracted from PE and sediment for cell-block staining, we performed a prospective study at one medical center and three teaching hospitals in Taiwan. We aimed to determine whether pleural cfDNA can be used to assess targetable mutations in patients with lung adenocarcinoma having MPE. This was a pioneering study on the clinical application of next-generation genetic testing for MPE in Taiwan.

## 2 Material and Methods

### 2.1 Study Design and Patient Recruitment

This multicenter study prospectively recruited 39 patients between January 2022 and August 2023 who were newly diagnosed with stage IV lung adenocarcinoma and had MPE. The study protocol was approved by the Institutional Review Boards of Far Eastern Memorial Hospital (IRB No.: 111267-F), National Yang Ming Chiao Tung University Hospital (IRB No.: 2022A011), National Taiwan University

Hospital Yunlin Branch (IRB No.: 202205102RIFD), and E-Da Hospital (IRB No.: EMRB-111-140). The study was conducted in accordance with the Declaration of Helsinki for biomedical research. All patients' biopsied tissue or cell-block samples underwent standard care genotyping sponsored by the National Health Insurance for hotspot variants, including *EGFR* (Cobas V2), *ALK* (IHC D5F3), and *ROS1* (IHC D4D6 or SP384). PE samples were obtained and sent to a CAP-certified laboratory for liquid biopsy NGS (IMBdx, Inc., South Korea). Written informed consent was obtained from all patients prior to any study-specific procedures. The *EGFR* (Cobas V2) detection range includes: Exon 18: c.2156G>C (p.G719A), c.2155G>A (p.G719S), or c.2155G>T (p.G719C). Exon 19: In-frame deletions. Exon 20: c.2303G>T (p.S768I), c.2369C>T (p.T790M), and in-frame insertions. Exon 21: c.2573T>G or c.2573\_2574TG>GT (p.L858R), and c.2582T>A (p.L861Q).

## 2.2 Pleural Effusion Collection and Cell-Free DNA Extraction

Each patient's PE sample was collected after the cytological confirmation of malignancy. All the PE samples (40 mL) were collected and transferred to Cell-Free DNA BCT tubes (Streck, USA). Each PE sample was centrifuged at 2000×g for 10 min. cfDNA was then extracted from the supernatant of PE using the Maxwell<sup>®</sup> RSC cfDNA Plasma Kit (Promega, USA) following the manufacturer's protocol. The cfDNA was quantified using a 4200 TapeStation (Agilent Technologies, Santa Clara, CA, USA).

## 2.3 Targeted Panel Sequencing

A DNA NGS library was established using an IMBdx NGS DNA Library Prep Kit. At IMBdx, Inc. (Seoul, South Korea), solution-based target enrichment was performed using the AlphaLiquid<sup>®</sup> 100 target capture panel [12–14]. This panel was specifically designed to cover all the exons of 118 cancer-related genes. Subsequently, the captured DNA libraries were subjected to sequencing on an Illumina Novaseq 6000 platform (Illumina, San Diego, CA, USA) in the 2 × 150 bp paired-end mode (<https://doi.org/10.1038/s41416-022-01837-z>). The initial output of the sequencing data from the samples was in the bcl format. Then, to process and analyze the data, the Burrows-Wheeler Aligner (BWA, version 0.7.17-r1188) was used for demultiplexing (<https://doi.org/10.1093/bioinformatics/btp324>). Adaptor sequences were trimmed from the fastq files, followed by alignment to the human reference genome (hg38) performed using BWA (version 0.7.10) with the “mem” algorithm. Reads that were mapped to the AlphaLiquid<sup>®</sup> 100 target regions were extracted. Initial variant calls were generated

based on fragment counts, which included both single-strand consensus sequences (SSCS) and duplex consensus sequences (DCS). Subsequently, a series of in-house filtering steps specific to IMBdx was applied. The variant calls obtained were then scored using a machine-learning model designed to distinguish true from false variants. Subsequently, the variants were annotated to predict their functional effects.

## 2.4 Identifying ctDNA Variants (SNV-INDEL, Fusion, and CNV)

After the initial variant calling process on the cell-free DNA (cfDNA) samples, a series of filtering steps were applied to improve the accuracy and specificity of the results.

To minimize the influence of noise, contamination, and sequencing errors, we set specific thresholds for ctDNA mutations. These thresholds required a variant allele frequency (VAF) of at least 0.1% and a minimum of four altered duplex consensus sequence (DCS) counts. These criteria ensured that only the mutations with sufficient evidence and confidence were retained for further analysis. Then, we performed a manual review and curation of unexpected false positives by visually examining the longitudinal ctDNA mutation profiles. This step allowed us to identify and exclude any potential false positives that may arise due to technical artifacts or other confounding factors, thus ensuring the accuracy and reliability of the final mutation calls.

For copy number variations (CNVs), we established specific criteria to identify amplifications and gains. Gains were defined as having a copy number (CN) of 4 or greater, or when the CN ranged from 2.2 to 4, applying statistical criteria with a p-value below 0.001. For gene fusion, the computational time was optimized by selecting paired-end reads that overlapped with the targeted regions. Candidate fusion genes were identified using the dual-fusion caller approach with two software tools, Genefuse (<https://doi.org/10.7150/ijbs.24626>) and SViCT (<https://doi.org/10.1093/nar/gkz067>). To ensure high-confidence fusion gene detection, only the fusions supported by two or more reads with a mapping quality of 60 were considered. In addition, the predicted transcript resulting from the fusion event must be considered functional.

## 3 Results

This study included 39 patients (30.8 % male) with stage IV non-small-cell lung cancer who presented with PE. The median age at diagnosis was 67.7 years (range 48–84 years), and 25 patients (64.1%) were non-smokers. Among them, 26 (66.7%) were diagnosed with distant metastases (stage IVB).

All 39 PE cfDNA samples were successfully sequenced. Among them, 32 (82.1%) had less than 10% tumor content in the PE. We analyzed these patients, of whom only 18 (46.2%) were able to undergo tissue biopsy successfully and 21 (53.8%) were unable to undergo tissue biopsy (Table 1).

Standard cell-block testing of PE sediments for *EGFR* (Cobas), *ALK* (IHC), and *ROS1* (IHC) identified 20 mutations (51.3%), whereas the AlphaLiquid 100 panel detected 25 mutations (64.1%). The five additional patients detected

using cfDNA technology included one with *EGFR* L858R mutation, two with *EGFR* L747\_P753 del-insS mutation, one with *EGFR* E746\_S752del-insV mutation, and one with *EGFR* E746\_A750 del-insIP mutation. This suggests the improved mutation detection capability of PE cfDNA compared with that of standard tissue testing methods. The AlphaLiquid 100 panel detected 36 patients (92.3%) with all oncogenic mutations, including non-hotspots in the *EGFR* (Cobas) test. Compared with traditional cell-block testing, the 11 additional patients included three with non-hotspot *EGFR* mutations, three with *KRAS* mutations, two with *Met-14 skipping*, one with an *NRAS* mutation, one with a *BRAF* mutation, and one with *ERBB2 amplification* (Table 2; Fig. 1). Moreover, even with a very low cfDNA input, high detection rates were achieved (Fig. 2). Similar to other tissue-based NGS studies, analysis of PE cfDNA revealed the main driver mutations and various co-occurring alterations (Fig. 3).

We simultaneously analyzed the response to *EGFR*-TKI treatment in patients with *EGFR* mutations 3 months after treatment. Among the 18 patients who tested positive for *EGFR* in both cell blocks and cfDNA, 17 (94.4%) showed a partial tumor response 3 months after receiving *EGFR*-TKI treatment. In contrast, among the eight patients who tested negative for *EGFR* in the cell block but positive for cfDNA, all (100%) showed a partial tumor response 3 months after receiving *EGFR*-TKI treatment (Table 3).

**Table 1** Patient and tumor characteristics

	N (39)	Percentage (%)
Age, years (mean)		
67.74		
Sex		
M	12	30.8
F	27	70.2
Tissue biopsy availability at Dx		
Available	18	46.2
Not available	21	53.8
Cytology (%)		
≥ 10%	7	17.9
< 10%	32	82.1
Smoking history		
Never	25	64.1
Ex-smoker	9	23.1
Current smoker	5	12.8
Stage		
IVA	13	33.3
IVB	26	66.7

**Table 2** Comparison of traditional cell-block test and PE cfDNA test (AlphaLiquid 100 panel)

Total samples (N = 39)	Cell block ( <i>EGFR</i> , <i>ALK</i> , <i>ROS1</i> )	AlphaLiquid 100 panel in MPE ( <i>EGFR</i> , <i>ALK</i> , <i>ROS1</i> ) ( <i>EGFR</i> was only compared to Cobas detectable sites)	AlphaLiquid 100 panel in MPE (all oncogenic mutations: <i>EGFR</i> , <i>ALK</i> , <i>ROS1</i> , <i>RAS</i> , <i>ERBB2</i> , <i>MET</i> , <i>BRAF</i> , <i>RET</i> )
Detected numbers	20	25	36
Detection rate	51.3%	64.1%	92.3%
Additional mutation detected MPE cfDNA		P03: <i>EGFR</i> L858R P14: <i>EGFR</i> L747_P753delinsS P19: <i>EGFR</i> E746_A750delinsIP P22: <i>EGFR</i> L747_P753delinsS P29: <i>EGFR</i> E746_S752delinsV	P02: <i>EGFR</i> N771dup P06: <i>NRAS</i> Q61L P09: <i>KRAS</i> G12V P12: <i>MET</i> c.2888-16_2888-4del (ex14skip) P13: <i>KRAS</i> G12A P15: <i>BRAF</i> V600E P20: <i>EGFR</i> H773L, V774M P31: <i>KRAS</i> G12V P34: <i>EGFR</i> R776H, L858R P36: <i>ERBB2</i> amp P39: <i>MET</i> D1010N (ex14skip)

Fig. 1 Mutation distribution

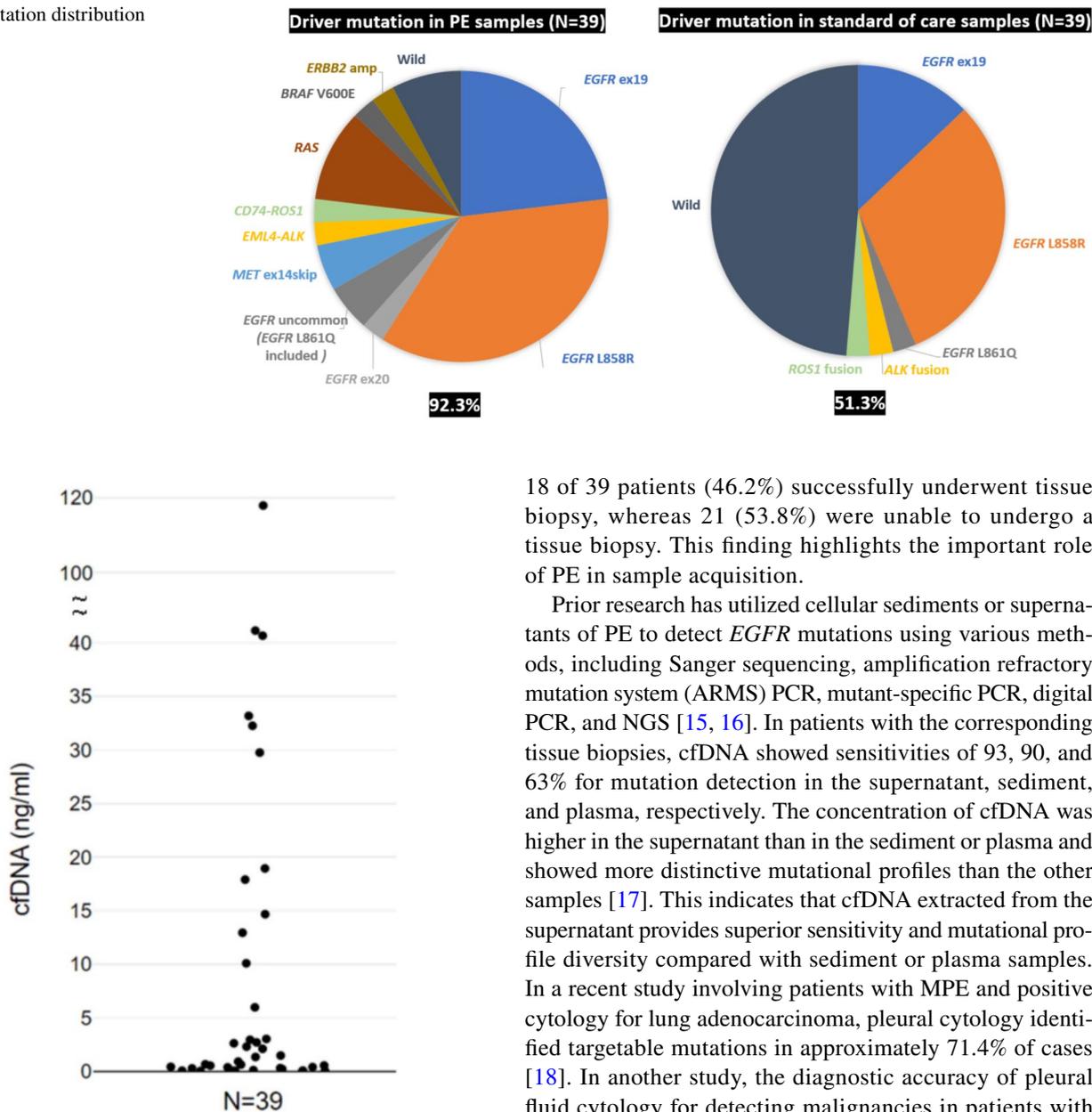


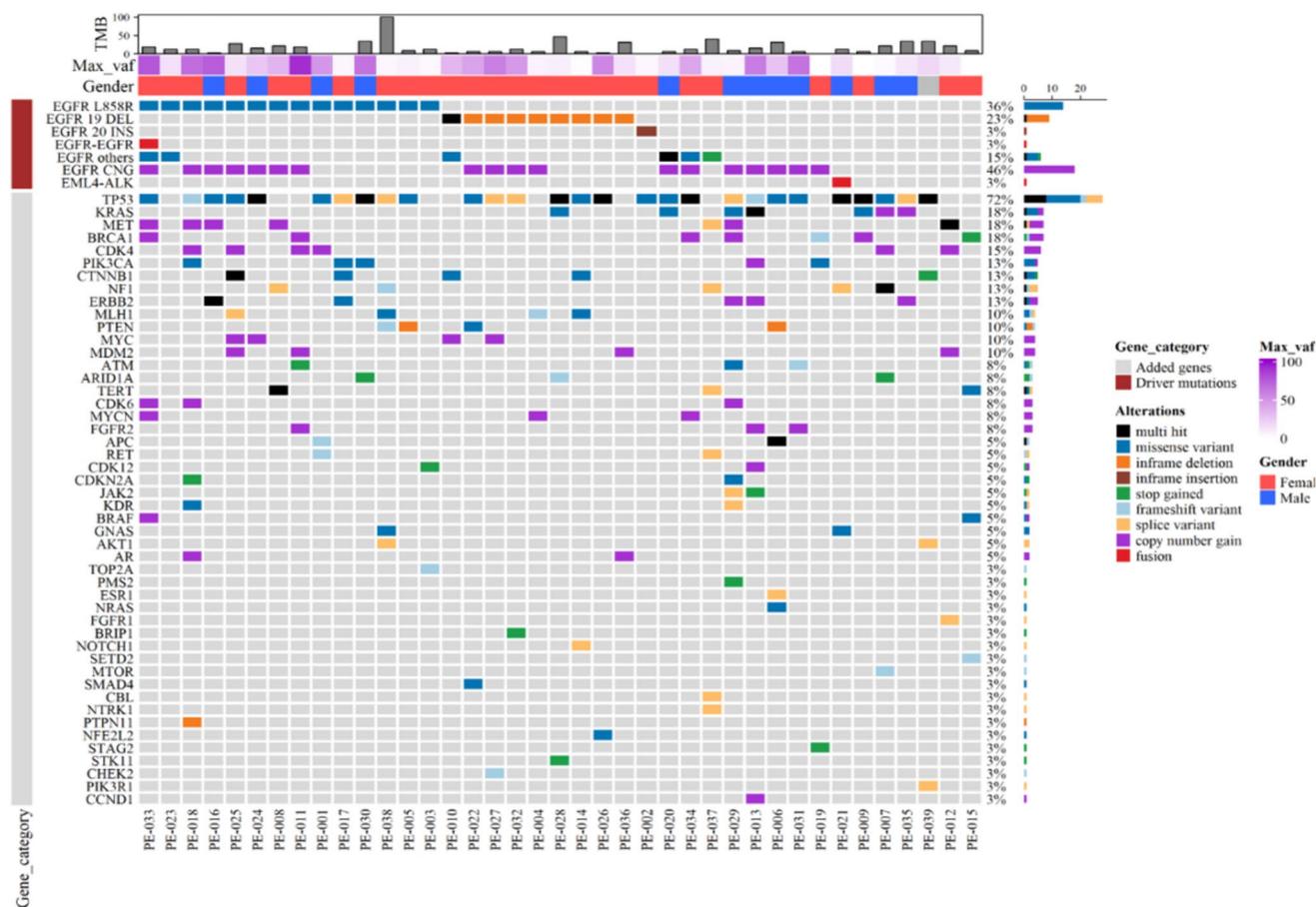
Fig. 2 Distribution of the input cfDNA amount

#### 4 Discussion

To the best of our knowledge, this is the first study to assess the diagnostic potential of oncogenic mutations in cfDNA extracted from PE. We compared these results with those of clinically available specimens from various centers across Taiwan. The prevailing method for identifying targetable genetic mutations in the pleural fluid of lung adenocarcinoma patients involves PCR or NGS, using malignant cells isolated from the sediment. In our study, if tissue samples were not obtained via PE, only

18 of 39 patients (46.2%) successfully underwent tissue biopsy, whereas 21 (53.8%) were unable to undergo a tissue biopsy. This finding highlights the important role of PE in sample acquisition.

Prior research has utilized cellular sediments or supernatants of PE to detect *EGFR* mutations using various methods, including Sanger sequencing, amplification refractory mutation system (ARMS) PCR, mutant-specific PCR, digital PCR, and NGS [15, 16]. In patients with the corresponding tissue biopsies, cfDNA showed sensitivities of 93, 90, and 63% for mutation detection in the supernatant, sediment, and plasma, respectively. The concentration of cfDNA was higher in the supernatant than in the sediment or plasma and showed more distinctive mutational profiles than the other samples [17]. This indicates that cfDNA extracted from the supernatant provides superior sensitivity and mutational profile diversity compared with sediment or plasma samples. In a recent study involving patients with MPE and positive cytology for lung adenocarcinoma, pleural cytology identified targetable mutations in approximately 71.4% of cases [18]. In another study, the diagnostic accuracy of pleural fluid cytology for detecting malignancies in patients with lung adenocarcinoma was 80.2% [19]. In our study, standard cell-block testing of PE sediments for *EGFR* (Cobas), *ALK* (IHC), and *ROS1* (IHC) identified mutations in approximately 51.3% of cases, whereas PE cfDNA analysis using the AlphaLiquid 100 panel detected mutations in 64.1% of cases. Under Taiwan’s National Health Insurance coverage, if only conventional tests were included, we might have missed many patients who could benefit from targeted drug therapies. An additional five patients were detected using cfDNA technology in the hotspots of *EGFR* mutations (L858R, L747\_P753 del-insS, E746\_S752del-insV, and *EGFR* E746\_A750 del-insIP). In other words, more oncogenic mutations were detected by the method of PE-cfDNA NGS than sediment cell block using Cobas *EGFR* mutation test. The overall rate of oncogenic mutations identified using



**Fig. 3** Landscape of heterogeneous mutations

**Table 3** Objective response rate after 3 months of EGFR-TKI treatment in patients with EGFR-mutant non-small-cell lung cancer

Assay	Patient with sensitizing EGFR mutation ( <i>n</i> )	Duration of response > 3 months
PE cfDNA (+) and tissue/cell block EGFR Cobas (+)	18	17/18 (94.4%) <sup>a</sup>
PE cfDNA (+) and tissue/cell block EGFR Cobas (-)	8	8/8 (100%)
Total	26	97.2%

<sup>a</sup>One primary resistance

the PE cfDNA was 92.3%. Therefore, cfDNA technology has greater clinical utility for detecting mutations in pleural effusion compared to using cell block Cobas test.

Additional patients included those with non-hotspot *EGFR* mutations, *KRAS*, *Met-14 skipping*, *NRAS*, *BRAF* mutations, and *ERBB2 amplification*. Rare mutations can be identified using cfDNA, enabling more effective

targeted therapies. Our study suggests that analyzing cfDNA extracted from PE can offer valuable genomic insights into an individual's cancer, complementing and enriching the data obtained from tissue biopsies.

A previous study reported that 5 mL of pleural fluid is sufficient to confirm malignancy [20]. In our study, 82.1% of the patients had PE with less than 10% tumor content; however, cfDNA mutational analyses were conducted successfully in all cases. Most patients in our study had PE with a low tumor burden; however, cfDNA analysis was still feasible.

Previous a study has shown that MPE is suitable for the detection of *EGFR* and *ALK* mutations in cell blocks [21]. To validate our accuracy, we observed tumor shrinkage in patients who tested positive after treatment with targeted drugs for 3 months. In our study, 59% of patients carried *EGFR* L858R or exon 19 deletion mutations, all of whom received treatment with either second- or third-generation *EGFR* inhibitors. Remarkably, 96.2% of these *EGFR*-positive patients exhibited a response duration exceeding 3 months, with only one patient exhibiting primary resistance. These results suggest that supernatants from MPE are as effective as precipitate samples in detecting

genetic alterations, despite the presence of gene mutation heterogeneity. Studies have shown that mutations in *TP53* and *CTNNB1* or amplifications in *EGFR*, *MET*, *MYC*, and *MDM2* may correlate with a shorter *EGFR*-TKI response duration and poorer clinical prognosis [22, 23]. Further analysis of these risk factors in relation to first-line *EGFR*-TKI treatment duration in our cohort is required.

Cytology remains the gold standard for diagnosing PE. However, establishing an MPE diagnosis in individuals with cytology-negative effusions can be challenging [24]. Cytology-negative PE specimens are often considered inadequate for *EGFR* testing because of the low number of tumor cells. Previous studies have shown that *EGFR* mutations cannot be detected in cfDNA samples from non-malignant cells [25]. However, our study found that although as many as 82.1% of patients had fewer than 10% tumor cells in the cytology cell block, the overall rate of oncogenic mutations identified by PE cfDNA achieved a high detection rate of 92.3%.

This study has some limitations that should be addressed. The study acknowledges that the small sample size (39 subjects) is a limitation, which may affect the generalizability of the findings. Future research with a larger cohort is needed to validate and confirm these results. Simultaneous NGS testing of both PE supernatant and PE sediment block was not performed due to limited research resources. We were unable to investigate the concordance among tissue DNA, plasma cfDNA, PE supernatant, and sediment. To validate the accuracy of our results, we observed tumor shrinkage and an objective treatment response rate of 97.2% in patients who tested positive for *EGFR* after being treated with *EGFR*-TKI for 3 months. However, not all patients with genetic alterations, except those with *EGFR* mutations, were treated with targeted therapy. Additional studies are required to validate these findings. Furthermore, we could not comprehensively compare the competencies of each sample type. The nature of the liquid cfDNA may limit the detection of rare fusion events. However, the amplification threshold for PE requires further validation.

## 5 Conclusions

This groundbreaking real-world study conducted in Taiwan verified that PE supernatants collected from patients with advanced NSCLC were highly reliable and effective in detecting mutations. Genotyping of PE supernatant cfDNA is feasible in clinical practice, in addition to plasma and tumor testing, to improve diagnostic yield and extend the patients' potential to benefit from targeted therapies.

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## Declarations

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**Conflict of interest** The authors S.C. Chang, C.Y. Chen, Y.F. Wei, Y.C. Lai, P.W. Hu, J.C. Hung, and C.Y. Chang declare that they have no conflicts of interest that might be relevant to the contents of this article.

**Ethics approval** The study protocol was approved by the Institutional Review Boards of Far Eastern Memorial Hospital (IRB No.: 111267-F), National Yang Ming Chiao Tung University Hospital (IRB No.: 2022A011), National Taiwan University Hospital Yunlin Branch (IRB No.: 202205102RIFD), and E-Da Hospital (IRB No.: EMRB-111-140). The study was conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

**Consent to participate** Informed consent was obtained from all individual participants included in the study.

**Consent for publication** Not applicable.

**Data availability statement** The data that support the findings of this study are available on request from the corresponding author.

**Code availability** Not applicable.

**Author contributions** Yu-Feng Wei, Chung-Yu Chen, and Shih-Chieh Chang contributed to data collection and analysis. Cheng-Yu Chang and Yu-Feng Wei helped finalize study concept and study design. Yi-Chun Lai, Po-Wei Hu, and Jui-Chi Hung contributed to acquisition of data. Cheng-Yu Chang and Shih-Chieh Chang drafted the manuscript. All authors contributed to the article and approved the submitted version.

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