

Research Article

## Molecular Mechanisms of Resistance to Osimertinib from Liquid Biopsies of Plasma and Pleura Effusion in EGFR Mutant NSCLC Patients

Ning Li<sup>1\*</sup>, Shuhang Wang<sup>1</sup>, Huiyao Huang<sup>1</sup>, Minghui Zhang<sup>2</sup>, Zicheng Yu<sup>3</sup>, Fend Xie<sup>4</sup>, Shidong Jia<sup>4</sup>, Yixuan Ren<sup>5</sup>, Danyi Wen<sup>5</sup>

<sup>1</sup>Clinical Trial Center, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

<sup>2</sup>Department of Medical Oncology, Harbin Medical University Cancer Hospital, Harbin, China

<sup>3</sup>GenePlus-Shenzhen, Shenzhen, China

<sup>4</sup>Huidu Shanghai Medical Sciences, Shanghai, China

<sup>5</sup>Shanghai LIDE Biotech, Shanghai, China

**\*Corresponding Author:** Ning Li, Clinical Trial Center, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 100021, Beijing, China, Tel: 010-67781331

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### Abstract

In the past decade, tyrosine kinase inhibitors (TKI) have been developed and widely-approved by countries to treat advanced lung cancer patients with sensitizing epidermal growth factor (*EGFR*) mutations, and substantially

improved patients' prognosis compared to traditional chemotherapies. However, a large proportion of patients who initially experienced ostensibly remarkable therapeutic outcomes still developed inevitable drug resistance eventually, including those received newly developed third-generation TKIs. Previous studies have demonstrated the

value of plasma cell-free DNA (cfDNA) in monitoring patients' response to therapies in lung cancer. However due to several physiological reasons and DNA degradation, DNA released from tumor cell apoptosis in lung was hard to maintain at a high concentration and good quality before being collected from venous blood, leading to the loss of pivotal mutational information. Pleural effusion is a potential substitute to overcome these problems.

However, limited studies have been reported to characterize the consistency of genetic alterations found in these two sample types, and due to the modest sample sizes, the mechanisms underlying third-generation TKI resistance remains elusive. Herein we drew 8ml venous blood and collected 20ml pleural effusion by pleural effusion drainage from 8 lung cancer patients, and performed hybrid-capture based next generation sequencing assays on 18 matched plasma cfDNA, pleural effusion cells genomic DNA and pleural effusion supernatant cfDNA samples, we have shown that only sequencing either plasma or pleural effusion sample resulted in the missing of mutations in key cancer drivers. Sequencing both pleural effusion and plasma yielded a more concrete genetic landscape for monitoring tumor response. We have also exhibited that somatic mutations and copy number changes in PI3K/AKT pathway, NOTCH signaling pathway, hedgehog signaling pathway and pathways involved in DNA damage repair might be potential mechanisms underlying TKI resistance, and might serve as candidate targets for developing novel therapies to circumvent patients' resistance.

**Keywords:** EGFR; TKI; Osimertinib; Liquid Biopsie

## 1. Introduction

In the past decade, tyrosine kinase inhibitors (TKI) have been developed and widely-approved by countries to treat advanced lung cancer patients with sensitizing epidermal growth factor (*EGFR*) mutations, and substantially improved patients' prognosis compared to traditional chemotherapies [1-4]. In clinical trials, first-generation TKIs including gefitinib, carboplatin-paclitaxel and second-generation TKIs including afatinib, dacomitinib both yielded remarkable response rates and therapeutic outcomes initially, nonetheless acquired resistance was observed in a large proportion of patients [5-8]. The commonest mechanism of acquired resistance was indicated to be *EGFR* T790M mutation, and based on this, third-generation TKIs were developed [1].

Third-generation TKIs include osimertinib, nazartinib, olmutinib, PF-06747775, YH5448, avitinib and rociletinib [1, 9], and in China, only osimertinib has been approved to date [10]. Objective response rates and progression-free survival of *EGFR* T790M positive patients were observed significantly improved with osimertinib [11, 12]. However, acquired resistance was again experienced eventually by many patients. Studies suggested potential molecular mechanisms underlying resistance encompassing *BRAF* V600E, *EGFR* G796D, *EGFR* C797S, *EGFR* L718Q mutations and *MET* amplification *etc* as reported [13-17]. Recent studies revealed that a wide range of other genetic changes might also contribute to the resistance to third-generation TKIs, such as *HER2* amplifications, *PIK3CA* mutations and several gene fusions [18, 19]. Given the highly heterogenous mechanisms behind the resistance to third-generation TKIs, a few studies published recently suggested that sequencing both plasma cfDNA and tissue

biopsies might result in an optimal sensitivity in predicting the resistance [20, 21]. Another recent case study reported primary resistance was observed in a patient who was detected with *EGFR* T790M mutation from sequencing traditional plasma liquid biopsy, suggesting plasma cfDNA might not be sufficient to detect these possible resistant mechanisms [22]. In addition, though studies have demonstrated multiple possible mechanisms underlying third-generation TKI resistance and even proposed potential fourth-generation inhibitor EAI045 to circumvent this issue [13-17, 23], our understandings were still largely limited by the modest sample sizes in these studies, and a whole picture still awaits exploration given that most of these studies were based on individual cases. Moreover, there is an urgent need to develop a novel tool to predict patients' response to third-generation TKIs and monitor the therapeutic outcomes in a real-time manner, considering tissue biopsies were believed to be Janus-faced due to their invasive nature and high risk of inducing implantation metastasis [24].

In this regard, non-invasive liquid biopsies have been developed. Cell-free tumor DNA (ctDNA) in plasma has gradually become a commonly used tool in the therapeutic arsenal for real-time clinical surveillance [25-30]. However, tumor-derived ctDNA in several specific cancer types might be restrained from entering the venous blood, for instance ctDNA from brain tumors is mostly unable to enter the blood because of the blood-brain-barrier, and ctDNA released from tumor cell apoptosis in lung is hard to maintain at a high concentration and good quality before being collected in venous blood [31]. Based on these, cell-free DNA in tissue-specific bodily fluids were also utilized, such as cerebrospinal fluid and pleura effusion [32, 33]. Previous reports have demonstrated the value of plasma

ctDNA in monitoring patients' response to therapies in lung cancer [34]. Nevertheless, whether plasma or pleura effusion could be solutions to monitor patients' resistance to third-generation TKIs still needs to be addressed. Herein by using targeted-capture next-generation sequencing (NGS), we profiled the genetic alterations in 15 matched plasma cfDNA, pleura effusion cells genomic DNA, pleura effusion supernatant cfDNA samples from 8 lung cancer patients, trying to answer whether comparable mutational landscapes could be obtained from plasma and pleural effusion biopsies, and reason the possible mechanisms underlying the acquired drug resistance, rendering it promising to guide personalized treatment decisions, screen suitable candidates for third-generation TKIs and develop novel drugs to overcome the drug resistance.

## 2. Methods and Materials

### 2.1 Patient enrollment and ethics

8 patients diagnosed with lung cancer were enrolled from Cancer Hospital Chinese Academy of Medical Sciences from 2014 to 2020. Detailed clinicopathological characteristics of all patients with the timing of progression and sample collections in relation to third-generation TKIs administration were shown in Table S1. All patients harbored *EGFR* T290M mutations determined by fluorescent PCR and underwent third-generation TKI therapies as indicated in Table S1 before developing resistance afterwards. Then matched peripheral plasma and pleura effusion samples were collected in EDTA Vacutainer tubes (BD Diagnostics, NJ, USA) for cfDNA extraction and purification within 3h. Written informed consent was collected and this study was approved by the Institutional Review Board (IRB) of Cancer Hospital Chinese Academy of Medical Sciences and conducted in accordance with the Declaration of Helsinki.

## 2.2 Plasma cfDNA NGS testing

Circulating cell-free DNA (cfDNA) testing was performed using the 600-gene PredicineaATLAS assay in a College of American Pathologist (CAP)-accredited laboratory in China (Huidu Shanghai Medical Sciences Ltd.) in 2019-2020.

## 2.3 Plasma cfDNA extraction

cfDNA was extracted using QIAamp circulating nucleic acid kit (Qiagen, Hilden, Germany) from plasma samples. Quantity and quality of the purified cfDNA were checked using Qubit fluorimeter and Bioanalyzer 2100 (Thermo Fisher Scientific, MA, USA).

## 2.4 Library preparation, capture and sequencing

5 to 20ng of extracted cfDNA was prepared for library construction including end-repair, dA-tailing, adapter ligation, and PCR amplification. The amplified DNA libraries with sufficient yields proceeded to hybrid capture. In brief, the library was hybridized overnight with the panel probes. Unbound fragments were then washed away. The purified libraries were QCed with Bioanalyzer 2100 and then paired-end 2x150bp sequenced using the Illumina sequencing platform.

## 2.5 Variant calling

Variants were called using a Predicine in-house developed analysis pipeline, which starts from the raw sequencing data and outputs the final mutation calls. Briefly, the pipeline first performed adapter trimming, barcode checking, and correction. Cleaned paired FASTQ files were outputted by the in-house pipeline and further aligned to the human reference genome build hg19 using BWA alignment tool. Consensus bam files were then derived by merging paired-end reads originated from the same molecules (based on mapping location and unique molecular identifiers) as

single strand fragments. Single strand fragments from the same double strand DNA molecules were further merged as double stranded. Both sequencing and PCR errors were deeply suppressed during this process.

Candidate variants, consisting of point mutations, small insertions and deletions, were identified across the targeted regions covered in the panel. A variant identified in cfDNA was considered a candidate somatic mutation only when (i) the number of distinct fragments containing a particular mutation was at least 0.25% of the total distinct fragments. For hotspot variants, the number of distinct fragments containing a particular mutation was at least 0.1% of the total distinct fragments; and (ii) the variant was not present in public databases of common germline variants including 1000 genomes, ExAC, gnomAD with population allele frequency >0.5%. Candidate somatic mutations were further filtered on the basis of gene annotation to identify those occurring in protein-coding regions. Intronic and silent changes were excluded, while mutations resulting in missense mutations, nonsense mutations, frameshifts, or splice site alterations were retained. Mutations annotated as benign or likely benign in the ClinVar database were also filtered out.

Copy number variations were estimated at the gene level. The pipeline calculated the on-target unique fragment coverage, which was first corrected for GC bias, and was then adjusted to the probe level bias (estimated from a pooled reference). Each adjusted coverage profile was self-normalized (assuming diploid of each sample) first and then compared against correspondingly adjusted coverages from a group of normal reference samples to estimate the significance of the copy number variant. Amplification or

deletion of gene copy number with an absolute z-score > 3 were called.

### 3. Result

#### 3.1 Somatic point mutation identified in liquid biopsies

Targeted-capture NGS yielded an average sequencing depth of 20,000x from all samples. A total of 161 somatic single nucleotide variations (sSNV) and small insertions and deletions (Indels) were identified. Within expectation, EGFR was detected with the highest mutation frequencies (15/18, 83.3%) among all samples, including all plasma, pleural effusion cells and pleural effusion supernatant samples. Other recurrent mutations with high prevalence include mutations in canonical cancer-associated genes, encompassing BCR (6/18, 33.3%), SUFU (6/18, 33.3%), DNMT3A (5/18, 27.8%), TP53 (5/18, 27.8%), CTNNB1 (4/18, 22.2%), FAT1 (4/18, 22.2%), CTSLTR2 (4/18, 22.2%) *etc* (Figure 1). As for mutation frequencies among all patients, a specific gene was considered mutated in a patient if this mutation was detected in any of the above three sample types from the patient. In this regard, EGFR was detected with mutations in 7/8 (87.5%) patients. Other recurrently mutated genes included TP53 (4/8, 50.0%), BCR (3/8, 37.5%), SUFU (3/8, 37.5%), DNMT3A (3/8, 37.5%), CTNNB1 (2/8, 25.0%), FAT1 (2/8, 25.0%), CYSLTR2 (2/8, 25.0%). Mapped into Reactome signaling pathway database (accessed in Oct. 2020), mutations were observed enriched in pathways of growth factor receptor (EGFR, CBL, CTNNB1, AKT1, CTNNA1, PGR, EP300, PIK3CA, PDGFRA, NTRK1, NTRK2) (7/8, 87.5%), DNA repair pathways (ERCC6, FANCC, BRCA1, APEX1, ERCC1, TP53) (5/8, 62.5%), hedgehog signaling pathway (SUFU, GLI1) (4/8, 50.0%), NOTCH signaling pathway (NOTCH1, NOTCH3) (2/8, 25.0%) and *etc*.

We then compared the consistency of mutation detection among three matched samples from individual patients. In result, a total of 16 mutations were shared by all three matched samples from the same patients. 7 mutations were found only shared between plasma and pleura effusion supernatant, while 4 mutations were shared between pleura effusion cells and supernatant. A total of 22, 9, and 21 mutations were private to plasma, pleura effusion cells and pleura effusion supernatant respectively. Specifically, in P1, four mutations were shared by all the three samples (BCR, SUFU, FAT1, CYSLTR2). DNMT3A mutation was detected in both plasma and pleura effusion supernatant, while DOT1L, C7orf76 and EGFR mutation was only detected in pleura effusion supernatant. 5 mutations were shared by all the samples in P2, including mutations in EGFR, TGFBR2, FLCN, ERBB3 and BRCA1. Mutations in APEX1, NTRK2 and TBX3 were observed in both plasma and pleura effusion supernatant. 10 mutations were private to the plasma, while 2 and 9 mutations were private to pleura effusion cells and supernatant respectively. In P3, 4 mutations were shared by all three samples. Mutations in DNMT3A and TET2 were identified in both plasma and pleura effusion supernatant, whereas NKX3-1 mutation was shared by pleura effusion cells and supernatant. In addition, 2 and 6 mutations were found private to the plasma and pleura effusion supernatant samples. P4 harbored mutations in EGFR and GLI1 in both of the plasma and pleura effusion supernatant samples, while 1, 2 and 3 mutations were detected privately in the plasma, pleura effusion cells and supernatant accordingly. In P6, only EGFR mutation was shared across all the three samples. BCR, SUFU and ALK mutations were shared between two pleura effusion samples whereas TP53 mutation was shared between plasma and pleura effusion supernatant. 10 and 5 mutation were observed private in plasma and pleura effusion cells

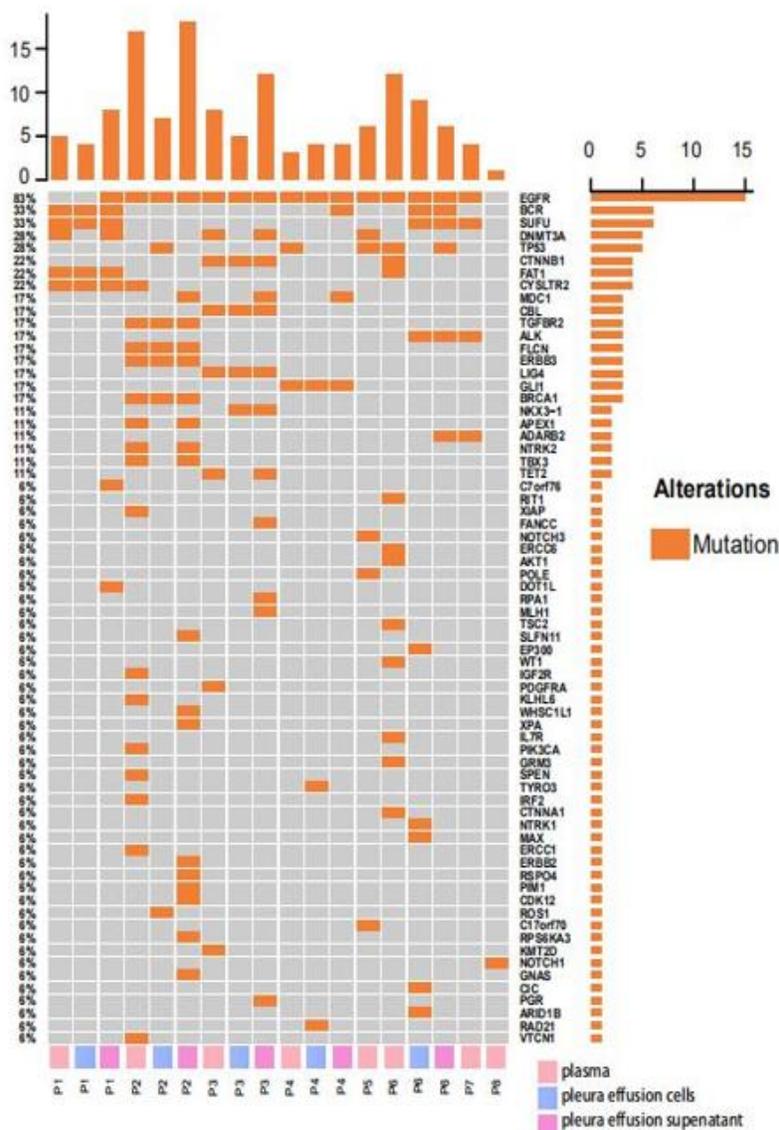
respectively. Among all patients excluding two with only plasma samples collected, 22/45 (number of private mutations in plasma/number of all mutations in plasma; 48.9%), 9/29 (number of private mutations in pleural effusion cells/number of all mutations in pleural effusion cells; 31.0%) and 21/48 (number of private mutations in pleural effusion supernatant/number of all mutations in pleural effusion supernatant; 43.8%) mutations were found private to plasma, pleural effusion cells and pleural effusion supernatant accordingly.

### 3.2 Somatic copy number variations identified in liquid biopsies

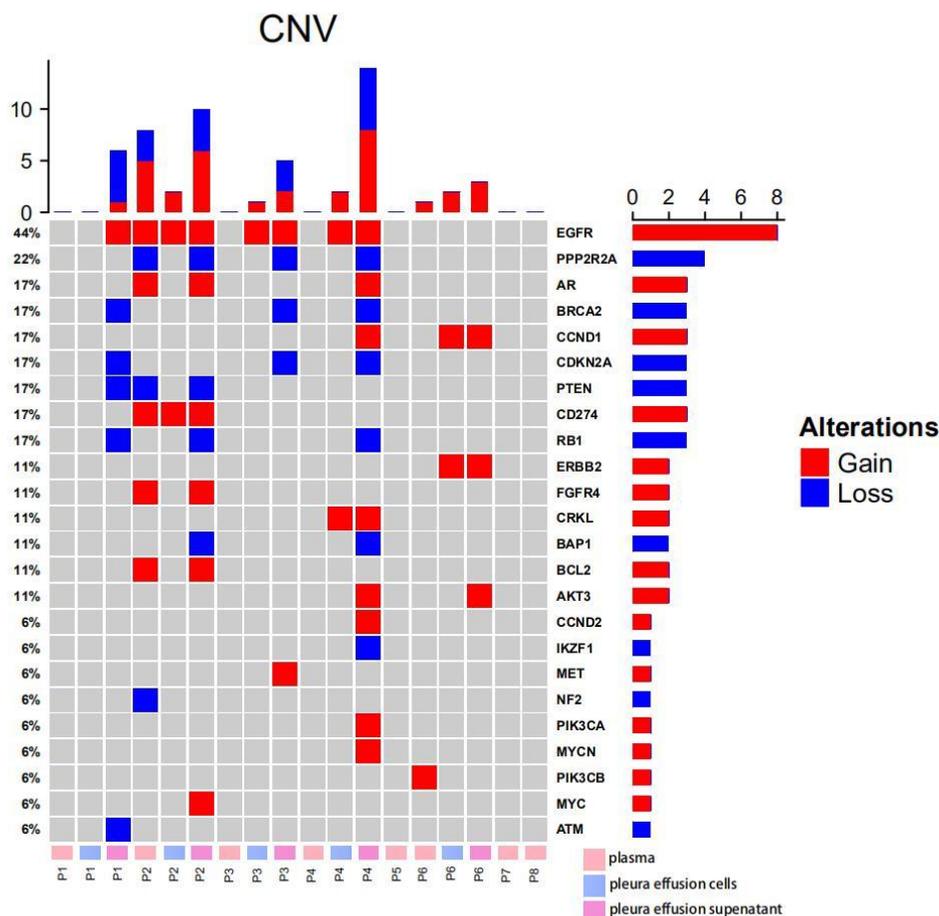
A total of 54 copy number variations were identified from all samples (Figure 2). EGFR was observed with the highest frequencies of variations (8/18, 44.4%), where all 8 changes were amplifications. Other recurrent amplifications were loss of PPP2R2A (4/18, 22.2%), gain of AR (3/18, 16.7%), loss of BRCA2 (3/18, 16.7%), gain of CCND1 (3/18, 16.7%), loss of CDKN2A (3/18, 16.7%), loss of PTEN (3/18, 16.7%), gain of CD274 (3/18, 16.7%), loss of RB1 (3/18, 16.7%) and *etc* (Figure 2). Among all patients, amplifications of EGFR were identified in 4/8 (50.0%) patients. Loss of PPP2R2A, BRCA2, CDKN2A, RB1 were all found in 3/8 (37.5%), while gain of AR, CCND1, AKT3 were identified in 2/8 (25.0%) patients. Pathway analysis revealed the enrichment of EGFR-dependent growth factor receptor pathway (BCL2, CCND1, AKT3, EGFR) (5/8, 62.5%) and PI3K/AKT signaling pathway (PIK3CB, FGFR4, PTEN, PIK3CA, AKT3, ERBB2, MET, EGFR) (5/8, 62.5%).

Surprisingly, relatively less consistent mutational patterns of somatic CNVs were observed among plasma, pleural effusion cells and pleural effusion supernatant. In P1,

somatic CNVs were only identified in pleural effusion supernatant, including gain of EGFR and loss of BRCA2, CDKN2A, PTEN, RB1 and ATM. P2 harbored amplifications of EGFR and CD274 in all of the three samples and loss of PPP2R2A, PTEN, gain of AR, FGFR4 and BCL2 in only plasma and pleural effusion supernatant. Loss of RB1, BAP1 were observed private in pleural effusion supernatant while loss of NF2 was specific to the plasma sample. In P3, only EGFR amplification was found shared between two pleural effusion samples while other CNVs including loss of PPP2R2A, BRCA2, CDKN2A and gain of MET were observed private to pleural effusion supernatant. Similarly, P4 harbored most of the somatic CNVs only in pleural effusion supernatant, including gain of AR, CCND1, AKT3, CCND2, PIK3CA, MYCN and loss of PPP2R2A, BRCA2, CDKN2A, RB1, BAP1, IKZF1, while gain of EGFR and CRKL were both identified in pleural effusion cells and pleural effusion supernatant. In P6, gain of CCND1 and ERBB2 were found in both of the two pleural effusion samples, while gain of AKT3 and PIK3CB were found specific to pleural effusion supernatant and plasma samples respectively. In summary, 26/38 (68.4%; number of private CNVs in pleural effusion supernatant/number of all CNVs in pleural effusion supernatant) somatic CNVs detected in pleural effusion supernatant were not detected in the other matched samples, and only 2/9 (22.2%; number of private CNVs in plasma/number of all CNVs in plasma) CNVs in plasma were found tissue-specific. While all 7 CNVs detected in pleural effusion cells could also be observed in either matched plasma or pleural effusion supernatant sample. In regard of CNVs, pleural effusion supernatant and plasma might be more suitable biopsies yielding more comprehensive mutational information.



**Figure 1:** Somatic SNVs and Indels detected from plasma, pleural effusion cells and pleural effusion supernatant from 8 EGFR-mutated lung cancer patients; panel on the top demonstrates the number of mutations detected in each sample; panel at the right indicates the numbers of mutations identified in respective genes; panel at the bottom shows the sample ID where P represents patient, and plasma, pleura effusion cells and pleura effusion supernatant were labeled in pink, blue and violet accordingly.



**Figure 2:** Somatic CNVs detected from plasma, pleural effusion cells and pleural effusion supernatant from 8 EGFR-mutated lung cancer patients; the panel on the top illustrates the number of CNV detected in each sample; the panel at the right indicates the number of CNV identified in each gene; the panel at the bottom exhibits the sample ID, where P stands for patient, and plasma, pleura effusion cells and pleura effusion supernatant were labeled in pink, blue and violet accordingly.

#### 4. Discussion

Osimertinib showed efficacy superior to that of standard EGFR-TKIs in the first-line treatment of EGFR mutation-positive advanced NSCLC [35]. However, all patients eventually developed resistance to osimertinib. Several mechanisms of resistance to osimertinib have been described, such as acquired KRAS mutations, EGFR

T790M loss and targetable gene fusions [36]. Basically, the resistance mechanisms are divided into EGFR-dependent and EGFR-independent. EGFR-dependent mechanisms include EGFR rare mutation [37] whereas examples of EGFR-independent mechanisms include alternative kinase activation [38, 39] and histological transformation [40]. Other reported mechanisms of acquired mechanisms

include mutations like BRAF V600E, EGFR G796D, EGFR C797S, EGFR L718Q, MET amplification *etc* [13-17]. Previous studies have characterized the consistency between pleural effusion and plasma samples in lung cancer patients. A real-world research published recently have demonstrated that pleural effusion supernatant detected actionable mutations with higher sensitivity than plasma samples in M1a disease but not in M1b/c disease, showing different mutational profiles in these two samples [41]. Besides, the study also showed that pleural supernatant outperformed plasma in identifying mutations that confer resistance to first and second generation TKIs [41]. These results were relatively consistent with the present studies, and our results further substantiated that pleural effusion might detect mutations with an improved sensitivity in some patients while plasma might be preferred in the others, indicating that sequencing both plasma and pleural effusion might yield optimal sensitivity. Also, we enriched Jin *et al's* findings that pleural effusion could be used in detecting resistance to first and second generation TKIs, by showing that possible mechanisms to third-generation TKI were also observed. Besides, studies exhibited only a low amount of circulating tumor cells could be detected in plasma of lung cancer patients compared to other cancer types [31], indicating that pleural effusion samples were indeed better choice for liquid biopsies in lung cancer patients. Recent researches also demonstrated the use of various types of liquid biopsies to characterize genetic changes in lung cancer patients. Song *et al* showed that comparable mutational landscapes were observed in pleural effusion-derived exosomal DNA and pleural effusion cfDNA, suggesting that pleural effusion exosomal DNA could be a potential alternative source for genetic testing [42]. Lee *et al* reported that extracellular vesicle-derived DNA (EV-DNA) from pleural effusion detected EGFR mutations with

higher sensitivity than pleural effusion cfDNA and tissue biopsies, indicating that pleural effusion EV-DNA might suitable in predicting resistance to TKIs [43]. In our study, we have showed in addition to plasma cfDNA and pleural effusion supernatant, pleural effusion cells could also be used in genetic testing, providing with an alternative source of biopsy for lung cancer.

In general, somatic SNVs detected across three sample types were considered consistent. Mutations in canonical cancer driver genes were mostly shared by matched samples, including EGFR, BCR, SUFU, FAT1, ERBB3 and BRCA1, indicating that these mutations might be clonal mutations that occurred at an early stage of tumorigenesis, so that they existed in most of the tumor cells making it easy to detect from either plasma or pleural effusion, and they might play pivotal roles in driving tumor progression. However, TP53, another essential cancer driver gene, was only identified in pleural effusion cells in P2 and plasma in P4, suggesting that some key drivers might be missed when sequencing only one of the sample types. Therefore, testing pleural effusion in addition to plasma might be able to provide a more comprehensive and concrete mutational landscape of tumors. Notably, we also noticed that in two patients, EGFR mutation was not observed in some of the samples, namely plasma and pleural effusion cells of P1 and the plasma sample of P8, while fluorescent PCR result indicated they harbored EGFR T290M mutation. This discrepancy was possibly due to the difference in the sequencing techniques utilized, and again indicated that sequencing only one liquid biopsy might not be sufficient to reveal the whole picture of genetic alterations in patients.

We also noted that the two components of pleural effusion samples harbored relatively different mutations in some

patients, such as P2 and P6, and this was conceivably reasonable, considering about the different source of DNA contained in these two components. DNA from the pleural effusion supernatant were mainly cell-free DNA that released from cell apoptosis, necrosis, exosome secretion, and other cellular activities [43, 44]. In comparison, DNA from pleural effusion cells were mainly genomic DNA from a variety of cells in pleural effusion [45], including immune cells, tumor cells *etc.* Sequencing both pleural effusion cells and supernatant might be able to avoid missing any key mutations that potentially contribute to drug resistance and compromise therapeutic outcomes, and also helps get a better knowledge of the detailed tumor micro-environment. Intriguingly, more mutations were found shared between matched plasma and pleural effusion supernatant than between plasma and pleural effusion cells, indicating that plasma and pleural effusion supernatant might shared a substantially similar contents, possibly released by tumor cell apoptosis. Also, more private mutations were found in these two sample types compared to pleural effusion cells, suggesting that compared to cellular gDNA, cfDNA could better reveal the heterogeneity of the tumor samples and yield a systemic overview of the genetic alterations in patients. Few CNVs were detected in plasma and pleural effusion cells samples. A possible explanation is the tumor DNA fraction in plasma and pleural effusion was considerably low, and targeted-capture sequencing could only cover limited chromosomal segments, therefore the detection of somatic CNVs might not be sensitive as the whole-exome sequencing [34] of tissue biopsies. Another limitation of this study was the modest sample size. Whether deep WES sequencing of a larger size of liquid biopsy cohort can accurately detect somatic CNVs awaits further investigations.

Looking at the somatic SNVs and CNVs, we proposed several potential mechanisms underlying patients' resistance to third-generation TKIs. First of all, mutations in PI3K-AKT signaling pathway was observed recurrently mutated, and copy number changes in related genes AKT3, ERBB2, PTEN *etc* were identified in several patients. In previous studies, PI3K/AKT pathway was indicated to be the down-stream signaling pathway orchestrated by EGFR. PI3K encodes proteins to function with RAS and ANG1/TIE2 and further regulate PTEN functions to induce ADK and mTOR signaling transduction, finally facilitating cellular survival and prevent apoptosis [46-50]. It was also suggested that PI3K-AKT pathway mutations were stringently associated with patients' resistance to targeted therapies in breast cancer [47]. PI3CA mutations were also an indicator of chemoresistance [51]. NOTCH signaling pathway is another pathway frequently mutated in our cohort, and was indicated to play pivotal roles in various cancer types. Essentially, NOTCH reacts to signaling from cyclin D and Sox-9 on the plasma membrane of the tumor cell, and triggering down-stream beta-catenin/Tcf-4 signaling and eventually enticing tumor cell proliferation [52-56]. Mutations in hedgehog pathway, regulated by interaction of Hh, Ptc and Smo proteins and functioning to trigger Kif/sufu/Gli signaling were also related to cell cycle, survival and proliferation in cancer [57-59]. Of particular interest, we also observed high prevalence of somatic SNVs and CNVs in genes involved in DNA damage repair, including ERCC6, FANCC, BRCA1, APEX1, ERCC1 *etc*, which was indicated to be a powerful indicator of patients' response to PARP inhibitors in ovarian and breast cancers [60-62] as well as therapies targeting DNA damage checkpoint in gastric cancer [63]. Based on these findings, we concluded that these signaling pathways might be potential mechanisms underlying patients' acquired

resistance to third-generation TKIs, and they might also be the candidate targets to develop novel therapies in third-generation TKI resistant patients.

In summary, mutations identified by pleural effusion supernatant was quite consistent to plasma samples. The small sample size is the major limitation of this study, more investigations with larger sample sizes are needed to gain a deeper understanding in the mechanisms of third-generation TKI resistance and the clinical practice of liquid biopsies of lung cancer. Private mutations detected in plasma, pleural effusion cells or pleural effusion supernatant indicated that sequencing single liquid biopsies might not be sufficient to obtain a concrete and comprehensive picture of the genetic alterations in lung cancer patients. Sequencing pleural effusion in addition to plasma can help avoid missing important alterations related to drug resistance. Somatic mutations and copy number changes in PI3K/AKT pathway, NOTCH signaling pathway, hedgehog signaling pathway and genes involved in DNA damage repair might be potential mechanisms underlying third-generation TKI resistance, serving as the potential therapeutic targets to develop novel drugs for TKI resistant patients.

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