Gene Alterations in Paired Supernatants and Precipitates from Malignant Pleural Effusions of Non-Squamous Non-Small Cell Lung Cancer

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ABSTRACT

OBJECTIVE: This study investigated the feasibility of using malignant pleural effusion (MPE) supernatant and paired cell blocks (precipitate) for gene profiling in patients with non-small cell lung cancer (NSCLC) using next-generation sequencing (NGS) technique. METHODS: Stage IV non-squamous NSCLC patients with MPE were eligible in this prospective study and recruited from Zhejiang Cancer Hospital between May 2014 and October 2015. MPE supernatant and paired precipitate sample gene alterations were determined with NGS containing 14 cancer-related genes. Progression free survival (PFS) was evaluated using Kaplan–Meier method and compared using log-rank test. RESULTS: A total of 102 patients were enrolled in the present study. All pleural effusions were confirmed as malignant with cytological smears. A total of 77 paired MPE supernatant and precipitate samples were acquired from the 102 patients. The results revealed that there were no statistically significant differences in the detection rate and maximum allelic fraction between supernatant and precipitate samples ($P = 1.0$ and $P = .6$). Collectively, 172 and 158 genomic alterations with 112 shared mutations were identified in supernatant and precipitate samples, respectively. Comparable PFS was found in EGFR mutation patients according to the supernatant and precipitate sample results (14.0 vs.13.9 months, $P = .90$). CONCLUSIONS: These results demonstrated that MPE supernatants were comparable to precipitate samples for detection of genetic alterations. However, gene mutation heterogeneity was found between these two media types.

Introduction

Malignant pleural effusion (MPE) is a common complication of malignancies, with an estimated rate of 10–15% in advanced non-small cell lung cancer (NSCLC) upon initial diagnosis and a higher rate in later treatment period [1]. There are several types of cells in MPE, including mesothelial cells, lymphocytes, and, most importantly, cancer cells. The cancer cells are considered a diagnostic gold standard for MPE [2].

Molecular therapeutics targeting driver genes, such as EGFR, ALK, and ROS1, are an appealing strategy for the treatment of advanced NSCLC [3–5]. A prolonged survival time has been identified in the subpopulation harboring these genetic alterations compared to patients without such mutations [6,7]. In the clinical practice, the eight-gene test that is recommended for advanced NSCLC by the NCCN guidelines is not routinely performed due to inadequate samples for genetic testing. A national survey

in China has shown that the EGFR mutation detection rate is less than 30% in NSCLC patients [8]. Currently, noninvasive genetic testing is widely conducted, especially in patients without sufficient tumor tissue samples. Circulating free DNA (cfDNA) is a small, double-stranded, fragmented DNA found in plasma. It is emerging as a powerful tool for liquid biopsy for noninvasive genetic testing in cancer patients [9,10]. However, circulating tumor DNA (ctDNA) only represents a very small fraction of cfDNA, which hinders tumor mutation sensitivity in cfDNA [11,12]. Different from ctDNA in plasma, much more DNA is released by the cancer cells in MPE, indicating that MPE supernatants may be alternative samples to plasma ctDNA for liquid biopsy. Although MPE is a common complication in NSCLC, few studies have investigated the value of MPE for liquid biopsy using next-generation sequencing (NGS).

To address this issue, an observational study was conducted to investigate the genetic alterations in MPE supernatants and paired cell blocks

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(precipitate) from patients with non-squamous NSCLC and to further evaluate the efficacy of targeted therapy based on the gene expression profiling.

Materials and Methods

Patient and Sample Collection

Patients were prospectively enrolled from Zhejiang Cancer Hospital between May 2014 and October 2015. Eligible patients were aged at least 18-years-old and had cytologically or histologically confirmed, advanced, non-squamous NSCLC with pleural effusion. All of the pleural effusion samples were confirmed as malignant by cytological smears. Paired formalin-fixed paraffin-embedded (FFPE) blocks were obtained from the pathology department of the hospital. At the time of enrollment, all patients were not treated by targeted inhibitors. Patients with squamous cell NSCLC, small-cell lung cancer, or other metastatic malignancies of the lung were excluded. Tumor diagnosis was performed by institutional pathologists in accordance with the 2004 World Health Organization classification. The present study was approved by the Ethics Committee of Zhejiang Cancer hospital. Written informed consent was obtained from all participants.

Preparation of Cell Block Samples from MPE

Ten-milliliter fluid specimens were centrifuged at 3000 rpm for 5 min. Cell sediments were harvested, fixed with three times the volume of 10% neutral-buffered formalin for 1 h, wrapped in filter paper, and processed in an automatic tissue processor. The cell block samples were embedded in paraffin and sectioned at a thickness of 5 mm after standard tissue processing. Histological diagnoses were independently performed by two experienced pathologists.

DNA Extraction and Quantification

Ten-milliliter pleural effusion samples were first centrifuged at a low speed. The supernatants were harvested and then centrifuged at a high speed to remove any residual debris. Cell-free DNA (cfDNA) from the final supernatants was extracted using QIAmp Circulating Nucleic Acid Kit (Qiagen). Size distribution of cfDNA was analyzed using Bioanalyzer 2100 with a High Sensitivity DNA kit (Agilent Technologies).

FFPE tumor samples (cell block samples) were de-paraffinized with xylene, followed by genomic DNA extraction using QIAamp DNA FFPE Tissue Kit (Qiagen). Purified genomic DNA was qualified using NanoDrop 2000 to obtain the A260/280 and A260/A230 ratios (Thermo Fisher Scientific). All DNA samples prepared above were quantified by Qubit 3.0 using the dsDNA HS Assay Kit (Life Technologies) according to the manufacturer's instructions. The FFPE-derived genomic DNA was sheared into 350-bp fragments using Bioruptor (Diagenode) following manufacturer's protocol.

Library Preparation and Target Enrichment

Libraries were constructed using the KAPA Hyper Prep Kit (KAPA Biosystem) with an optimized manufacturer's protocol for different types of samples. In brief, an appropriate amount of cfDNA or fragmented FFPE-derived DNA underwent end-repair, 3′A-tailing, and indexed adapter ligation sequentially, followed by size selection using Agencourt AMPure XP beads (Beckman Coulter). Finally, libraries were amplified by PCR and purified using Agencourt AMPure XP beads.

Hybridization-based target enrichment was carried out with Geneseeq One pan-cancer gene panel (14 cancer-relevant genes) using a Hybridization and Wash Reagents Kit (Integrated DNA Technologies). After hybridization was complete, the captured targets were selected by pulling-down the biotinylated probe/target hybrids using Dynabeads M-270 (Life Technologies). Then, the off-target libraries were washed out. Captured libraries were amplified in KAPA HiFi HotStart ReadyMix (KAPA Biosystems) and quantified by qPCR using the KAPA Library Quantification Kit (KAPA Biosystems) for sequencing.

Sequencing and Data Processing

The libraries were paired-end sequenced on the HiSeq4000 platform (Illumina) according to the manufacturer's instructions. The mean coverage depth was >500× for tumor tissues after removing PCR duplicates. For cfDNA samples, the original mean sequencing depth was >3000×. Base calling was performed on bcl2fastq v2.16.0.10 (Illumina, Inc.) to generate sequence reads in FASTQ format (Illumina 1.8+ encoding). Quality control (QC) was performed with Trimmomatic (below 15 or N bases were removed). High quality reads were mapped to the human genome hg19 (GRCh37) using Burrows-Wheeler Aligner (BWA-mem, v0.7.12; https://github.com/lh3/bwa/tree/master/bwakit) with default parameters. The Genome Analysis Toolkit (GATK, version 3.4–0) was used for local indel realignment and base quality score recalibration.

VarScan2 software was employed for detection of single nucleotide variants (SNVs) and short insertions/deletions (indels). Common SNPs were filtered out using dbSNP (v137) and the 1000 Genomes database, followed by annotation using ANNOVAR. Copy-number variations (CNVs) were detected using ADTEx (http://adtex.sourceforge.net) with default parameters by comparing to the healthy individuals control pool with the cut-off of 0.65 for copy number loss and 1.80 for copy number gain. Genomic fusions were identified by FACTERA with default parameters.

Evaluation of the Response to Treatment with Targeted Therapy

The Response Evaluation Criteria in Solid Tumors (RECIST; version 1.1) was used to evaluate the efficacy of treatment, which was designated as either a complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD).

Statistical Analysis

The relationship between gene variability and clinicopathological variables was analyzed using the chi-squared test. Progression-free survival (PFS) with targeted inhibitor therapy was defined as the time from initiation of treatment to documented progression or death from any cause. PFS values were plotted using the Kaplan–Meier method. All analyses were performed using SPSS® version 18.0 (SPSS Inc., Chicago, IL, USA). The last follow-up date was Dec 31, 2018. No patients were lost to follow-up.

Results

Patient Characteristics

This study enrolled 102 advanced NSCLC patients (stage IV with malignant pleural effusions) with a median age of 57 ± 10.5 years (range, 28–81 years) and included 50 males and 52 females. Among them, 77 cases provided paired MPE supernatant and precipitate samples. The clinical characteristics of the present study are listed in Table 1.

MPE Supernatants are Comparable to MPE Precipitates in Detecting Gene Mutations

Capture-based targeted sequencing was performed to detect and quantify MPE supernatant and precipitate mutations using a panel consisting of 14 genes, including *EGFR, ALK, BRAF, AKT1, ERBB2, KRAS, MAP2K1, MET, NF1, NRAS, PIK3CA, PTEN, RET,* and *ROS1*. In total, 96.1% (74/77) of both supernatants and precipitates were detected with at least one mutation in the 14 genes (Figure 1A). The results revealed that there was no statistically significant difference in the detection rate between supernatants and precipitates from MPE ($P = 1.0$). Next, the maximum allelic fraction (maxAF) was compared in supernatants and precipitates from MPE. The median MAF of supernatants and precipitates was 20% and 15.6%, respectively, with no significant differences ($P = .6$, Figure 1B). The most frequently mutated gene in *EGFR* was identified for each gene in the panel.

Table 1
Clinicopathological features of present study

	All subject (n = 102)	Patients with matched samples (n = 77)
Age, years		
median	57 ± 10.5	55 ± 9. 0
range	28–81	28–79
Gender, n (%)		
Male	50	44
Female	52	33
Smoking status, n (%)		
Current or Former	35	21
Never	67	56
TKI treatment		
Yes	0	0
No	102	77
Chemotherapy before enrollment		
Yes	32	24
No	70	53

The values were 70% and 64% in the supernatants and precipitates from MPE, respectively (Figure 1C and D). *KRAS, ALK, BRAF, RET,* and *PIK3CA* mutations occurred in 16.9%, 16.9%, 13.0%, 7.8% and 6.5% of supernatants and 15.6%, 11.7%, 13.0%, 13.0%, and 6.5% of precipitates. Collectively, these data demonstrated that the detection rate of gene mutations was comparable in the MPE supernatants and precipitates in advanced NSCLC.

Genomic Profile is Comparable in MPE Supernatants and Precipitates in Advanced NSCLC

Next, the genomic profile associated with MPE supernatants and precipitates was investigated. In total, 172 and 158 genomic alterations were identified from MPE supernatant and precipitate samples. Specifically, 148 mutations were detected in MPE supernatants, including 97 SNVs, 37 indels, 24 copy-number amplification (CNA), and 14 rearrangements. The

most frequently mutated gene was *EGFR*, occurring in 70% of patients, followed by *KRAS,* occurring in 17% of patients (Figure S1A). In addition, mutations in other classic NSCLC drivers were also observed in MPE supernatants, including nine cases with *ALK* fusion, two cases with *RET* fusion, three cases with *MET* amplification, and one case with *ERBB2* amplification. Moreover, 149 mutations were identified in MPE precipitates, including 101 SNVs, 36 indels, nine CNAs, and 12 rearrangements. Other driver mutations included eight cases with *ALK* fusion, two cases with *RET* fusion, and one case with *MET* amplification (Figure S1B). There were 60 and 46 mutations that were only present in MPE supernatants and precipitates, respectively (Figure 2).

EGFR Status in MPE Supernatants and Precipitates

Next, the study analyzed the common and distinct *EGFR* mutations in MPE supernatants and precipitates. Consequently, *EGFR*-activating mutations (EGFR_19del, EGFR_L858R, EGFR_T790M, and EGFR_20ins) were detected in 78% (60/77) and 64% (49/77) of MPE supernatants and precipitates, respectively. The concordance rate was significantly higher in *EGFR* mutations (70%, 45/64) than that in all 14 gene mutations (51%, 112/218, $P = .01$) between MPE supernatants and precipitates (Figure 3). Interestingly, *EGFR* T790M was the least concordant, with a concordance rate of 42.9% (Figure S2A and S2B). Collectively, these data demonstrated that *EGFR* mutations were comparable in the MPE supernatants and precipitates.

PFS Data in Patients Who Received EGFR-TKI

In total, 45 of 77 patients with paired MPE supernatants and precipitates received the EGFR-TKI treatment. Among them, positive *EGFR* mutations were detected in 33 MPE supernatants and in 32 precipitates. Next, the study explored whether there is a difference in the efficacy of *EGFR*-TKI therapy between supernatant- and precipitate-*EGFR* positive patients. As a result, the data revealed comparable PFS (14.0 vs. 13.9 months, $P = .90$) between the two groups.
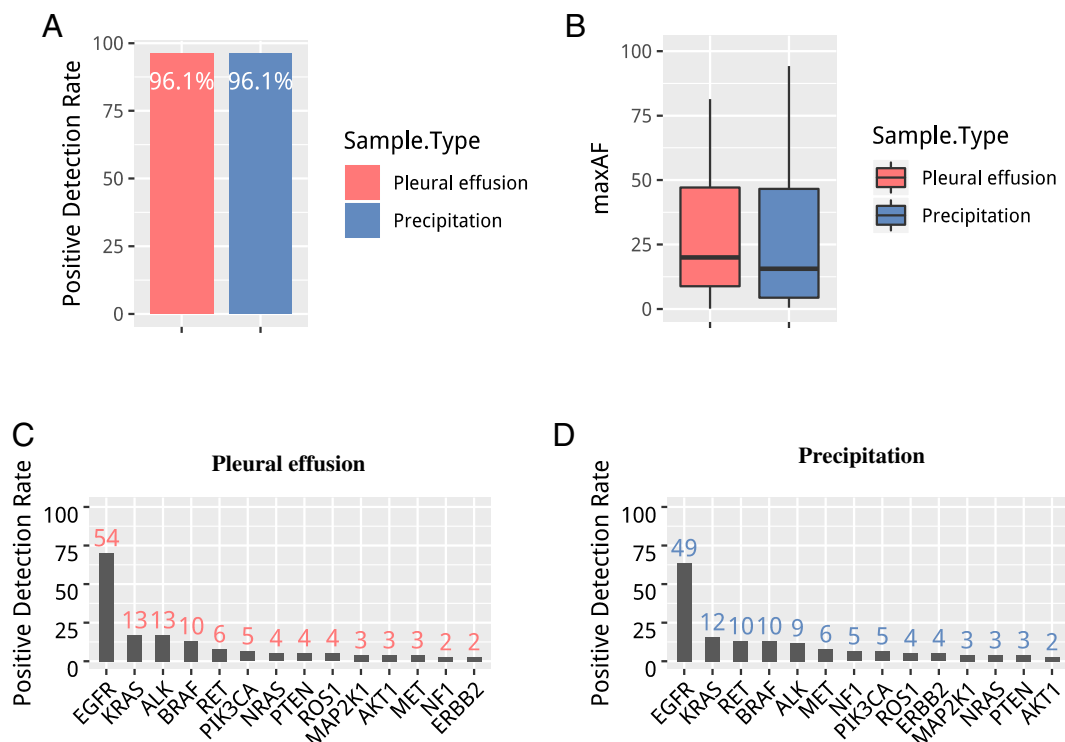
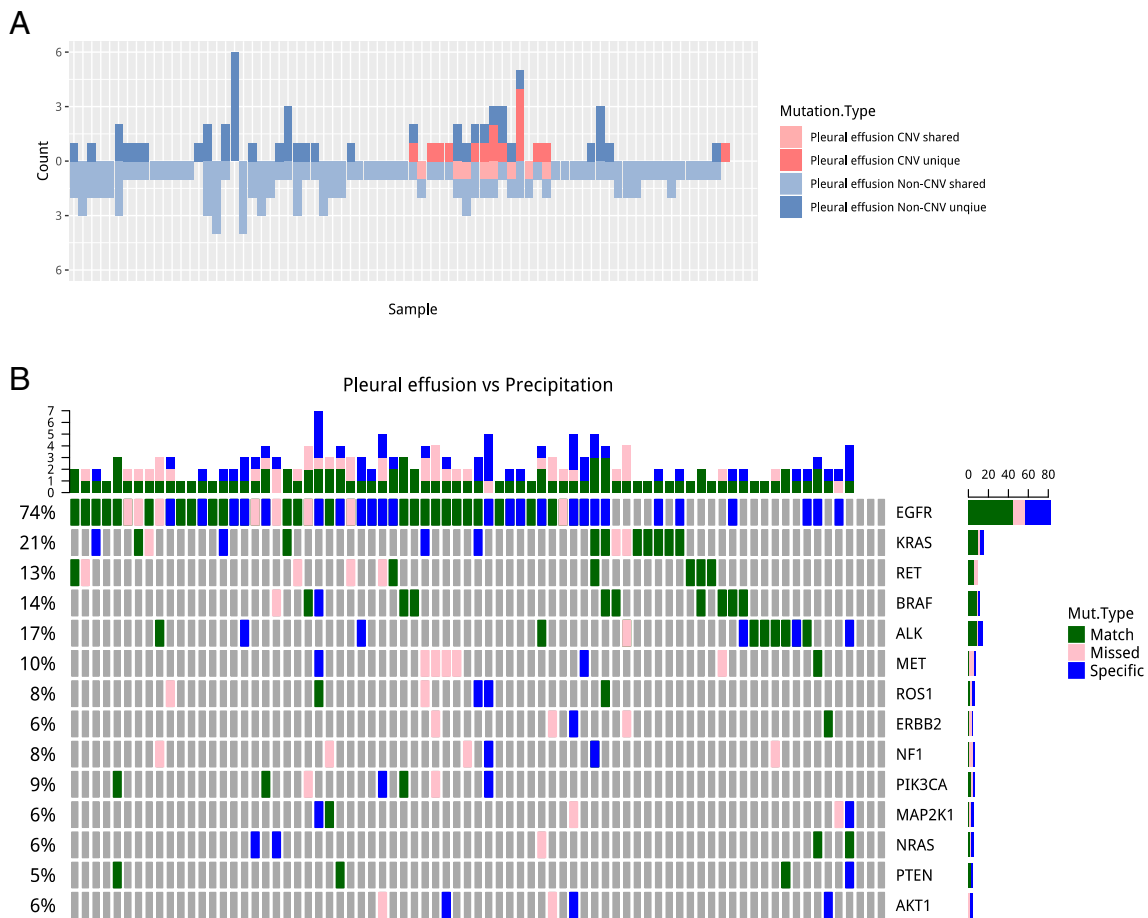**Figure 1.** Gene mutation list for supernatant and paired precipitate samples.

Figure 2. Genomic profile comparison in MPE supernatant and paired precipitate samples.

Discussion

This prospective observational study compared the feasibility of NGS in supernatant and paired precipitate samples in MPE and further evaluated the treatment outcome based on genetic alteration in precipitates and supernatants in NSCLC patients. Study findings supported the value of supernatants as a liquid biopsy for NGS detection. These results demonstrated that supernatants from MPE were comparable to precipitate samples for detection of genetic alterations, although gene mutation heterogeneity was found. Moreover, common driver genes were increasingly identified in MPE supernatants and paired precipitates with combination analysis.

Tissue biopsy is usually not suitable for some patients due to the tumor location or size. In the recent year, liquid biopsy has represented an alternative approach for cancer diagnosis. Tumor cfDNA is found in blood, lymph, spinal fluid, urine, and other types of body fluids. The cfDNA in plasma is mostly studies [13–15]. However, there is a major challenge associated with plasma cfDNA sequencing because gene mutations are at very low allele frequencies, usually lower than 0.5%. In previous studies, NGS technique has been often applied to compare the performance of EGFR testing in cfDNA vs. tumor tissue, with a sensitivity ranging from 70% to 80% [16,17]. Different from plasma, it has been recognized that more ctDNA is released in MPE, in which abundant cancer cells are present. In previous studies, MPE has been found to be suitable for detection of EGFR and ALK mutations in cell blocks of MPE [18–22],while, studies that compared cell blocks with supernatants were scarce by NGS technique.

Accumulating evidence supports tumor cell heterogeneity in lung cancer. Consistently, genetic heterogeneity was also detected in supernatants and cell blocks from MPE samples in NSCLC patients. Interestingly, gene mutations were more frequently detected in supernatants than in cell blocks. It should be highlighted that combined detection of genetic alterations in different samples should be considered due to the mutually complementary mutations.

All of the MPE samples were confirmed to have cancer cells in the present study. In fact, more than 20% to 30% of pleural effusions could not be confirmed as malignant. It was found that cytologically-negative pleural effusion could be an alternative liquid biopsy media for detection of EGFR mutation compared to cytologically positive pleural effusion in our previous study [23]. This interesting finding indicated that NGS has a superior detection sensitivity in supernatants compared to cell blocks. In addition, unlike cell blocks, embedding is not necessary for supernatant samples. Collectively, body fluid supernatants may have more advantages for NGS than paired cytological samples.

There were several limitations in this study. First, only 102 patients were enrolled. Thus, more patients are needed to validate these findings. Second, this study focused on genetic mutations in the MPE samples and no paired tumor tissues were obtained. Last, not all patients with genetic alterations were treated with targeted therapy given the nature of the non-intervention study. Thus, gene mutations in the present study were not fully validated.

In conclusion, this study demonstrated that MPE supernatants served as alternative samples for NGS compared to paired precipitate samples, although genetic heterogeneity was found between MPE supernatants and precipitates. The data supported the idea that combined detection with MPE supernatants and precipitates should be considered in clinical practice.

Contributions

(I) Conception and design: Z Song; (II) provision of study materials or patients: Z Song, Y Zhang; (III) collection and assembly of data: Wenxian
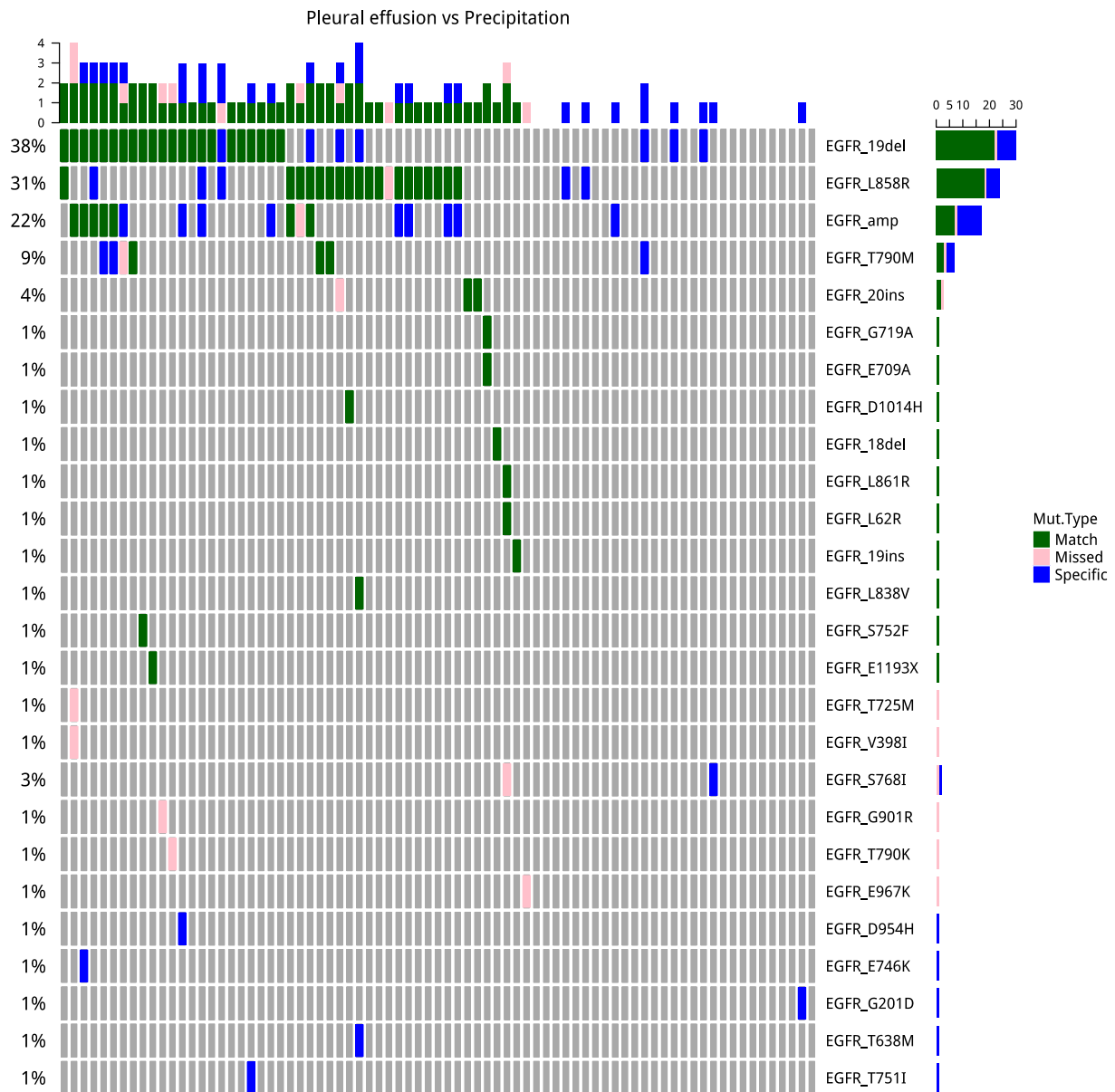
Figure 3. EGFR mutation comparison in MPE supernatant and paired precipitate samples.

Wang; (IV) data analysis and interpretation: YW Shao; (V) manuscript writing: all authors; Revised manuscript: J Li (VI) final approval of manuscript: all authors.

Conflict of Interest Disclosures

The authors report no conflicts of interest.

Ethical Statement

The authors are accountable for all aspects of the work and ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All patients provided written informed consent in compliance with ethical regulations of the Zhejiang Cancer Hospital. This study was approved by the Ethics Committee of the Zhejiang Cancer Hospital (No. IRB2014–03-032).

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2020.100784.

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