European Journal of Cancer 217 (2025) 115224

Contents lists available at ScienceDirect

European Journal of Cancer

journal homepage: www.ejcancer.com

Original research

Enhanced detection of actionable mutations in NSCLC through pleural effusion cell-free DNA sequencing: A prospective study

Hsin-Yi Wang [a,b], Wei-Yu Liao [c], Chao-Chi Ho [c], Shang-Gin Wu [d], Ching-Yao Yang [c], Chia-Lin Hsu [e], Yen-Ting Lin [d], James Chih-Hsin Yang [d], Jin-Yuan Shih [b,c,*]

[a] Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, National Taiwan University Hospital Yunlin Branch, No. 579, Section 2, Yunlin Rd., Yunlin County, Douliu City 640, Taiwan
[b] Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, No. 1, Section 4, Roosevelt Rd., Taipei, Taiwan
[c] Department of Internal Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Zhongzheng Dist., Taipei City 100, Taiwan
[d] National Taiwan University Cancer Centre, Taipei City 106, Taiwan
[e] Department of Internal Medicine, National Taiwan University Hospital Hsin-Chu Branch, Taiwan

ARTICLE INFO

Keywords:
Next generation sequencing
Pleural effusion cfDNA
CtDNA
Liquid biopsy
NSCLC

ABSTRACT

Background: Inadequate tumour samples often hinder molecular testing in non-small cell lung cancer (NSCLC). Plasma-based cell-free DNA (cfDNA) sequencing has shown promise in bypassing these tissue limitations. Nevertheless, pleural effusion (PE) samples may offer a richer cfDNA source for mutation detection in patients with malignant PE.

Methods: This prospective study enrolled newly diagnosed advanced NSCLC patients with malignant PE. PE samples were collected for cfDNA NGS analysis. Meanwhile, PE cell pellet RNA was extracted for reverse transcription polymerase chain reaction (RT-PCR) for clinically relevant actionable mutations and then confirmed by Sanger sequencing. The concordance between PE cell pellet RT-PCR and PE cfDNA NGS analyses was analysed.

Results: Fifty patients were enrolled. The median age was 68.5 years, and the female-to-male ratio was 29:21. Most patients (74 %) were non-smokers. Notably, 45/50 patients (90 %) had actionable mutations, including EGFR exon 19 deletions (24 %), EGFR L858R mutations (36 %), HER2 exon20 insertions (10 %), ROS1 rearrangements (4 %), EGFR exon20 insertions (2 %), ALK rearrangements (4 %), RET rearrangements (2 %), KRAS G12C mutations (2 %), and CD74-NRG1 fusions (2 %). Among the 50 enrolled patients, actionable mutations were detected in 44 (88 %) by PE cfDNA NGS, 39 (78 %) by PE cell pellet Sanger sequencing, and 33 (66 %) by clinical tissue genetic testing ($P = 0.031$). The detection of actionable mutations from PE cfDNA NGS remained consistently high across M1a to M1c stages.

Conclusions: PE cfDNA genotyping has clinical applicability for NSCLC patients and can serve as an additional source for molecular testing. Incorporating PE NGS cfDNA analysis into genetic testing enhances diagnostic yield and aids in identifying actionable mutations in clinical practice.

1. Introduction

Molecular testing of non-small cell lung cancer (NSCLC) specimens is important for the identification of potentially efficacious targeted therapies, but it is frequently limited by inadequate tumour samples. The limitations associated with tissue collection and tissue-based testing can be overcome by cell-free DNA (cfDNA) next-generation sequencing (NGS). Studies have demonstrated the feasibility of mutation detection in NSCLC through clinical plasma-based circulating tumour DNA (ctDNA) NGS [1–4]. The NSCLC treatment guidelines recognize the clinical utility of cfDNA assays but only for plasma cfDNA NGS as a complement to tissue testing for molecular diagnosis in advanced NSCLC [5,6]. However, the amount of tumour-derived DNA in the bloodstream can be low, especially in patients with low tumour burden, which may lead to false-negative results. Plasma ctDNA testing is also prone to lower sensitivity, depending on the tumour stage and burden, and this is a significant concern in treatment decisions making [7].

Pleural effusion (PE) samples may provide a more abundant source

* Corresponding author at: Department of Internal Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Zhongzheng Dist., Taipei 100, Taiwan.
E-mail address: shihjy@ntu.edu.tw (J.-Y. Shih).

https://doi.org/10.1016/j.ejca.2025.115224
Received 11 October 2024; Received in revised form 27 December 2024; Accepted 2 January 2025
Available online 7 January 2025

of ctDNA in NSCLC patients with malignant PE. CfDNA in PE, even in haemorrhagic or cytologically negative samples, contains substantial tumour-derived genetic material [8]. The PE supernatant cfDNA may be a potentially superior source for molecular testing than traditional PE sediment for cell-block preparations in lung adenocarcinoma. Furthermore, cfDNA in PE can be utilised to accurately detect epidermal growth factor receptor (EGFR) mutations, including those involved in resistance to tyrosine kinase inhibitor (TKI) treatment (e.g., EGFR T790M mutation) [9,10]. PE cfDNA has demonstrated comparable diagnostic accuracy compared to tumour tissue, plasma cfDNA, or PE cell block for oncogenic mutation detection in lung adenocarcinoma [11]. Compared to DNA, RNA-based testing methodologies, such as targeted RNA sequencing and hybrid-capture RNA sequencing, play a pivotal role in identifying key alterations, such as gene fusions and splice variants. However, cell-free RNA (cfRNA) is yet to be clinically applied for NSCLC genetic alteration detection due to the lack of standardized protocols, unvalidated biomarker panels, and interference from non-cancer factors. Challenges such as cfRNA degradation, extraction, and detection limitations hinder reliability and highlight the need for improved techniques and reference genes in the future [12].

Although PE cfDNA can provide a high yield of tumour DNA, significant clinical gaps persist in the implementation of molecular analysis using physiological fluids. Testing protocols for biological fluids, such as pleural effusion and cerebrospinal fluid, lack standardization and challenges remain in achieving optimal sensitivity and specificity. Additionally, data on the clinical utility of fluid-based testing in real-world settings are sparse. Given the growing importance of molecular testing throughout the course of lung cancer, optimizing the use of diagnostic minute specimens such as PE or cytology is critical for advancing individualised therapy. To gather more real-world clinical experience, we initiated a prospective trial to evaluate the diagnostic performance and feasibility of using PE cfDNA NGS in molecular profiling for lung adenocarcinoma with malignant PE. Previous studies have shown that RNA-based mutation analysis enhances sensitivity in malignant PE cytology samples by minimizing interference from non-tumor cells with low or absent mutant gene expression [13]. Building on this, our study aims to utilize PE cell pellets for RT-PCR and Sanger sequencing and compare the diagnostic yield to PE cfDNA NGS in identifying genetic alterations in treatment-naïve lung adenocarcinoma patients.

2. Material and methods

2.1. Study design and patients

This prospective study was conducted between February 2023 and May 2024 at National Taiwan University Hospital (NTUH) and Cancer Centre. Patients with treatment-naïve advanced lung adenocarcinoma and aged $\geq$ 20 years were enrolled. All patients were cytologically confirmed to have malignant PE. The pleural fluids were acquired aseptically in vacuum bottles by thoracentesis in the ultrasonography examination room, and the presence of adenocarcinoma was confirmed by the pathologist. Driver mutations of cancer cells from PE samples were assessed using both cell pellet Sanger sequencing and PE cfDNA NGS. The clinical characteristics and tissue or plasma genetic testing results, as determined by the clinician in routine clinical practice, were collected from medical records. Tissue-based genetic testing in the standard-of-care clinical setting at NTUH included EGFR, ALK, ROS1, and BRAF mutation analysis. Tissue NGS was optional based on the clinician's and patient's decisions [3]. (Supplementary Table 1)

This study was approved by the Institutional Review Board of National Taiwan University Hospital. (202212007RIPB). Informed consent was obtained from all patients enrolled.

2.2. Pleural effusion samples preparation and genetic testing

2.2.1. Cell pellet for Sanger sequencing

Cancer cells obtained from the PE samples of patients diagnosed with lung adenocarcinoma were collected and centrifuged at 800 $\times$ *g* for 10 min at room temperature. The cell pellet was washed with red blood cell lysis buffer until it was clear of blood. It was then immersed in RNAlater (Qiagen) for storage until RNA isolation, which was carried out using the TRIozl reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. Exons of *EGFR, HER2, ALK, ROS1, RET, KRAS, BRAF*, and *MET* were amplified using specific forward and reverse primers (Supplementary Table 2). Reverse transcription-polymerase chain reaction (RT-PCR) was conducted utilizing the Qiagen One-Step RT-PCR Kit. Amplicons were purified and subjected to Sanger sequencing using the BigDye Terminator Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing products were subjected to electrophoresis on an automatic ABI PRISM 3730 genetic analyser (Applied Biosystems). The sequencing results were analysed using the Vector NTI Advance 10 software (Invitrogen, Thermo Fisher Scientific Inc.) (Figure1a). The procedure was performed as previously reported [14].

2.2.2. PE supernatant cfDNA for NGS

PE fluid (~40 ml) was collected in Streck Cell-Free DNA BCT® (Streck, USA). The PE sample processing was performed consistently within 1 day of PE arrival to prevent cfDNA degradation and release of genomic DNA from blood cell lysis. Each PE sample Cell-Free was checked before proceeding with the experimental protocol and separated by centrifugation at 2000 $\times$g for 10 min to remove cell debris. PE cfDNA was extracted using a Maxwell® RSC cfDNA Plasma Kit (Promega, USA) following the manufacturer's instructions. They were then quantified using a 4200 TapeStation System (Agilent Technologies, USA). DNA NGS library construction and target enrichment were performed at IMBdx, Inc. (Seoul, South Korea) using the AlphaLiquid® 100 target capture panel. The targeted gene panel included 118 cancer-related genes (Supplementary Table 3) and was designed to cover the entire exon of the genes. Captured DNA libraries were sequenced using the NovaSeq™ 6000 platform (Illumina, USA) in 150 bp paired-end mode (Figure1b). The cfDNA variant, including SNV/InDel, fusion and copy number variations, were identified as previously described [15].

2.2.3. Data and statistical analysis

Clinically relevant actionable mutations were identified for each patient through comprehensive genetic profiling. They were defined as tier-1 variants according to the European Society of Medical Oncology Scale for Clinical Actionability of Molecular Targets classifications and the joint Associated of Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists [16].

Descriptive statistics were employed to summarize the clinical characteristics of the patients. Continuous variables were reported as medians with their corresponding ranges, while categorical variables were expressed as percentages. The concordance in detecting driver mutations between cell pellet Sanger sequencing and PE cfDNA NGS testing was analyzed. Kappa statistics were calculated to measure the agreement between different testing methods. The detection rates of clinically relevant actionable mutations by cell pellet Sanger sequencing, PE cfDNA NGS, and standard clinical tissue genetic testing were calculated and compared using a chi-square test. Failure of mutation detection in one test indicated that the mutation was identified by one or more alternative genetic testing methods.

Treatment response to targeted therapy based on the detected actionable mutations was evaluated using the Response Evaluation Criteria in Solid Tumors1.1 criteria. Statistical analyses were performed using IBM SPSS Statistics, version 22.00 (SPSS Inc., Chicago, IL, USA). A two-sided p-value of < 0.05 was considered statistically significant.

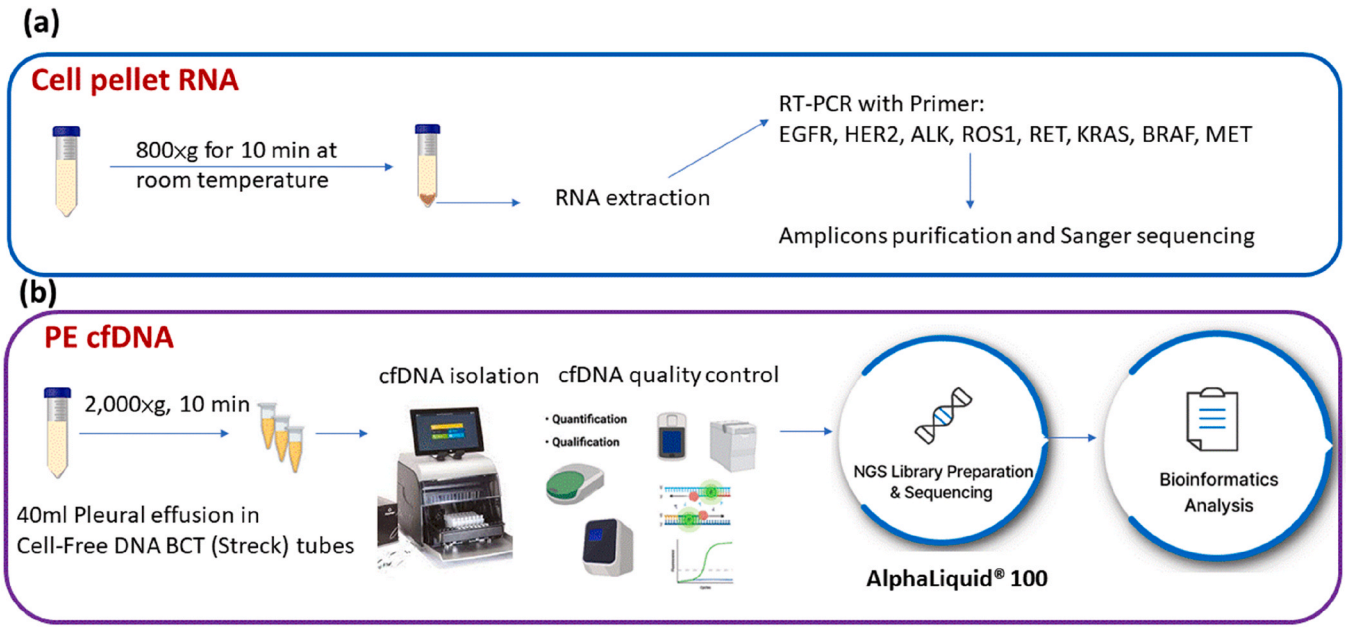

Fig. 1. Study design. This is a prospective trial to evaluate the diagnostic performance and feasibility of using (b) PE cfDNA NGS for molecular profiling in lung adenocarcinoma with malignant pleural effusion in comparison to (a) Sanger sequencing using cell pellet RNA. Pleural effusion samples from patients with newly diagnosed lung adenocarcinoma are collected and simultaneously analysed using both methods.

3. Results

3.1. Patient characteristics

Altogether, 51 patients with malignant PE were enrolled in the study. However, one patient who was initially included based on positive cytology suggesting adenocarcinoma in PE was subsequently excluded upon confirmation of colon cancer. Thus, the final analysis involved 50 patients. The median patient age was 68.5 years, 29 (58 %) patients were female, and 37 (74 %) patients were nonsmokers. The distribution of M stages was as follows: M1a, 38 %; M1b, 4 %; and M1c, 58 %. Histologically, all patients had adenocarcinoma based on PE cytology at enrolment, although one patient (2 %) was ultimately diagnosed with large cell NSCLC based on tissue pathology. Table 1 shows the baseline patient characteristics.

Table 1
Baseline patient characteristics.

		Value
Total		50
Age (years), median (range)		68.5 (44.5–91.2)
Sex		
	Female	29
	Male	21
Smoking		
	Nonsmokers	37
	Former/current smokers	13
Stage		
	IVa	21
	IVb	29
T		
	1	2
	2	9
	3	11
	4	28
N		
	0	12
	1	5
	2	13
	3	20
M		
	1a	19
	1b	2
	1c	29

3.2. Actionable mutation

Clinically actionable mutations were identified in 45 (90 %) patients (Fig. 2a). EGFR mutations were found in 31 patients (62 %): exon 19 deletions, 12 (24 %); L858R mutations, 16 (32 %); L858R compound mutations, 2 (4 %); and exon 20 insertion, 1 (2 %). HER2 exon 20 insertion mutation was detected in 5 patients (10 %). Other rare mutations of ALK, ROS1, RET, KRAS, and NRG1 were found in two (4 %), two (4 %), one (2 %), three (6 %), and one (2 %) patient, respectively. The genomic alterations, including the main actionable mutations and pathogenic or likely pathogenic co-occurring mutations, identified in PE cfDNA NGS analysis are summarised in Figure 3.

3.3. Mutation detection performance

The detailed genetic mutations detected through different methods are shown in Supplementary Table 4.

Among the 50 enrolled patients, actionable mutations were detected in 44 (88 %) by PE cfDNA NGS, 39 (78 %) by PE cell pellet Sanger sequencing, and 33 (66 %) by clinical tissue genetic testing ($P = 0.031$, Chi-square). (Table 2 and Fig. 2b)

For the PE cfDNA NGS assays, the median total reads per sample were 11766, with a range of 4124–58316. The median target coverage depth was 10406X. The median variant allele frequency (VAF) was 23.22 % (range: 0.07–95.3 %).

The concordance rate and Cohen's kappa coefficient were 90 % and 0.65 (95 % confidence interval [CI], 0.38–0.92, substantial agreement) between PE cfDNA and cell pellet Sanger sequencing, and 78 % and 0.42 (95 % CI, 0.17–0.67, moderate agreement) between PE cfDNA and clinical tissue genetic testing, respectively. For the discordant cases, a total of five discrepancies were observed, where mutations were detected by PE cfDNA NGS analysis but were not identified in the cell pellet Sanger sequencing. In one case, L858R, and in another case, RET fusion, the presence of these mutations was confirmed by additional plasma cfDNA analysis ordered by the clinician. A case with KRAS G12C

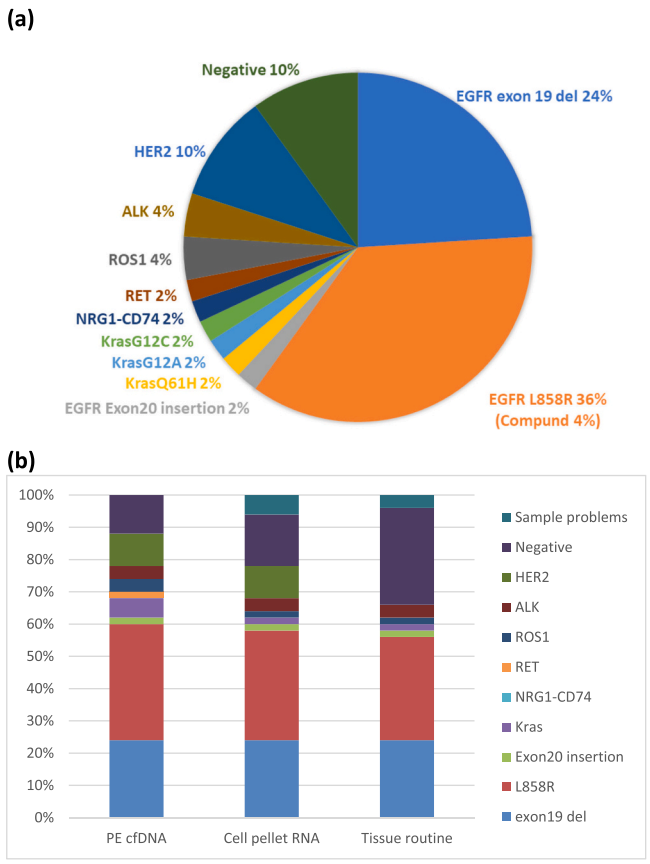

Fig. 2. (a)Distribution and frequency of clinically relevant actionable mutations in the study cohort (b) Rate of actionable mutations detected by different methods.

was confirmed following repeated PE RNA extraction and RT-PCR analysis, after improving RNA quality. Additionally, a rare ROS1 fusion variant was validated by tissue ROS1 fluorescence in situ hybridization and PE cell pellet Sanger sequencing, following a redesign of the primer location (Figure 4). Only in one case with KRAS Q61H, the presence of this mutation could not be confirmed after repeating PE Sanger sequencing, and there was no suitable clinical tissue available for further examination.

The influencing factors of false negative results for genetic mutations varied. In PE cfDNA NGS analysis, only one patient had a non-detected result for actionable mutations. This patient was ultimately found to have the CD74-NRG1 fusion, which was not covered by the AlphaLiquid® 100 target capture panel and was detected through FoundationOne Liquid CDx plasma cfDNA analysis as assigned by the clinician in the clinical setting. In PE cell pellet Sanger sequencing, three patients showed no bands on RT-PCR sequencing, likely owing to RNA quality issues. In one patient with the CD74-NRG1 fusion, the mutation was not identified because NGR1 was not included in the initial primer design for RT-PCR. Two-thirds of the patients with KRAS mutations also had false negative results. For clinical tissue genetic testing, two patients did not have sufficient tissue available for genetic analysis. Additionally, some rare mutations, such as NRG fusion, RET fusion, ERBB2, and KRAS, were not tested in routine practice.

3.4. Treatment response to targeted therapy based on PE cfDNA analysis

All 30 patients with EGFR-mutated NSCLC received EGFR-targeted therapy, equally distributed across first-, second-, and third-generation TKIs. Overall, 26 patients achieved a partial response, and 3 patients showed stable disease, yielding an objective response rate of 86.7 %. All patients with ALK, RET, and ROS1 fusions showed partial response to targeted therapy. Among the 5 patients with HER2 mutations, two of three patients who received trastuzumab deruxtecan as either first- or second-line therapy showed partial response, while the other had stable disease. (Supplementary Table 4)

3.5. A case with rare SLC34A2-ROS1 fusion

We presented a case of a rare ROS1 fusion variant detected by PE cfDNA NGS, which was not identified by other methods, to emphasize its clinical utility. A 44-year-old nonsmoker woman was diagnosed with stage IV adenocarcinoma (T4N3M1c) with multiple brain, pleural, and bone metastases. Following a computed tomography-guided biopsy, formalin-fixed paraffin-embedded tumour tissues were analysed using the IntelliPlex Lung Cancer Panel assay (PlexBio, Taipei, Taiwan). No actionable mutation was detected. Cell pellet Sanger sequencing of malignant PE sample yielded negative results, but PE cfDNA NGS detected a SLC34A2-ROS1 fusion with breakpoint at exon 13 of SLC34A2 and intron 32 of ROS1 (Figure 4a). Reviewing the IntelliPlex Lung Cancer Panel datasheet revealed that this panel covers SLC34A2-ROS1 fusion variants with breakpoints at SL4;R32 and SL4;R34 only. The forward and reverse primers used for cell pellet RNA RT-PCR were also located at SLC34A2 exon 4 and ROS1 exon 34 (Figure 4b). This explained the undetected results in these two assays. After redesigning the forward primer to target SLC34A2 exon 12 and exon 13, cell pellet Sanger sequencing confirmed the presence of the SLC34A2-ROS1 fusion transcript, joining SLC34A2 exon 13 to ROS1 exon 34 (Figure 4c,d). The patient received entrectinib and initially showed a partial response, but the tumour progressed after approximately 6 months of therapy (Figure 4e).

4. Discussion

This prospective study shows the high diagnostic yield of PE cfDNA NGS analysis, confirming the feasibility of its clinical utility. PE cfDNA NGS analysis demonstrated higher mutation detection performance than PE cell pellet Sanger sequencing and real-world clinical tissue genetic testing. Additionally, the response rates were as expected for targeted therapies determined based on actionable mutations identified through PE cfDNA NGS. These findings establish PE cfDNA NGS as a reliable and effective method for detecting genetic mutations in lung adenocarcinoma.

In our study, 90 % of the patients tested positive for clinically relevant actionable mutations, exceeding the previously reported prevalence of 68–84 % in Asian patients with NSCLC [17–20]. Specifically, a tissue-based targeted NGS analysis in Taiwan identified driver mutations in 69 % of cases, while a plasma cfDNA NGS analysis reported an incidence of 76 % [3,21]. Previous studies on patients with malignant PE, have reported that targetable mutations, including EGFR, KRAS, BRAF, ALK, and ROS1 rearrangements, were detected in approximately 59–79.6 % of cases [11,15,22–24]. However, these findings were significantly influenced by variations in testing methods, the types of mutations included, and the characteristics of the enrolled populations. By employing a prospective design and highly sensitive NGS techniques, our study may provide a more accurate estimate of the mutation prevalence in NSCLC patients with malignant PE within the Asian population. Several factors may explain the high mutation rate detected in NSCLC patients with malignant PE in our study. First, we exclusively enrolled patients with adenocarcinoma, a subtype more likely to harbor driver mutations. Additionally, malignant PE is more common in NSCLC with EGFR mutations [25–27]. The presence of actionable mutations is often linked with a more aggressive disease course, which can lead to a higher risk of PE development [28,29]. We cannot also rule out the possibility that clinicians tend to enroll patients with a higher likelihood of actionable mutations, based on clinical characteristics, in genetic testing trials.

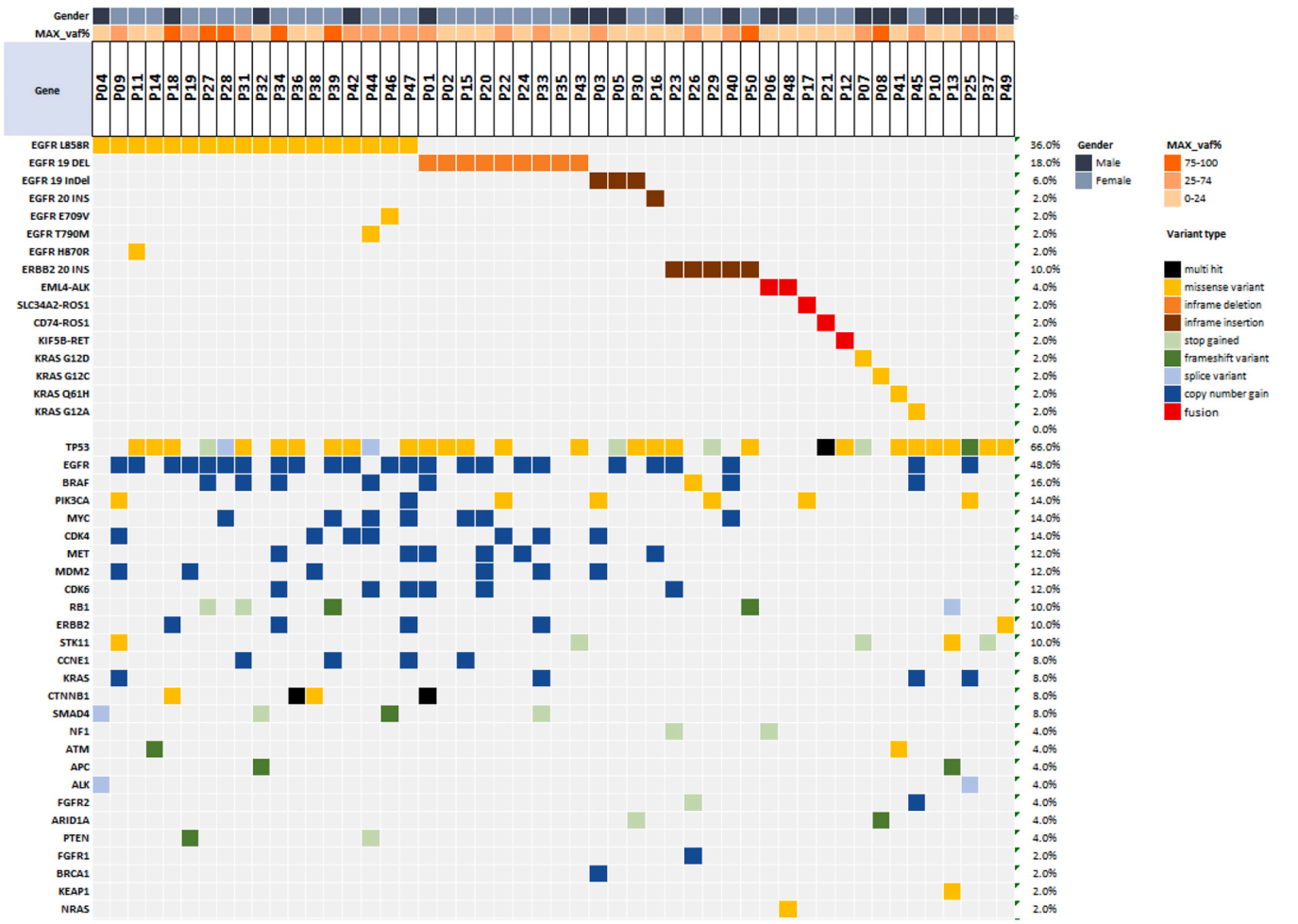

Fig. 3. Overview of genomic alterations identified by PE ctDNA NGS analysis. Oncoplot showing the genomic alterations identified in each sample through PE ctDNA NGS analysis, highlighting the main actionable mutations (middle row) and pathogenic or likely pathogenic co-occurring mutations (lower row).

Table 2
Diagnostic performance for detecting actionable mutations.

	PE-ctDNA NGS	Cell pellet Sanger sequencing	Clinical testing
Total number of samples	50	50	50
Positive no.	44 (88 %)	39 (78 %)	33(66 %)
Failure of mutation detection[a]	1 (2 %)	6 (12 %)	12 (24 %)
Insufficient sample[b]	0 (0 %)	3 (6 %)	2 (4 %)

[a] Failure of mutation detection in one test indicated that the mutation was identified by one or more alternative genetic testing methods.

[b] Three patients had inadequate RNA quality for RT-PCR and two patients had no tissue for genetic testing.

The EGFR L858R mutation was more prevalent than exon 19 deletion in the this study, consistent with previous results in studies evaluating the EGFR mutation rate in malignant PE samples of lung adenocarcinoma. The L858R mutation is more frequently observed in malignant PE, while exon 19 deletions are more common in surgically resected solid NSCLCs [25]. From a biological behaviour perspective, the EGFR L858R mutation enhances cell invasive ability and promotes malignant PE formation through activation of the CXCL12-CXCR4 pathway [30]. These differences in EGFR mutations may reflect the distinct nature of carcinogenesis, which poses varying risks of pleural involvement and malignant effusion development.

Liquid biopsy has emerged as an effective alternative for detecting mutations in NSCLC, particularly when tissue samples are unavailable. Blood samples are now a commonly used source for liquid biopsy. However, plasma cfDNA genetic testing in advanced NSCLC demonstrates sensitivities between 70 % and 90 %, and they are strongly correlated with the stage of metastasis [3,31,32]. In a Taiwanese cohort evaluated using the FoundationOne Liquid CDx, driver mutations were present but not detected in plasma cfDNA analysis in 32 %, 21.9 %, and 3 % of patients with M1a, M1b, and M1c diseases, respectively. This high rate of non-detection in patients with limited metastasis presents a substantial challenge to the routine use of plasma cfDNA analysis in clinical practice [3]. Our study revealed that the detection of actionable mutations from PE cfDNA analysis via NGS remained consistently high across M1a to M1c stages. All patients with M1a and M1b disease with actionable mutations were successfully identified through PE cfDNA NGS analysis. Only one M1c patient had mutations that went undetected and this was owing to limitations in the NGS panel design. These findings support that PE cfDNA is an ideal alternative source to plasma for genetic mutation identification, particularly in patients with M1a disease with limited metastasis.

Our study have some strengths. This is the first study to prospectively compare the use of PE cfDNA NGS in real-life clinical practice. The study design mirrors typical clinical processes, demonstrating the utility of PE cfDNA NGS across various stages of metastasis. This robustly supports integrating PE cfDNA NGS into routine clinical workflows. This study has some limitations. First, NGS was employed for the PE cfDNA analysis, whereas Sanger sequencing was used for the cell pellet analysis. The mutations analysed via the Sanger sequencing approach were

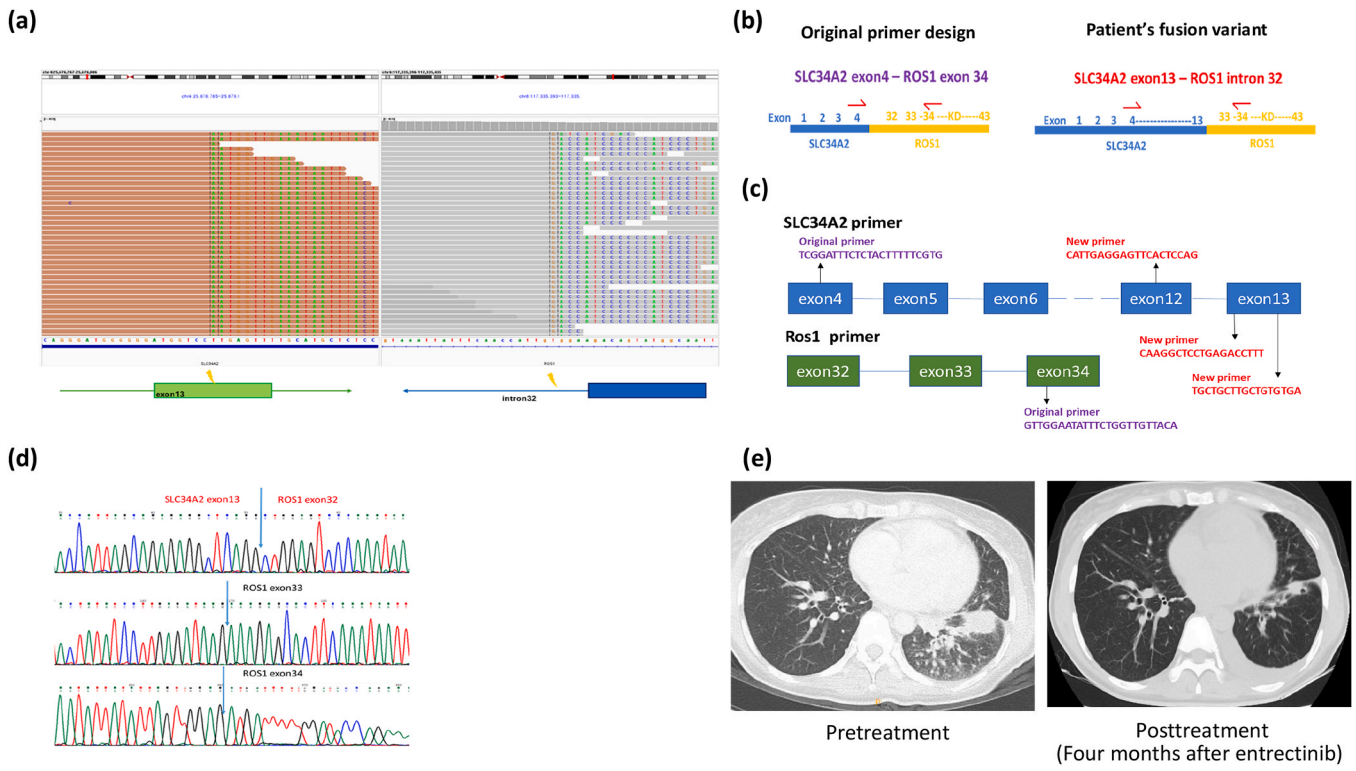

Fig. 4. A case of uncommon SLC34A2-ROS1 fusion breakpoints detected by NGS. (a) PE cfDNA NGS reveals that the SLC34A2 gene breakpoint is at exon 13 (chr4:25676785), and the ROS1 gene breakpoint is at Intron 32 (chr6:117335413). (b) The original forward and reverse primers for cell pellet RNA RT-PCR are located at SLC34A2 exon 4 and ROS1 exon 34, which are over 2000 bases apart in the patient's fusion variant. (C) To validate the presence of the SLC34A2-ROS1 fusion in the patient, new forward primers spanning SLC34A2 exon 12 and exon 13 are designed. (d) After redesign of the primers, cell pellet Sanger sequencing confirms the presence of SLC34A2-ROS1 fusion transcript, joining SLC34A2 exon 13 to ROS1 exon 34. (e) ROS1 fusion-targeted therapy with entrectinib is administered, and the tumour decreases in size.

limited to EGFR, HER2, ALK, ROS1, RET, KRAS, BRAF, and MET, as determined by our primer designs. The higher performance of PE cfDNA NGS than of cell pellet analysis via Sanger sequencing can be partly attributed to the differing methodologies applied to these two specimens. Ideally, a comprehensive comparison would involve performing both cell pellet DNA/RNA NGS and PE cfDNA NGS. However, due to economic and funding constraints, we could not perform NGS on all cell pellet samples. Thus, Sanger sequencing was utilized for cell pellet RNA as a more cost-effective alternative. Further, several critical questions remain unresolved in our study. We included only patients who presented with positive malignant cytology in their PEs. Consequently, we were unable to determine the diagnostic yield of actionable mutation detection in PEs exhibiting negative cytology. Furthermore, our cohort exclusively included treatment-naïve patients. Thus, the clinical utility and value of PE-cfDNA in detecting acquired resistance during the course of disease progression remain unclear. Additionally, clonal haematopoiesis of indeterminate potential (CHIP) mutations have emerged as potential sources of background noise, leading to false positives in plasma ctDNA analyses and complicating the interpretation of true somatic mutations in advanced NSCLC [33–36]. However, the presence and impact of CHIP mutations in PE cfDNA analyses for calling NSCLC somatic mutations have yet to be determined.

5. Conclusions

PE cfDNA NGS has superior mutation detection performance compared to PE cell pellet Sanger sequencing for detecting actionable mutations in NSCLC. As an easily accessible and valuable source for genetic testing, PE samples provide a promising alternative for identifying clinically relevant mutations. Although tissue-based genetic testing remains the standard in routine clinical practice, our findings strongly support the integration of PE cfDNA NGS as a reliable and effective tool for molecular diagnosis in treatment-naïve lung adenocarcinoma.

Funding

The study was sponsored by TSH Biopharm Corporation Ltd. TSH Biopharm provided NGS tests and analyses. This work was also supported by the National Taiwan University Hospital [grant number NTUH 113-S0284]. The funding source had no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

CRediT authorship contribution statement

Ching-Yao Yang: Resources. **Chia-Lin Hsu:** Resources. **Chao-Chi Ho:** Resources. **Shang-Gin Wu:** Resources. **Wei-Yu Liao:** Resources. **Hsinyi Wang:** Writing – original draft, Methodology, Formal analysis, Data curation. **Jin-Yuan Shih:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Yen-Ting Lin:** Resources. **James Chih-Hsin Yang:** Resources.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jin-Yuan Shih has served as an advisory board member from AstraZeneca, Roche, Boehringer Ingelheim, Eli Lilly, Pfizer, Novartis, Merck Sharp & Dohme, Chugai Pharma, Ono Pharmaceutical, Takeda, CStone Pharmaceuticals, Janssen, and Bristol-Myers Squibb and received speaking honoraria from AstraZeneca, Roche, Boehringer Ingelheim, Eli

Lilly, Pfizer, Novartis, Merck Sharp & Dohme, Chugai Pharma, Ono Pharmaceutical, and Bristol-Myers Squibb, as well as grant from Roche. SG Wu has received speaking honoraria from Roche, AstraZeneca, and Pfizer. YTL has received speaking honoraria from ACT Genomics, Amgen, AstraZeneca, Boehringer Ingelhleim, Bristol-Myers Squibb, Chugai Pharmaceutical, Daiichi Sankyo, Eli Lilly, Illumina, Janssen, Lotus, Merck, Merck Sharp & Dohme, Novartis, Pfizer, Roche, Sanofi and Takeda. James Chih-Hsin Yang served as an advisory board member from Boehringer Ingelheim, Novartis, AstraZeneca, Clovis Oncology, Lilly (Inst), MSD Oncology, Celgene, Bayer, Pfizer, Ono Pharmaceutical, Bristol Myers Squibb, Boehringer Ingelheim (Inst), Yuhan, Hansoh, Blueprint Medicines, Daiichi Sankyo, G1 Therapeutics, AbbVie, Takeda, Amgen, Incyte, GlaxoSmithKline (Inst), Amgen (Inst), Takeda (Inst), AstraZeneca (Inst), Novartis (Inst), MSD Oncology (Inst), Janssen Oncology (Inst), Merck KGaA (Inst), Daiichi Sankyo/Astra Zeneca (Inst), Puma Biotechnology (Inst), Gilead Sciences (Inst), Pfizer (Inst), Taiho Pharmaceutical (Inst), Bayer (Inst), Roche/Genentech (Inst), Sanofi (Inst) and received speaking honoraria from Boehringer Ingelheim, Roche, MSD, AstraZeneca, Novartis, Bristol Myers Squibb, Ono Pharmaceutical, Takeda, Lilly, Pfizer, Amgen (Inst), AstraZeneca/MedImmune (Inst), Boehringer Ingelheim (Inst), Dizal Pharma (Inst), Taiho Pharmaceutical (Inst), Pfizer (Inst), Takeda (Inst), Roche/Genentech (Inst), Daiichi Sankyo/Astra Zeneca (Inst), MSD Oncology (Inst), BeiGene (Inst), Gilead Sciences (Inst), Sanofi/Regeneron (Inst).The remaining authors declare no conflict of interest.

Acknowledgments

We thank the staff of the Third Core Lab, Department of Medical Research, National Taiwan University Hospital for technical support during the study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2025.115224.

References

[1] Aggarwal C, Thompson JC, Black TA, Katz SI, Fan R, Yee SS, et al. Clinical implications of plasma-based genotyping with the delivery of personalized therapy in metastatic non–small cell lung cancer. JAMA Oncol 2019;5(2):173–80.
[2] Cui W, Milner-Watts C, O'Sullivan H, Lyons H, Minchom A, Bhosle J, et al. Up-front cell-free DNA next generation sequencing improves target identification in UK first line advanced non-small cell lung cancer (NSCLC) patients. Eur J Cancer 2022;171:44–54.
[3] Wang H-Y, Ho C-C, Lin Y-T, Liao W-Y, Chen C-Y, Shih J-Y, et al. Comprehensive genomic analysis of patients with non–small-cell lung cancer using blood-based circulating tumor DNA assay: findings from the BFAST database of a single center in Taiwan. JCO Precis Oncol 2024;8:e2300314.
[4] Yang C-Y, Shih J-Y, Liao W-Y, Ho C-C, Hsu C-L, Tsai T-H, et al. Upfront liquid next-generation sequencing in treatment-naïve advanced non-small cell lung cancer patients: a prospective randomised study in the Taiwanese health system. Eur J Cancer 2023:193.
[5] Pascual J, Attard G, Bidard FC, Curigliano G, De Mattos-Arruda L, Diehn M, et al. ESMO recommendations on the use of circulating tumour DNA assays for patients with cancer: a report from the ESMO Precision Medicine Working Group. Ann Oncol 2022;33(8):750–68.
[6] Rolfo C, Mack P, Scagliotti GV, Aggarwal C, Arcila ME, Barlesi F, et al. Liquid biopsy for advanced NSCLC: a consensus statement from the International Association for the Study of Lung Cancer. J Thorac Oncol 2021;16(10):1647–62.
[7] Fernandes MGO, Cruz-Martins N, Machado JC, Costa JL, Hespanhol V. The value of cell-free circulating tumour DNA profiling in advanced non-small cell lung cancer (NSCLC) management. Cancer Cell Int 2021;21(1):675.
[8] Tong L, Ding N, Tong X, Li J, Zhang Y, Wang X, et al. Tumor-derived DNA from pleural effusion supernatant as a promising alternative to tumor tissue in genomic profiling of advanced lung cancer. Theranostics 2019;9(19):5532–41.
[9] Hummelink K, Muller M, Linders TC, van der Noort V, Nederlof PM, Baas P, et al. Cell-free DNA in the supernatant of pleural effusion can be used to detect driver and resistance mutations, and can guide tyrosine kinase inhibitor treatment decisions. ERJ Open Res 2019;5(1). 00016-2019.
[10] Lee KWC, Li MSC, Gai W, Lau YM, Chan AKC, Chan OSH, et al. Testing for EGFR variants in pleural and pericardial effusion cell-free DNA in patients with non-small cell lung cancer. JAMA Oncol 2023;9(2):261–5.
[11] Mahmood K, Jampani P, Clarke JM, Wolf S, Wang X, Wahidi MM, et al. High yield of pleural cell-free DNA for diagnosis of oncogenic mutations in lung adenocarcinoma. Chest 2023;164(1):252–61.
[12] Kolenda T, Guglas K, Baranowski D, Sobocińska J, Kopczyńska M, Teresiak A, et al. cfRNAs as biomarkers in oncology - still experimental or applied tool for personalized medicine already? Rep Pr Oncol Radio 2020;25(5):783–92.
[13] Tsai TH, Su KY, Wu SG, Chang YL, Luo SC, Jan IS, et al. RNA is favourable for analysing EGFR mutations in malignant pleural effusion of lung cancer. Eur Respir J 2012;39(3):677–84.
[14] Wu SG, Liu YN, Yu CJ, Yang JC, Shih JY. Driver mutations of young lung adenocarcinoma patients with malignant pleural effusion. Genes Chromosomes Cancer 2018;57(10):513–21.
[15] Chang SC, Wei YF, Chen CY, Lai YC, Hu PW, Hung JC, et al. Profiling cell-free DNA from malignant pleural effusion for oncogenic driver mutations in patients with treatment-naive stage IV adenocarcinoma: a multicenter prospective study. Mol Diagn Ther 2024.
[16] Mateo J, Chakravarty D, Dienstmann R, Jezdic S, Gonzalez-Perez A, Lopez-Bigas N, et al. A framework to rank genomic alterations as targets for cancer precision medicine: the ESMO scale for clinical actionability of molecular targets (ESCAT). Ann Oncol 2018;29(9):1895–902.
[17] Cho BC, Loong HHF, Tsai C-M, Teo MLP, Kim HR, Lim SM, et al. Genomic landscape of non-small cell lung cancer (NSCLC) in East Asia using circulating tumor DNA (ctDNA) in clinical practice. Curr Oncol 2022;29(3):2154–64.
[18] Meng H, Guo X, Sun D, Liang Y, Lang J, Han Y, et al. Genomic profiling of driver gene mutations in Chinese patients with non-small cell lung cancer. Front Genet 2019:10.
[19] Si X, Pan R, Ma S, Li L, Liang L, Zhang P, et al. Genomic characteristics of driver genes in Chinese patients with non-small cell lung cancer. Thorac Cancer 2021;12(3):357–63.
[20] Lee VHF, Mok TSK, Goto Y, Hsue VCC, Yang L, Jiang Y, et al. Differences between the east and the west in managing advanced-stage non-small cell lung cancer. Clin Oncol 2020;32(1):e1–9.
[21] Kuo CHS, Yoh K, Yang CT, Wang CC, Yen TC, Lin KJ, et al. 519P - Initial results of lung cancer genomic screening project for individualized medicine in Asia: LC-SCRUM-Asia. Ann Oncol 2019;30:ix174–5.
[22] DeMaio A, Clarke JM, Dash R, Sebastian S, Wahidi MM, Shofer SL, et al. Yield of malignant pleural effusion for detection of oncogenic driver mutations in lung adenocarcinoma. J Bronchol Inter Pulmonol 2019;26(2):96–101.
[23] Carter J, Miller JA, Feller-Kopman D, Ettinger D, Sidransky D, Maleki Z. Molecular profiling of malignant pleural effusion in metastatic non-small-cell lung carcinoma. The effect of preanalytical factors. Ann Am Thorac Soc 2017;14(7):1169–76.
[24] Akamatsu H, Koh Y, Kenmotsu H, Naito T, Serizawa M, Kimura M, et al. Multiplexed molecular profiling of lung cancer using pleural effusion. J Thorac Oncol 2014;9(7):1048–52.
[25] Wu S-G, Gow C-H, Yu C-J, Chang Y-L, Yang C-H, Hsu Y-C, et al. Frequent epidermal growth factor receptor gene mutations in malignant pleural effusion of lung adenocarcinoma. Eur Respir J 2008;32(4):924–30.
[26] Smits AJ, Kummer JA, Hinrichs JW, Herder GJ, Scheidel-Jacobse KC, Jiwa NM, et al. EGFR and KRAS mutations in lung carcinomas in the Dutch population: increased EGFR mutation frequency in malignant pleural effusion of lung adenocarcinoma. Cell Oncol (Dordr) 2012;35(3):189–96.
[27] Zou J, Bella AE, Chen Z, Han X, Su C, Lei Y, et al. Frequency of EGFR mutations in lung adenocarcinoma with malignant pleural effusion: implication of cancer biological behaviour regulated by EGFR mutation. J Int Med Res 2014;42(5):1110–7.
[28] Yang H, Liu Z, Wang H, Chen L, Wang J, Wen W, et al. Relationship between EGFR, ALK gene mutation and imaging and pathological features in invasive lung adenocarcinoma. Zhongguo Fei Ai Za Zhi 2022;25(3):147–55.
[29] Zeng H, Zhang Y, Tan S, Huang Q, Pu X, Tian P, et al. Efficacy of bevacizumab through an indwelling pleural catheter in non-small cell lung cancer patients with symptomatic malignant pleural effusion. BMC Pulm Med 2024;24(1):89.
[30] Tsai MF, Chang TH, Wu SG, Yang HY, Hsu YC, Yang PC, et al. EGFR-L858R mutant enhances lung adenocarcinoma cell invasive ability and promotes malignant pleural effusion formation through activation of the CXCL12-CXCR4 pathway. Sci Rep 2015;5:13574.
[31] Schwaederlé MC, Patel SP, Husain H, Ikeda M, Lanman RB, Banks KC, et al. Utility of genomic assessment of blood-derived circulating tumor DNA (ctDNA) in patients with advanced lung adenocarcinoma. Clin Cancer Res 2017;23(17):5101–11.
[32] Luo J, Shen L, Zheng D. Diagnostic value of circulating free DNA for the detection of EGFR mutation status in NSCLC: a systematic review and meta-analysis. Sci Rep 2014;4(1):6269.
[33] Hu Y, Ulrich BC, Supplee J, Kuang Y, Lizotte PH, Feeney NB, et al. False-positive plasma genotyping due to clonal hematopoiesis. Clin Cancer Res 2018;24(18):4437–43.
[34] Ptashkin RN, Mandelker DL, Coombs CC, Bolton K, Yelskaya Z, Hyman DM, et al. Prevalence of clonal hematopoiesis mutations in tumor-only clinical genomic profiling of solid tumors. JAMA Oncol 2018;4(11):1589–93.
[35] Yaung SJ, Fuhlbrück F, Peterson M, Zou W, Palma JF, Patil NS, et al. Clonal hematopoiesis in late-stage non-small-cell lung cancer and its impact on targeted panel next-generation sequencing. JCO Precis Oncol 2020;4:1271–9.
[36] Yang X, Wang Y, Li Y, Liu D, Shao M, Liu L, et al. Clonal hematopoiesis in lung cancer and its impact on liquid biopsy based on targeted deep sequencing. J Clin Oncol 2022;40(16_):e20532-e.