Thoracic Cancer

Open Access

Thoracic Cancer ISSN 1759-7706

ORIGINAL ARTICLE

Malignant pleural effusion supernatant is an alternative liquid biopsy specimen for comprehensive mutational profiling

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Keywords
Cell-free DNA; liquid biopsy; lung cancer; malignant pleural effusion; MPE supernatant.

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Received: 6 December 2018;
Accepted: 16 January 2019.

doi: 10.1111/1759-7714.13006

Thoracic Cancer **10** (2019) 823–831

Abstract

Background: The clinical utility of malignant pleural effusion (MPE) to detect mutation has been well documented; however, routine practice of the use of MPE involves collection of the cell pellet to detect mutation, and limited studies have interrogated the MPE supernatant as an alternative source of tumor-derived DNA for mutation profiling. In this study, we investigated the potential of MPE supernatant as a liquid biopsy specimen by comparing its mutation profile with that of matched MPE cell pellets, tissue, and plasma samples.

Methods: Sequencing data from 17 patients with matched lung tissue, plasma, and MPE samples were retrospectively analyzed. Capture-based targeted sequencing was performed on matched plasma and MPE supernatant samples obtained from 154 patients with advanced lung adenocarcinoma.

Results: MPE supernatants had significantly higher median maximum allelic fractions (maxAFs) than their corresponding cell pellets ($P = 0.008$) and plasma samples ($P = 0.036$), and a comparable maxAF value to that of tissue samples ($P = 0.675$). Comparison of MPE supernatant and matched plasma samples from the larger cohort ($n = 154$) revealed a comparable mutation detection rate; however, MPE supernatant had a significantly higher median maxAF than plasma (20.3% vs. 1.13%; $P < 0.001$). Furthermore, the concordance rates between MPE supernatant and plasma for single-nucleotide and copy number variations were 56% and 18%, respectively, suggesting that MPE supernatant reveals a more comprehensive mutation spectrum, particularly for copy number variations.

Conclusion: Overall, our study shows that MPE supernatant is an optimal alternative source of tumor-derived DNA for comprehensive mutation profiling.

Introduction

In the past decade, the development of therapies directed at specific genomic alterations, including *EGFR*, *ALK*, and *ROS1*, has revolutionized the treatment of non-small cell lung cancer (NSCLC). Thus, molecular testing is essential to select patient subsets that can benefit from targeted therapy.[1] The gold standard specimen for molecular testing is tissue biopsy; however, obtaining tissue samples requires an invasive procedure, and sampling is also biased because of temporal and spatial heterogeneity. In contrast, blood-based liquid biopsies, consisting of circulating tumor DNA (ctDNA) released from apoptotic or necrotic tumor cells

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have the potential to comprehensively reflect tumor genomic profiles.[2,3] Because of the convenience of sample collection, blood has now been integrated as a routine specimen for mutation profiling, particularly for monitoring treatment responses and assessment of mechanisms of drug resistance; however, challenges remain because of the extremely limited amount of tumor DNA circulating in the plasma. Hence, other malignant biological fluids that likely contain tumor-derived DNA, including malignant pleural effusion, cerebrospinal fluid, and ascites, are being explored as potential alternative liquid biopsy specimens.

Malignant pleural effusion (MPE) is excess fluid that accumulates between the lung and the chest wall, and results from invasion of the pleural space by malignant cells that disrupt the drainage of pleural fluid.[4,5] The development of MPE is a common complication in advanced malignancies, including lung and breast cancer, lymphoma, and cancers with unknown primaries. Lung cancer is the most common cause of MPE, with approximately 15% of patients presenting with MPE at initial diagnosis and the condition ultimately affecting 40% of patients during the course of the disease.[6–8] Development of MPE is associated with poor prognosis, with a median survival of 5.5 months.[6] To relieve the symptoms associated with MPE, including dyspnea on exertion, shortness of breath, cough, and chest pain, excessive pleural effusion is drained by ultrasound-guided thoracentesis.[5] Because MPE contains malignant cells and its collection involves a less invasive procedure than tissue biopsy, its suitability as a liquid biopsy specimen has been explored. The first case report that used MPE as a liquid biopsy specimen for the detection of an *EGFR* mutation was in 2005,[9] and MPE is now used as an alternative specimen for molecular diagnosis. Genomic alterations, such as *EGFR* mutations, including exon 19 deletion, L858R, and T790M, can be detected in MPE samples by PCR[10–15] or next-generation sequencing (NGS)[16–20] however, a majority of studies have used the malignant cells sedimented from MPE (termed cell blocks or cell pellets), concluding that such samples generate results with high concordance compared to tissue samples in terms of sensitivity and specificity. In contrast, only a limited number of studies have evaluated the use of the remaining MPE supernatant for amplification refractory mutation system PCR, real-time PCR and/or Sanger sequencing[11,13–15] and even fewer studies have investigated the suitability of MPE supernatants as specimens for NGS-based mutation profiling.

In the present study, we performed capture-based targeted sequencing of matched tissue, plasma, and MPE samples from 17 patients to explore the feasibility of using MPE supernatant as an alternative liquid biopsy sample. We further validated our initial results in a larger cohort of 154 patients with matched plasma and MPE supernatant samples to demonstrate the suitability of MPE supernatants as an alternative source of tumor-derived DNA for comprehensive mutation profiling.

Methods

Patients

Matched tissue, plasma, and MPE samples from patients with advanced lung adenocarcinoma ($n = 17$) were sequenced. Additionally, 154 patients with advanced lung adenocarcinoma and MPE diagnosed at The First Affiliated Hospital of Guangzhou Medical University, The General Hospital of Guangzhou Military Command, or The Second Affiliated Hospital of Hainan Medical University between December 2016 and May 2018, were enrolled in the study. MPE and blood samples were collected from each patient at either diagnosis or disease progression. MPE samples were collected by ultrasound-guided thoracentesis, according to the guidelines of the British Thoracic Society.[21,22] Patients with no mutations detected via a sequencing panel were excluded from further analysis. The Ethical Committees of the three participating hospitals approved this study. All patients provided written informed consent for the use of their tissue, MPE, and/or plasma samples.

Preparation of malignant pleural effusion (MPE), tissue, and plasma samples

MPE cell pellets were separated from MPE supernatants by centrifugation at 1500 × g at 4°C for 10 minutes. MPE cell pellets were further processed into formalin-fixed paraffin-embedded (FFPE) cellblocks, while supernatant fractions were further centrifuged (16 000 × g, 4°C, 10 minutes) to remove cell debris, and 10 mL aliquots were stored at −80°C until DNA extraction. Lung cancer tissue samples were obtained by biopsy and processed into FFPE cellblocks. Plasma was separated from blood samples collected in ethylene-diamine-tetraacetic acid-treated tubes by centrifugation (1500 × g, 4°C, 10 minutes). Plasma fractions were transferred into fresh tubes, centrifuged (16 000 × g, 4°C, 10 minutes) to remove cell debris, aliquoted into fresh tubes, and stored at −80°C until DNA extraction.

DNA extraction

Genomic DNA was purified from FFPE tumor and MPE cell pellet samples using a QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany). Cell-free DNA (cfDNA) was isolated from MPE supernatant and plasma samples using a QIAamp Circulating Nucleic Acid kit, according to the manufacturer's standard protocol (Qiagen).[23] Quantification of DNA obtained from tissue, plasma, MPE supernatant, and cell pellet samples was performed

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using the Qubit dsDNA assay (Life Technologies, Carlsbad, CA, USA).

Next generation sequencing library preparation and sequencing

NGS libraries were prepared according to a previous publication.[24] A minimum of 50 ng of DNA was required for NGS library construction. Tissue DNA was sheared using an M220 Focused-Ultrasonicator (Covaris, Woburn, MA, USA). Fragments of 200–400 bp from cfDNA and sheared tissue/MPE cell pellet DNA were selected using beads (Agencourt AMPure XP Kit, Beckman Coulter, Brea, CA, USA), followed by hybridization with capture bait probes, hybrid selection with magnetic beads, and PCR amplification. The quality and size of the fragments were assessed using a Qubit 2.0 Fluorimeter with a dsDNA high-sensitivity assay kit (Life Technologies). Paired samples were sequenced using capture-based targeted sequencing panels (Burning Rock Biotech, Guangzhou, China) on Nextseq500 (Illumina, Inc., Madison, WI, USA) with paired-end reads. Sequencing data were mapped to the reference human genome (hg19) and genomic mutations were analyzed using Burrows–Wheeler aligner.

Statistical analysis

Statistical analyses were performed using Fisher's exact and Student's *t* tests in R software (R Foundation for Statistical Computing, Vienna, Austria); $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

Matched primary lung tissue, plasma, and MPE samples from 17 patients with stage IV lung adenocarcinoma (median age 63.4 years), were retrospectively sequenced and analyzed to explore the feasibility of using MPE supernatant as liquid biopsy. In this cohort, five patients had stage IV M1a disease and the remaining patients had stage IV M1b disease. Eight patients were female and nine male. In addition, 154 patients with stage IV lung adenocarcinoma (48 stage IV M1a and 106 stage IV M1b) who developed MPE, including 75 men and 79 women, were enrolled to validate the potential of MPE supernatant as a liquid biopsy. The median age of the validation cohort was 62.2 (range: 27–91) years, 13 patients were treatment naïve, and the remaining 141 had previously been treated at the time of sample collection. Aired MPE and plasma samples were collected from each patient.

MPE supernatant is superior to MPE cell pellet to detect mutation

First, we investigated the optimal MPE fraction for use to detect mutation. Using a panel consisting of eight classic NSCLC driver genes, including *EGFR*, *KRAS*, *MET*, *BRAF*, *ERBB2*, *ALK*, *ROS1*, and *RET*, we compared the detection rate and maximum allelic fraction (maxAF) between MPE supernatant and MPE cell pellet samples from 17 patients. The detection rate was defined as any mutation detected from the panel used, while maxAF was defined as the highest mutation allelic fraction among all mutations detected from the panel used. Genomic mutations were detected in 94.1% (16/17) of MPE supernatant and 70.6% (12/17) of MPE cell pellet samples ($P = 0.175$) (Fig 1a). Although there was no statistically significant difference in the detection rate between MPE supernatants and cell pellets, the median maxAF was significantly higher in MPE supernatants (median 26.4% vs. 2%; $P = 0.008$) (Fig 1b).

We then compared the mutation profiles derived from MPE supernatant and cell pellet samples. Collectively, 32 and 16 variants were identified from MPE supernatants and cell pellets, respectively (MPE supernatant, Fig 1c; comparison of MPE supernatant and cell pellet, Fig 1d). Among these mutations, 15 were detected in both MPE supernatant and cell pellet samples, while 17 were detected only in the MPE supernatant and 1 only in the MPE cell pellet (Fig 1d). *EGFR* mutations, the most frequently occurring alteration, were detected in 64.7% of MPE supernatant cfDNA and in 52.9% of MPE cell pellets. Overall, our results show that MPE supernatant samples are superior to MPE cell pellets for the identification of genomic alterations.

MPE supernatant is comparable to tumor tissue to detect somatic mutations

Because tumor tissue is often regarded as the best source material for mutation profiling, we compared the performance of matched MPE supernatant, lung tissue, and plasma samples for mutation detection. The detection rates for MPE supernatant, lung tissue, and plasma were 100%, 100%, and 82.3%, respectively (Fig 2a). There were no significant differences in the detection rates among the three sample types. Moreover, the median maxAF values for MPE supernatant, lung tissue, and plasma samples were 12.9%, 26.4%, and 5.0%, respectively (Fig 2b–c). The median maxAF values of MPE supernatant and tissue samples were significantly higher than that of plasma samples (MPE supernatant vs. plasma, $P = 0.036$; tissue vs. plasma, $P = 0.003$). The median maxAF values for MPE supernatant and lung tissue samples were comparable ($P = 0.675$) (Fig 2c).

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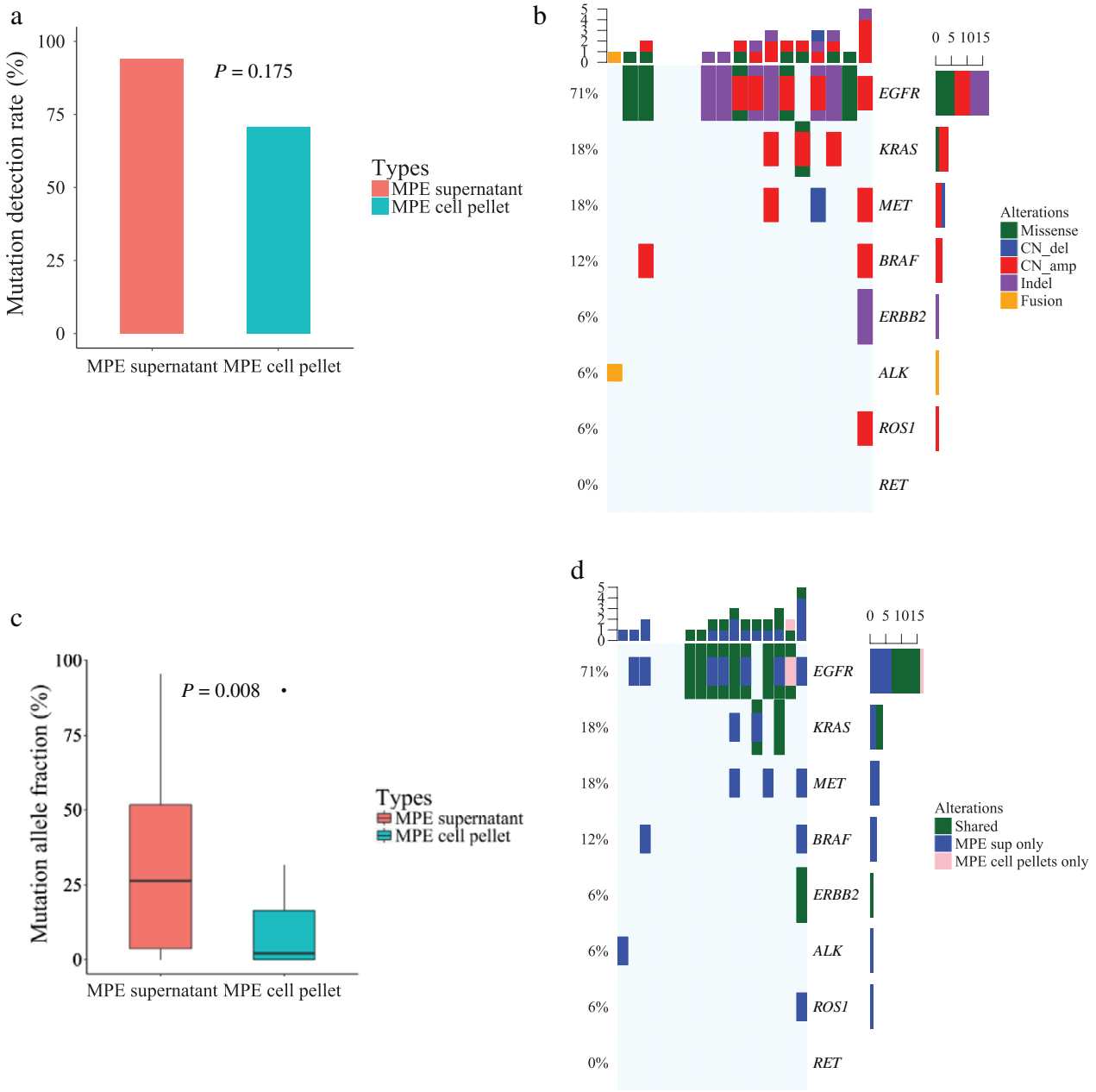

Figure 1 Malignant pleural effusion (MPE) supernatant (sup) is superior to cell pellets for detecting mutation. (**a**) Comparison of mutation detection rates in MPE sup and cell pellets. (**b**) The maximum allele frequencies (maxAF) were plotted. The maxAF were significantly higher in MPE sup than in cell pellets (P = 0.008). (**c**) Genomic profile of MPE sup. Each column represents one patient. Different types of mutations are represented by different colors. Each row represents a gene. The top bar denotes the number of mutations detected in each patient; the side bar represents the number of patients with mutation in a certain gene. (**d**) Comparison of genomic profiles derived from MPE sup and cell pellets. Green represents mutations detected in both MPE sup and cell pellets, pink represents mutations detected only in MPE cell pellets, and blue represents mutations detected only in MPE sup.

The mutation profiles derived from matched MPE supernatant, lung tissue, and plasma samples from each of the 14 patients were then compared; no mutation was detected in the MPE supernatant or cell pellet samples in the three remaining patients (Fig 1c,d) (matched tissue and plasma were excluded from this analysis). Using mutations detected from lung tissue as a reference, MPE supernatant achieved an 83% by-variant concordance rate, defined as the same variant detected in both types of sample (Fig 2d). A total of 36 and 37 single-nucleotide variations (SNVs)

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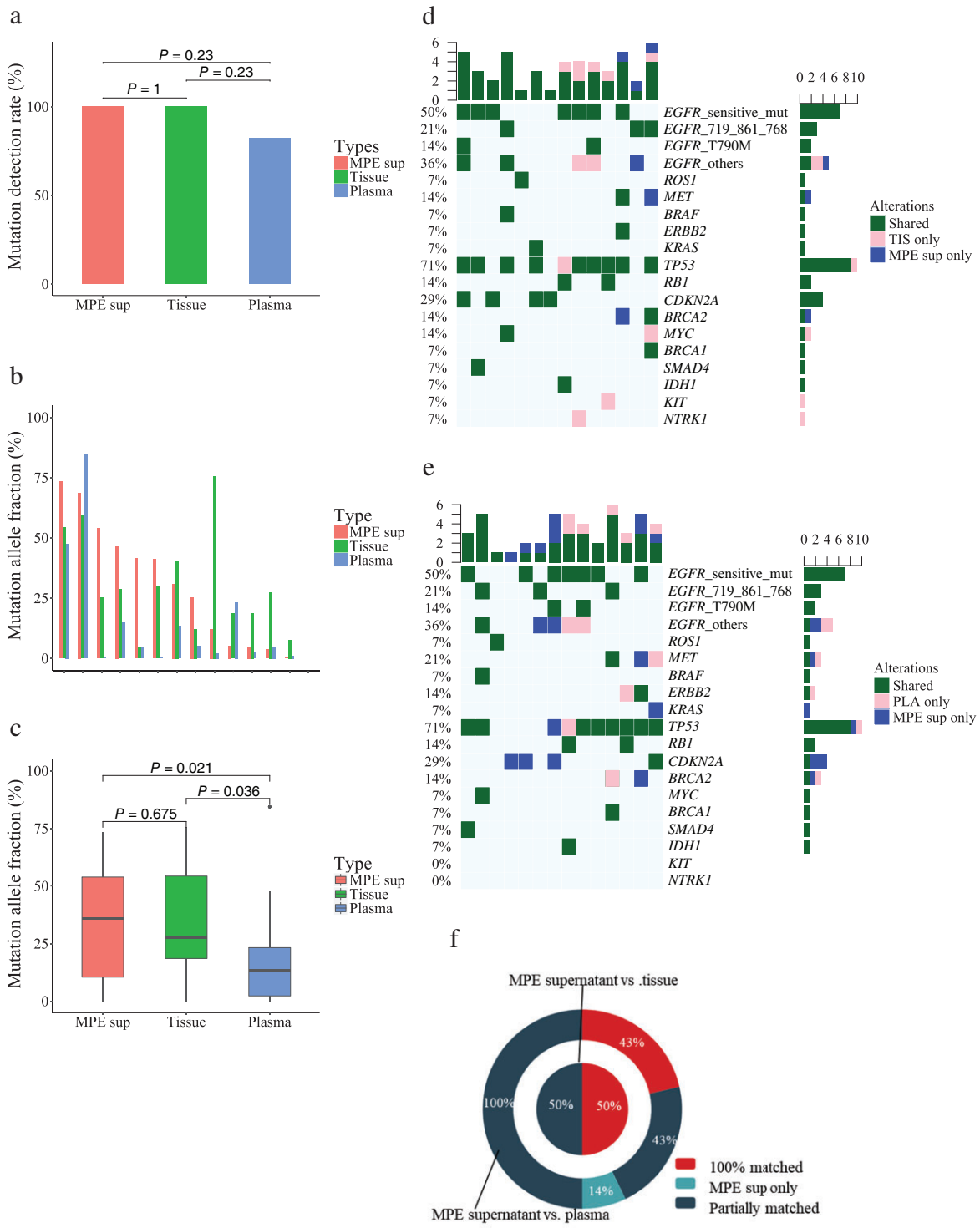

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were detected in MPE supernatant and tissue samples, respectively. Among them, 35 SNVs were detected in both MPE supernatant and tissue samples, representing a 92% concordance rate. In addition, a total of 10 and 12 copy number variations (CNVs) were detected in MPE supernatant and tissue samples, respectively. Among them, eight CNVs were detected in both MPE supernatant and tissue samples, achieving a concordance rate of 57% (Fig 2d).

We then compared the mutation spectrum derived from matched MPE supernatant and plasma samples. A total of 48 and 52 variants (SNVs and CNVs) were detected in MPE supernatant and plasma samples, respectively. Among these, 38 were detected in both MPE supernatant and plasma samples, achieving a concordance rate of 61% (Fig 2e). We also investigated the concordance of the mutation distributions between MPE supernatant versus tissue and MPE supernatant versus plasma samples. Half (7/14) of the patients had the exact same mutation profile derived from both tissue and MPE supernatant samples (red half, inner circle, Fig 2f). Moreover, among these seven patients with identical mutation profiles in tissue and MPE supernatants, only three (43%, 3/7) exhibited identical mutation profiles in plasma. A further three patients exhibited partial concordance between MPE supernatant and plasma samples, while no mutations were detected in the plasma from one patient, with variants detected only in MPE supernatant (outer circle, Fig 2f). Consistent with these data, among the seven patients with partially matched mutation profiles between tissue and MPE supernatant samples, their MPE supernatant and plasma mutation profiles also partially matched (blue half inner and outer circle, Fig 2f). These data show that MPE supernatant is comparable to tissue samples for use in the identification of genomic alterations.

MPE supernatant is an alternative sample source to detect mutation

Our data revealed that MPE supernatant is superior to its corresponding cell pellet, marginally better than plasma, and comparable to tissue as a sample to detect mutation. We further evaluated the suitability of MPE supernatants in a large cohort of 154 patients with advanced lung adenocarcinoma using a panel comprising 168 lung cancer-related genes.

Detection rates were comparable between MPE supernatant and plasma samples, with 87% (134/154) and 83.1% (128/154) positive mutation detection rates, respectively ($P = 0.424$) (Fig 3a). No mutations were detected in either MPE supernatant or plasma samples from seven patients. Despite the comparable mutation detection rates between matched MPE supernatant and plasma samples, the median maxAF value was significantly higher for MPE supernatant than plasma (20.3% vs. 1.13%; $P < 0.001$) (Fig 3b). Furthermore, the mutation profile derived from MPE supernatants revealed that *EGFR* and *TP53* were the two most frequently mutated genes, with variants detected in 66% and 58% of patients, respectively (Fig 3c). Other frequently mutated genes included *MET* (11%), *ALK* (10%), *KRAS* (9%), *PIK3CA* (8%), and *CDKN2A* (8%). Interestingly, two patients harbored concurrent *ALK* rearrangements and *EGFR* exon 19 deletions (Fig 3c). Next, we compared the mutation profiles derived from matched MPE supernatant and plasma samples. In total, 584 and 451 variants were detected in MPE supernatants and plasma, respectively. Among them, 331 variants were detected in both MPE supernatants and plasma, 253 variants only in MPE supernatant samples, and 120 only in plasma samples, representing a 47% concordance rate (Fig 3d; Table 1). Among the 253 mutations detected only in MPE supernatants, 30% (75/253) were driver mutations. Meanwhile, among the 120 mutations only detected in plasma samples, 26% (31/120) were driver mutations, with a 58% concordance rate between the two sample types for driver gene mutation detection. Furthermore, analysis of the concordance rates, based on mutation types, between MPE supernatant and plasma samples revealed that the concordance for SNVs (56%) was higher than for CNVs (18%) (Table 1). Because the detection of CNVs is dependent on the quantity and quality of DNA present in a given sample, these data suggest

FIGURE 2 Malignant pleural effusion (MPE) supernatant (sup) is comparable to tissue samples for detecting mutation. (**a**) Comparison of mutation detection rate in matched MPE sup, tissue, and plasma samples. (**b**) The maximum allele frequencies (maxAF) detected in matched MPE sup, tissue, and plasma samples of each patient was plotted. Comparison of (**c**) maxAF among matched MPE sup, tissue, and plasma samples and (**d**) mutation profiles derived from matched MPE sup and tissue samples. Mutations detected in both MPE sup and tissue samples are shown in green, mutations found in tissue (TIS) and MPE sup only are shown in pink and blue, respectively. *EGFR* sensitive mutation (mut) includes sensitizing mutations L858R and exon 19 deletion. (**e**) Comparison of mutation profiles derived from matched MPE supernatant and plasma samples. Common mutations are shown in green, mutations found in plasma (PLA) and MPE sup only are shown in pink and blue, respectively. (**f**) Correlation among mutations derived from matched tissue, MPE sup, and plasma samples. The three patients with no mutation detected in their plasma samples were excluded. The inner circle illustrates the association between MPE sup and tissue. Half (7/14) of the patients had the exact mutation profile detected in both MPE sup and tissue. The outer ring represents the association between MPE sup and plasma samples. Red represents a 100% identical mutation profile between MPE sup and either tissue or plasma samples, dark blue represents a partial match in mutation profile between MPE sup and either tissue or plasma samples, and blue green represents detection of mutation only in the MPE sup sample. *EGFR* sensitive mutation (mut) includes sensitizing mutations L858R and exon 19 deletion.

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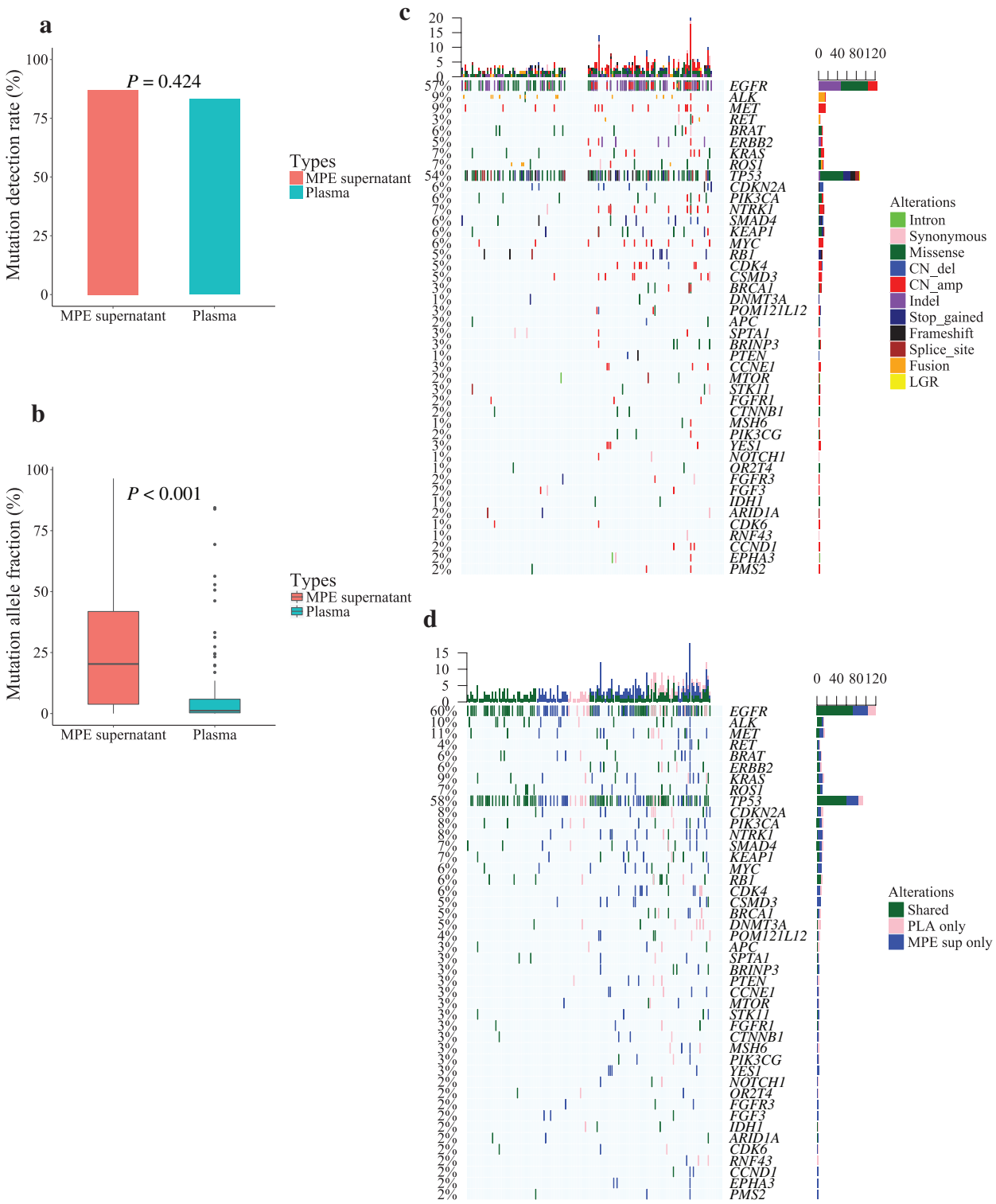

Figure 3 A comparison of mutation profiles derived from matched malignant pleural effusion (MPE) supernatants (sup) and plasma samples in 154 advanced non-small cell lung cancer patients. (**a**) Mutation detection rates in MPE sup and plasma. (**b**) The maximum allele frequencies (maxAF) of MPE sup and plasma. (**c**) Mutations derived from MPE sup of each of the 154 patients. Different types of mutations are represented by different colors. Each row represents a gene. The top bar denotes the number of mutations detected in each patient; the side bar represents the number of patients with mutation in a certain gene. (**d**) Comparison of mutations revealed from MPE sup and plasma samples. Mutations detected in both MPE sup and plasma samples are shown in green, mutations found in plasma (PLA) and MPE sup only are shown in pink and blue, respectively.

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Table 1 Comparison of number of mutations detected in matched MPE supernatant and plasma samples in 154 advanced NSCLC patients

Genes/Mutation types	Total # of mutations in MPE supernatant	Total # of mutations in plasma	# of mutations shared	# of mutations detected only in MPE supernatant	# of mutations detected only in plasma	Concordance rate (%)
All genes	583	451	331	253	120	47
SNV	441	393	301	140	92	56
CNV	143	48	30	113	28	18
Driver Genes	221	177	146	75	31	58

CNV, copy number variation; MPE, malignant pleural effusion; SNV, single-nucleotide variation.

that MPE supernatant samples harbor more tumor-derived DNA than plasma samples. Our data show that MPE supernatant is a potential alternative liquid biopsy specimen as it exhibits a higher median maxAF value and a better CNV detection rate than plasma.

Discussion

The gold standard sample for molecular genotyping remains tissue. Blood-based liquid biopsies are now integrated into clinical practice to complement tumor samples, or even as a substitute, particularly when tumors are unavailable and in situations when longitudinal sample collection is required, such as for treatment and disease monitoring. Nevertheless, detection of ctDNA derived from plasma remains challenging because of the limited amount of ctDNA in the circulation.[25] Hence, alternative sources of ct DNA, such as malignant fluids, including pleural effusion, cerebrospinal fluid (CSF), and ascites, are being actively explored.[24,26] These malignant fluids could provide a superior representation of the real-time tumor status compared to archived tumor samples obtained during initial diagnostic biopsy or surgery, and are a potential alternative source of tumor-derived DNA for mutation profiling.

MPE is a common complication of advanced lung cancer, affecting approximately 40% of patients during the course of the disease.[4] Because excessive MPE needs to be drained regularly to maintain patient breathing, it is a convenient alternative liquid biopsy specimen. Over the years, multiple studies have shown that MPE can be used as a liquid biopsy media for mutation detection by PCR[10–15] and NGS[16–20] however, most of these studies used MPE cell pellets, with only a few evaluating the suitability of MPE supernatant to detect mutation, often from a single gene perspective. Liu *et al.* reported that *EGFR* mutations could be detected in 46.5% of tumor samples, 48.1% of MPE cell pellets, 33.3% of MPE supernatants, and 31.4% of plasma samples, using the amplification refractory mutation system PCR method. Compared to tumor samples, the sensitivity and specificity were 81.8% and 80% for MPE cell pellets and 63.6% and 100% for MPE supernatants, respectively. In addition, Lin *et al.* showed that *EGFR* mutations could be detected in 92.3% and 69.2% of MPE supernatant and cell pellet samples, respectively, using high resolution melting and Sanger sequencing analysis, and concluded that MPE supernatant is a superior sample to its corresponding cell pellet.[14] In the present study, consistent with previously reported findings, our results also reveal that MPE supernatant is a superior sample to MPE cell pellet, based on both mutation detection rate and median maxAF value. Moreover, our findings are also in agreement with a recent report of the superiority of cell-free DNA derived from CSF, compared to its corresponding cell pellet.[24,27] It has been reported that the fraction of tumor-derived DNA is significantly higher in the supernatant than its corresponding cell pellet in CSF.[27] This may also be the case for MPE samples.

Overall, using capture-based ultra-deep sequencing to interrogate the mutation profiles of matched tissue, plasma, MPE cell pellet, and supernatant samples, the present study reveals that MPE supernatant is superior to MPE cell pellet, comparable to tissue samples, and provides a more comprehensive mutation profile and higher median maxAF value than plasma samples. Thus, MPE supernatant represents an ideal alternative source of tumor-derived DNA for NGS-based mutation profiling, while MPE cell pellets can be processed into FFPE blocks for use in cytology assays.

Acknowledgments

The authors thank all of the patients who participated in this study and their families. We also thank the investigators, study coordinators, operation staff, and the project team who worked on this study.

Disclosure

No authors report any conflict of interest.

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