Lung Cancer 136 (2019) 23–29

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Cytological-negative pleural effusion can be an alternative liquid biopsy media for detection of EGFR mutation in NSCLC patients

Zhengbo Song[a], Wenxian Wang[a], Min Li[b], Junjun Liu[b], Yiping Zhang[a,*]

[a] Department of Chemotherapy, Zhejiang Cancer Hospital, Hangzhou, 310022, China
[b] Burning Rock Biotech, Guangzhou, 510300, China

ARTICLE INFO

Keywords:
Cytological-negative pleural effusions
Malignant pleural effusions
Epidermal growth factor receptor
Mutation
Lung adenocarcinoma

ABSTRACT

Objective: Though the possibility of using malignant pleural effusions (MPEs) as alternatives for tumor tissues in epidermal growth factor receptor (*EGFR*) mutation test has been examined, the diagnosis of MPE is often clinically challenging, especially if the cytology is negative for malignancy. The aim of this study was to examine whether cytological-negative PE (CNPE) is useful in detecting *EGFR* mutation and evaluated its feasibility for predicting clinical outcomes.

Method: In this study, we performed capture-based targeted sequencing using a panel consisting of 520 lung cancer-related genes to detect *EGFR* mutation status in 121 MPEs and 40 CNPE samples from 161 advanced lung adenocarcinoma patients. Patients underwent TKI treatment with gefitinib, icotinib or erlotinib if *EGFR* sensitizing mutations were detected at their tumor biopsies or pleural effusion sediment.

Results: We revealed a mutation detection rate of 99.2% and 100% for MPE and CNPE, respectively ($p = 1$). The maximum allelic fraction (maxAF) of MPE and CNPE were 57.4% and 56.8%, respectively ($p = 0.77$). CNPE supernatant is comparable to MPE in reflecting the mutational profile of lung adenocarcinoma. *EGFR* activating mutations were detected in 47.5% (19/40) of CNPE supernatant sample and 32.5% (13/40) of matched tumor biopsies. CNPE sample is superior to tumor tissues in identifying *EFGR* mutation. Among the 72 *EGFR*-TKI treated patients, 51 were cytology positive and the remaining 21 were cytology negative. Our data showed that MPE patients exhibited comparable PFS ($p = 0.41$) and OS ($p = 0.26$) with CNPE patients treated with *EGFR*-TKI. Among the 21 CNPE patients received TKI treatment, patients harboring either *L858R* or *exon 19 deletion* had longer PFS than patients without a detectable mutation ($p = 0.036$).

Conclusion: Collectively, we demonstrated that CNPE supernatant provided a comprehensive profile of NSCLC, and can serve as a reliable lipid biopsy media for *EGFR* mutational detection.

1. Introduction

Malignant pleural effusions (MPEs) can be observed in patients with various types of neoplasm, especially lung adenocarcinoma, since it grows in the periphery of the lung and easily invades the pleural cavity [1,2].Although DNA from tissue can better reflect the genomic mutation in various cancers, the inadequate amount of tumor specimens and the risky invasive procedure have limited the availability of tumor tissue for molecular analysis [3,4].Instead, MPE sample may be an attractive alternative to lung biopsy for genotyping, as the collecting method is less invasive, and mutations of *EGFR* can also be detected in pleural effusion samples [5–7].Various studies have compared the diagnostic accuracy of MPE samples *versus* tumor tissue for detection of *EGFR* mutations, and suggested that MPE is a valid surrogate for non-small cell lung cancer (NSCLC) tumor *EGFR* mutation detection when tissue is not available [8–10]. In addition, *EGFR* mutation screening in MPE may be useful for the prediction of the clinical outcome of lung cancer patients treated with gefitinib [11–14].

Cell-free circulating tumor DNA (ctDNA) released from primary tumors or metastases represents genomic aberrations in cancer cells and has potential as a *liquid biopsy* to monitor tumors in real time [15–17]. Numerous studies have shown ctDNA derived from plasma can be used as a surrogate for reflecting the genomic alterations present in tumors as well as for diagnosis, monitor tumor progression and response to treatments [18,19].Moreover, ctDNA in cerebrospinal fluid (CSF) better represents the genomic landscape of brain tumors than plasma, thus may serve as a liquid biopsy to monitor brain tumor evolution in *EGFR*-mutant NSCLC [20–22]. Recently, studies have successfully detected

* Corresponding author at: Department of Chemotherapy, Zhejiang Cancer Hospital, 38 guangji road, Gongshu District, Hangzhou, 310022, China.
E-mail address: zjzlyy16@163.com (Y. Zhang).

https://doi.org/10.1016/j.lungcan.2019.08.004
Received 24 April 2019; Received in revised form 20 July 2019; Accepted 2 August 2019

EGFR mutations using ctDNA derived from MPE supernatant samples [23–25] and demonstrated that PE supernatant had significantly higher tumor-specific mutation detection rate and sensitivity compared to PE sediment containing tumor cells [26].Thus, DNA from the supernatant of PE is a promising source forgenetic testing to guide treatment-decision making in lung cancer. While the diagnosis of MPE is often a vexing problem because cytology findings are positive in only an average of 60–80% for adenocarcinoma cases [27–29]. And the application of PE in cases where pleural aspirate cytology is negative has been largely unexplored in the clinical diagnostic setting. Cytological-negative PE (CNPE) were commonly discarded in practice, maybe a rich source of fresh ctNDA. We hypothesized that the identification of genetic alterations may be particularly useful in this widely available biospecimen, and could guide the decision to undertake more invasive definitive testing such as thoracoscopy or thoracotomy.

In the present study, we performed capture-based targeted sequencing on 121 MPE supernatant and 40 CNPE supernatants to examine the potential of utilizing CNPE as an alternative liquid biopsy media and to detect *EGFR* mutation status in PE and evaluated its feasibility for predicting clinical outcomes.

2. Materials and methods

2.1. Patients and samples

The study cohort includes 161NSCLC patients with pleural effusion who were undergoing tumor biopsies or pleural drainage at Zhejiang Cancer Hospital between Jan 2015 to Apr 2017. The study was approved by the Institutional Ethics Committee of Zhejiang Cancer Hospital. All study subjects provided written informed consent. These patients were all diagnosed with stage IV NSCLC and underwent TKI treatment with gefitinib, icotinib or erlotinib based on the *EGFR* status at tumor biopsies or pleural effusion sediment. According to cytological diagnosis, samples were separated into MPE and CNPE. Genomic profiles derived from the supernatant of the two groups were compared.

2.2. Collection of pleural effusion fluid

Pleural effusion samples of up to 250–500 mL were collected from all patients. A 20 mL sample of the fluid was centrifuged at 1000*g* for 10 min at 4 °C within 1 h of collection, and the cell pellets were smeared for detection of tumor cells. The cytological results was confirmed by two independent pathologist.

2.3. EGFR detection by PCR

RNA was extracted from cell pellets with a Qiamp RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was extracted using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Reverse transcription and detection of the *EGFR* sensitizing gene was performed with the *EGFR* Super-ARMS® *EGFR* Mutation Detection Kit (Amoy Dx, Xiamen, China).

2.4. DNA extraction

PE supernatant ctNA was extracted with the QIAamp Circulating Nucleic Acid Kit (Qiagen). DNA was quantified using the Qubit dsDNA assay (Life Technologies).

2.5. Capture-based targeted DNA sequencing

DNA was profiled using a commercially available capture-based sequencing panel (Burning Rock Biotech Ltd, Guangzhou, China), targeting 520 genes and spanning 1.6MB of human genome. DNA was hybridized with the capture probes baits, selected with magnetic beads, and PCR amplified. A bioanalyzer high-sensitivity DNA assay was then performed to assess the quality and size of the fragments and indexed samples were sequenced on Nextseq500 sequencer (Illumina, Inc., California, US) with pair-end reads.

2.6. Sequence data analysis

Burrows-Wheeler aligner 0.7.10 [30] was used for mapping the pair-end reads to the human genome (hg19). Local alignment optimization, variant calling, and annotation were performed using GATK 3.2, MuTect, and VarScan. DNA translocation analysis was performed using both Tophat2 and Factera 1.4.3. Variants were filtered using the VarScan filter pipeline. Loci with depth less than 100 were filtered out. White blood cells were sequenced to filter out germline mutations. At least 2 and 5 supporting reads were needed for INDELs in plasma and CSF samples, respectively; while 8 supporting reads were needed for SNVs to be called in both plasma and CSF samples. According to the ExAC, 1000 Genomes, dbSNP, ESP6500SI-V2 database, variants with population frequency over 0.1% were grouped as SNP and excluded from further analysis. Remaining variants were annotated with ANNOVAR and SnpEff v3.6. DNA translocation analysis was performed using both Tophat2 and Factera 1.4.3.

Copy number variation was detected by in-house analysis scripts based on the depth of coverage data of capture intervals. Coverage data were corrected against sequencing bias resulting from GC content and probe design. The average coverage of all captured regions was utilized to normalize the coverage of different samples to comparable scales. Copy number was calculated based on the ratio between the depth of coverage in tumor samples and average coverage of an adequate number ($n > 50$) of samples without copy number variation as references as to each capture interval. Copy number variation is called if the coverage data of the gene region was quantitatively and statistically significantly different from its reference control. The limit of detection for CNVs is 1.5 for deletion and 2.64 for amplification.

2.7. Statistical analysis

Progression-free survival (PFS), was defined as the time from commencement of *EGFR*-TKI treatment to progressive disease (PD) according to RECIST criteria [31]. OS was calculated as the period from the date of first-line systemic treatment to the date of any cause of death or the last follow-up visit. PFS and OS were analyzed using the Kaplan-Meier method and compared between different groups using the log-rank test.

Continuous variables were summarized as either means ± standard deviations (SD) or medians with interquartile ranges and categorical variables by frequencies. Unpaired Wilcoxon signed-rank test was used for continuous variable comparison and two-sided Fisher's exact test was used to compare categorical data, as appropriate. $P < 0.05$ was considered statistically significant. All bioinformatics analyses were performed with R (version 3.3.3, the R Foundation for Statistical Computing, Vienna, Austria) and RStudio (version 1.1.383).

3. Results

3.1. Patient characteristics

Cytology analysis was performed on 167 PE samples, atypical tumor cells were found in 121 patients and 46 without tumor cell morphology in smear samples. Finally, 121 MPE and 40 CNPE samples was successfully used for gene detection. Six CNPE samples were failed for the detection due to insufficient ctDNA. At last, this study enrolled 161 *EGFR*-TKI treatment-naïve metastatic NSCLC patients. The median age was 60.33 ± 11.56 years old (range, 28–87 years). Among them, 86 patients were male and 75 were females. Seventy-two patients had *EGFR* sensitizing mutation detected from their MPE, tissue biopsy samples and subsequently treated with the first generation of *EGFR*-

Table 1
Clinicopathological features of study subjects.

	Malignant pleural effusions (n = 121)	Cytological-negative pleural effusions (n = 40)
Age, years		
Median	60.31 ± 12.17	60.38 ± 9.60
Range	28–87	41–79
Gender, n (%)		
Male	60	26
Female	61	14
Smoking status, n (%)		
Current or Former	45	18
Never	76	22
EGFR status (by ARMS-PCR)	In pleural effusions sediment	In tissue biopsies or plasma
Wild-type	68	
Exon 19 deletion	26	10
L858R	21	12
T790M	0	0
Exon 20 insertion	3	1
Exon 19 deletion + T790M	3	0

TKIs, erlotinib, gefitinib or icotinib. Their characteristics were summarized in Table 1.

3.2. Pleural effusion with negative cytology can also be used as a media for mutation detection

We performed capture-based targeted sequencing to detect and quantify mutations in MPE and CNPE samples. The supernatant of CNPE samples was used for mutation detection. We achieved detection rates, defined as harboring any mutation from the panel used, of 99.2% (120/121) and 100% (40/40) for MPE and CNPE samples, respectively (Fig. 1A).Our results revealed that there was no statistically significant difference in the detection rate between MPE and CNPE samples ($p = 1$). And then we compared the maximum allelic fraction (maxAF) of MPE and CNPE. The median maxAF of MPE and CNPE supernatant were 57.4% and 56.8%, respectively, demonstrating that there was no significant difference in the maxAF between MPE and CNPE samples ($p = 0.77$) (Fig. 1B).

Next, we investigated the genomic profile associated with CNPE. Collectively, 1665 and 388 genomic alterations were identified from 121 MPE and 40 CNPE samples, respectively. As for CNPE samples, we identified 311 single-nucleotide variants (SNVs), 39 insertion/deletions (indels), 22 copy-number amplification (CNA), and 9 rearrangements. And the most frequently mutated gene was *EGFR*, occurring in 52% of patients, followed by *TP53,* occurring in 42% of patients (Fig. 2A). In addition, other classic NSCLC driver mutations were also observed, including 14 cases with *ALK* fusion, 3 cases with *RET* fusion,3 cases with *MET* amplification, and 1 case with *ERBB2* amplification. For MPE samples, we identified 1164 SNVs, 221 indels, 229 CNA, and 29 rearrangements. And the most frequently mutated gene was *EGFR*, occurring in 69% of patients, other driver mutations including 1 case with *ALK* fusion, 1 case with *RET* fusion, and 1 case with *MET* amplification (Fig. 2B).Collectively, our data demonstrate that CNPE supernatant is comparable to MPE in identifying mutations of advanced NSCLC.

3.3. CNPE supernatant is superior to tumor biopsy in detecting EGFR mutations

Cytology negative patients underwent tissue biopsy for treatment guidance. Next, we compared *EGFR* status obtained from CNPE supernatant to that of matched tumor tissue, in which *EGFR* status was confirmed by PCR. *EGFR* activating mutations were detected in 47.5% (19/40) of CNPE supernatant sample and 32.5% (13/40) of tumor biopsies, resulting in a concordance rate of 22.5% (9/40) (Fig. 3). It is worth to note that we identified *EGFR* sensitizing mutations from 10 patients whose matched tumor biopsy samples did not reveal such mutations. Collectively, our data demonstrate that the CNPE sample is superior to tumor tissues in identifying *EGFR* mutation.

3.4. EGFR mutation status in CNPE supernatant predicted TKI treatment

Treatment guidance was based on molecular testing performed on MPE for cytology positive patients; in contrast, tissue biopsies results were used for treatment guidance for cytology negative patients. The PFS and overall survival were analyzed for 72 *EGFR*-TKI treated patients. Among them, 51 were cytology positive and the remaining 21 were cytology negative. Our data showed that MPE patients exhibited comparable PFS ($p = 0.41$) and OS ($p = 0.26$) with CNPE patients treated with *EGFR*-TKI (Fig. 4A and B).

Next, we evaluated the efficacy of *EGFR*-TKI in term of PFS and OS among the 21 cytology negative patients. Among the 21 CNPE patients, 15 had *EGFR* sensitizing mutation detected from their corresponding pleural effusion samples, and the remaining 6 were not. Patients harboring either *L858R* or *exon 19 deletion* had longer PFS (mean 10.4 months) than patients without a detectable mutation (mean 5.1 months, $p = 0.036$) (Fig. 4C and D). Taken together, these results showed that *EGFR* status in CNPE specimen is highly associated with clinical outcomes, and appears to be a reliable way for predicting the efficacy of an *EGFR*-targeted therapy.

At last, we evaluated the efficacy of EGFR-TKIs in 10 patients with EGFR mutation in CNPE specimen but without in matched tumor biopsy samples. Among them, 7 received EGFR-TKIs and three with other treatment. All of the 7 patients developed disease progression with EGFR-TKIs treatment and 6 patients died during the follow-up. The median progression-free survival (PFS) and overall survival (OS) of the cohort were 4.1 months and 12.7 months, ranging from 1 to 20.9 and

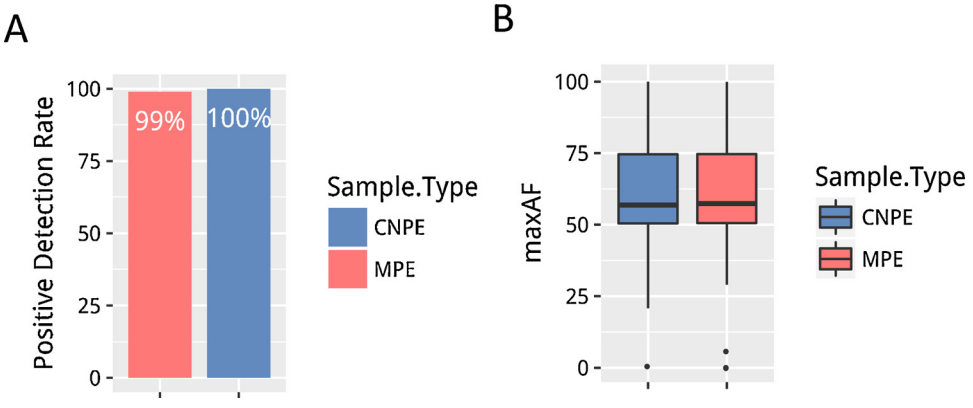

Fig. 1. Mutationdetection rates and maximum allelic fraction (maxAF) in CNPE supernatant and MPE. (A) Detection rates, defined as harboring any mutation from the panel used, in CNPE supernatant (blue) and MPE (red). (B) The median maxAF in CNPE supernatant (blue) and MPE (red) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

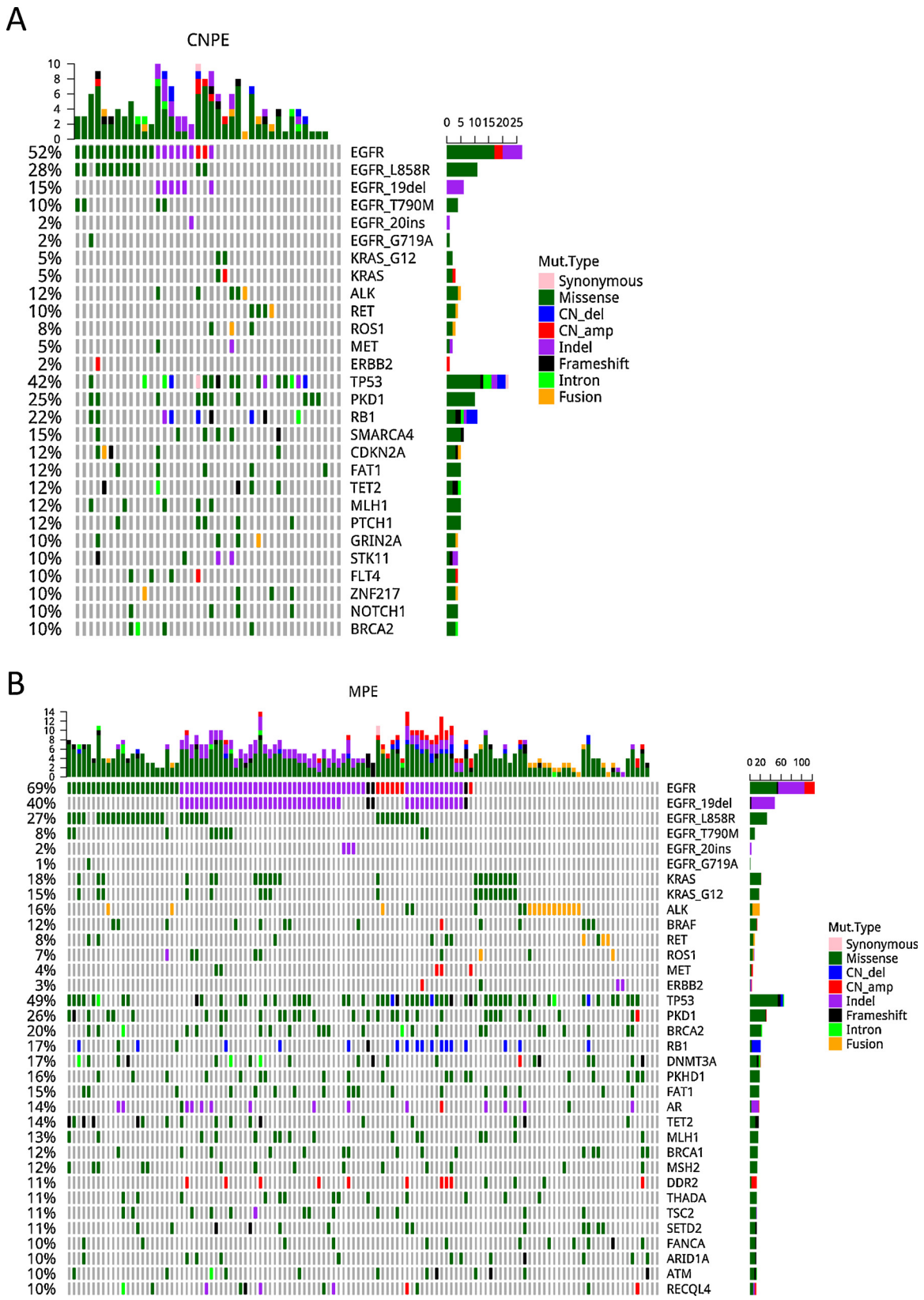

Fig. 2. Oncoprint of CNPE supernatant (A) and MPE (B). Each column represents a patient; each row represents a gene. Different types of mutations were denoted in different colors. The total number of mutations in a patient is summarized by the top bar. The frequency of a mutation in this cohort is summarized by the sidebar.

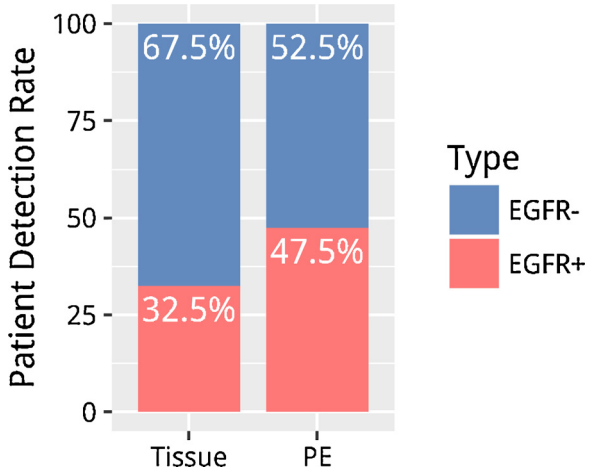

Fig. 3. Detection rates of *EGFR* mutation in the PE supernatant *vs*. tissue biopsy of cytologically negative NSCLC patients.

7.2–33 months, respectively.

4. Discussion

ctDNA in MPE can be used to detect *EGFR* mutations, and this can be useful not only at the beginning of treatment but also for monitoring to determine appropriate ongoing treatment [32]. Numerous studies have demonstrated the usefulness of *EGFR*mutation screening in MPE specimens including supernatant to predict the clinical outcome in cases treated with EGFR-TKI [33–35]. However, cytology fails to detect neoplastic cells in approximately 20–40% of MPE. In the current study, we hypothesized that the CNPE, commonly discarded in practice, maybe an alternative liquid biopsy media to detect *EGFR* mutation status. We observed that the mutation detection rate and maxAF in CNPE supernatant are comparable to MPE in advanced NSCLC. In CNPE supernatant, the high sensitivity of *EGFR* mutations detection in 47.5% of samples (*vs*. 32.5% in tumor biopsy) and in 25% of the cases that were negative in tumor biopsy, indicating that supernatant ctDNA could be useful in clinical practice for accurate detection of *EGFR* mutations in NSCLC patients with CNPE. Furthermore, we also revealed there was no difference in PFS and OS betweenMPE and CNPEpatients treated with *EGFR*-TKI. Among the 21 CNPE patients treated with *EGFR*-TKI, comparable PFS and OS were observed between the tissue biopsy EGFR-positive and CNPE supernatant EGFR-positive groups.

The difficulties of collecting sufficient amount of tumor sample for *EGFR* mutation detection have stimulated interest in analyses using liquid samples, such as plasma [36], urine [37,38],saliva [39], cerebrospinal fluid [40], and pleural fluid [41], which frequently contain ctDNA. In pleural effusions, genomic ctDNA was successfully purified and a high detection rate of mutant *EGFR* was achieved in samples with the positive cytological diagnosis.Therefore, many studies have demonstrated the feasibility of applying MPE supernatant for detecting

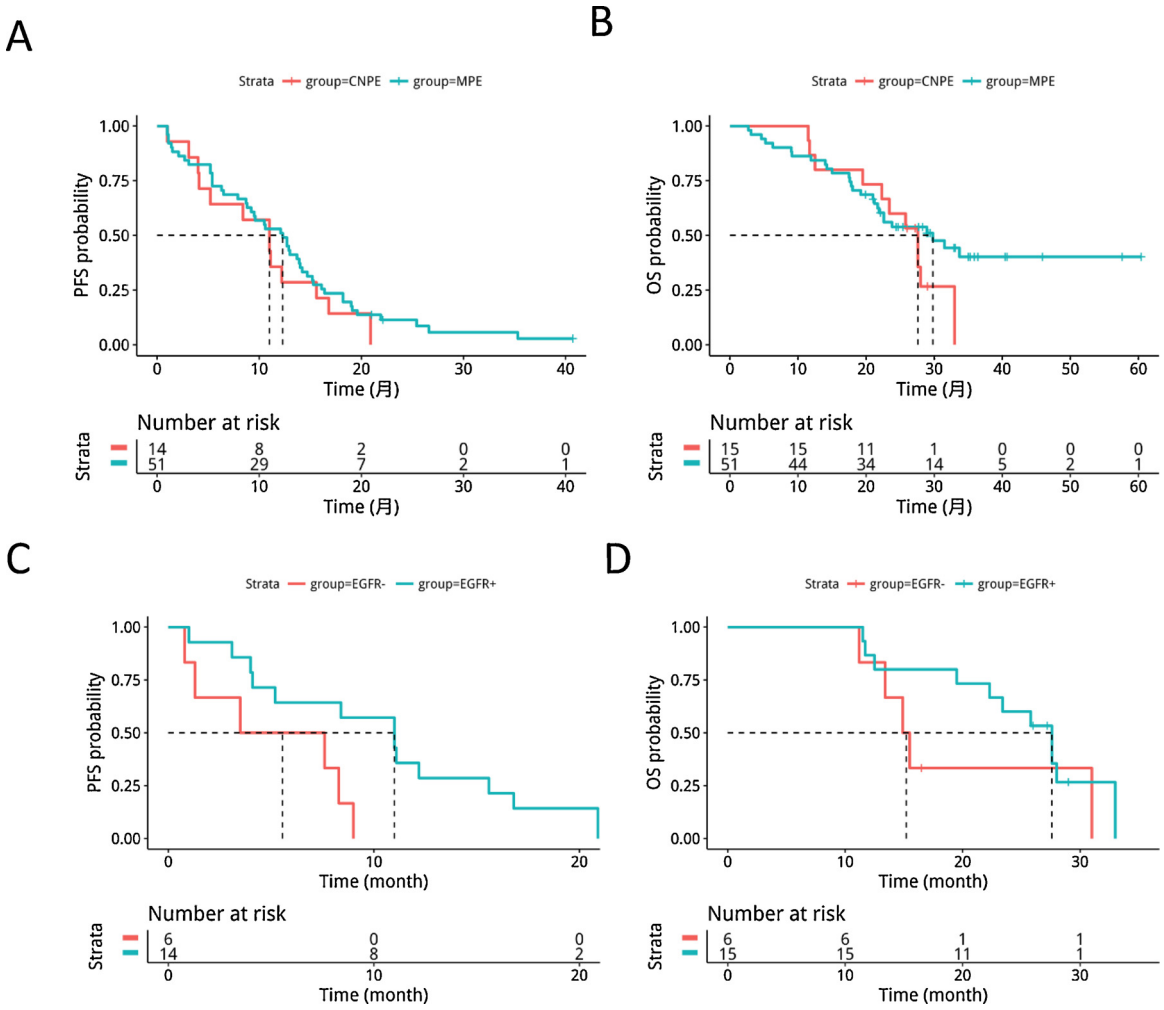

Fig. 4. Comparison of OS and PFS in patients treated withthe first generation of *EGFR*-TKI. Kaplan-Meier curves for OS (A) and PFS (B) in patients with NCPE*vs*MPE group. Kaplan-Meier curves for OS (C) and PFS (D) of patients with NCPE in the tissue *EGFR*+ *vs* PE supernatant *EGFR*+ group.

EGFR mutations, and compared to the conventional materials [23,35,42]. For instance, Liu et al. [42] reported that MPE supernatants are substitutes for metastatic pleural tumor tissues in *EGFR* mutation testing,Jian et al [35] reported that the difference in *EGFR* mutation rate was not significant between plasma and PE supernatant samples, and Lin et al [23] concluded that the cell-free supernatant of PE might be a better resource for mutation detection than cell pellets.In this study, we also reported that *EGFR* activating mutations can be detected in cfDNA extracted from the supernatant of MPE samples *via* NGS assay, and MPE supernatant is comparable to precipitate sample in terms of identifying *EFGR* mutation or reflecting the genomic profile.These data suggested that MPE supernatant could be a valid surrogate for *EGFR* analysis in NSCLC patients.

Cytology remains the diagnostic standard for evaluating pleural effusion samples. However, it is can be difficult to establish a diagnosis of MPE in subjects with cytology-negative effusions [43], since it requires invasive pleural biopsy tests, such as thoracoscopy. In many cases, CNPE specimens are judged inadequate for *EGFR* testing due to few tumor cells. In fact, Kimura et al. [33] reported that no *EGFR* mutations were detected in PE that did not contain malignant cells. In addition, Kawahara et al. [44] found that *EGFR* mutations could not be detected in cfDNA samples from nonmalignant cells. By contrast, Shin et al [45] reported that among 24 cytology negative or cell paucity samples, mutations were found in eight samples by PCR. Therefore, in this study, we conducted a detailed investigation of *EGFR* mutations in advanced NSCLC patients with PE of opposite cytological diagnoses. Interestingly, we observed *EGFR* mutations in 47.5% of the samples without cytopathologic evidence of neoplastic cells. Although the reason for these false negative diagnoses in *EGFR* mutation positive samples is unclear, cytologically, the tumor cells in these cases might be smaller, cannot be distinguished from reactive or inflammatory mesothelial cells, and cause false negative results. In addition, we also speculate that tumor DNA was present as cfDNA, which has potential application value in differential diagnosis of benign and malignant pleural effusion. This finding further indicates CNPE open to other important applications, such as the possibility to detect somatic mutations of frequently mutated genes in NSCLC (*i.e.*, *p53, EGFR,KRAS*) for early diagnosis and treatment.

Recently, a number of studies indicate that various solid tumors, particularly lung cancers, showed intra-tumoral heterogeneity as well as diverse mutation profiles between primary tumor and metastatic lesion [46,47]. Thus, when it comes to detecting the existing mutations, PE may be a better material than a localized tissue biopsy sample. In this series, 10 CNPE supernatant samples showed the *EGFR* mutations corresponding to that observed in matching tumor tissue, we identified *EGFR* mutations from 10 patients whose matched tumor biopsy samples did not reveal such mutation,and we were unable to detect *EGFR* mutations in 3CNPE samples as compared with tumor biopsy sample. This could be ascribed to poor sampling and/or tumor heterogeneity.

In conclusion, our study interrogated the genomic profiles and *EGFR* mutation status in 121 MPE and 40 CNPE supernatant samples, demonstrated that CNPE supernatant also provided a comprehensive profile of NSCLC since it identified a significant number of CNV events and mutations. CNPE supernatant can serve as a reliable lipid biopsy media for *EGFR* mutational detection, evidenced by a comparable detection rate with tumor biopsy. Furthermore, *EGFR* status in CNPE supernatant is highly associated with clinical outcome.

Authors' contributions

Concept and design: ZS and YZ; Data collection: ZS, WW and YZ; Data analysis and interpretation: ZS, ML, and JL; Manuscript writing: ZS and JL; Final approval of manuscript: All authors; Accountable for all aspects of the work: All authors.

Funding

This work was supported by the National Natural Science Foundation of China (Grant no: 81802276).

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Zhejiang Cancer Hospital. Informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated from the patients during the current study are not publicly available in accordance with the local health research ethics protocols, but might be available from the corresponding author.

Competing interests

Min Li, and Junjun Liu are the employee of Burning Rock Biotech Inc.

Declaration of Competing Interest

The contents of this manuscript have never been copyrighted or previously published. And it is not under consideration for publication elsewhere. All authors participated in its planning, execution, analysis, composition and critical review. And the final version has been approved. All authors have no conflict of Interest.

Acknowledgements

Not applicable.

References

[1] J. Chen, Z. Li, N. Xu, X. Zhang, Y. Wang, D. Lin, Efficacy of medical thoracoscopic talc pleurodesis in malignant pleural effusion caused by different types of tumors and different pathological classifications of lung cancer, Int. J. Clin. Exp. Med. 8 (10) (2015) 18945.

[2] A. Kawahara, K. Azuma, A. Sumi, T. Taira, K. Nakashima, E. Aikawa, H. Abe, T. Yamaguchi, S. Takamori, J. Akiba, Identification of non-small-cell lung cancer with activating EGFR mutations in malignant effusion and cerebrospinal fluid: rapid and sensitive detection of exon 19 deletion E746-A750 and exon 21 L858R mutation by immunocytochemistry, Lung Cancer 74 (1) (2011) 35–40.

[3] W.D. Travis, N. Rekhtman, G.J. Riley, K.R. Geisinger, H. Asamura, E. Brambilla, K. Garg, F.R. Hirsch, M. Noguchi, C.A. Powell, Pathologic diagnosis of advanced lung cancer based on small biopsies and cytology: a paradigm shift, J. Thoracic Oncol. Off. Publ. Int. Assoc. Study Lung Cancer 5 (4) (2010) 411–414.

[4] G.D.C. Santos, M.A. Saieg, W. Geddie, N. Leighl, EGFR gene status in cytological samples of nonsmall cell lung carcinoma, Cancer Cytopathol. 119 (2) (2011) 80–91.

[5] L. Jie, G. Ye, D. Rui, D. Min, L. Yaodan, D. Yanqing, Detection of EGFR mutation in supernatant, cell pellets of pleural effusion and tumor tissues from non-small cell lung cancer patients by high resolution melting analysis and sequencing, Int. J. Clin. Exp. Pathol. 7 (12) (2014) 8813–8822.

[6] H. Kimura, Y. Fujiwara, T. Sone, H. Kunitoh, T. Tamura, K. Kasahara, K. Nishio, High sensitivity detection of epidermal growth factor receptor mutations in the pleural effusion of non-small cell lung cancer patients, Cancer Sci. 97 (7) (2010) 642–648.

[7] S. Wu, C.-H. Gow, C. Yu, Y. Chang, C.-H. Yang, Hsu Y-c, J. Shih, Y. Lee, P. Yang, Frequent EGFR mutations in malignant pleural effusion of lung adenocarcinoma, Eur. Respir. J. (2008).

[8] X. Liu, Y. Lu, G. Zhu, Y. Lei, L. Zheng, H. Qin, C. Tang, G. Ellison, R. McCormack, Q. Ji, The diagnostic accuracy of pleural effusion and plasma samples versus tumour tissue for detection of EGFR mutation in patients with advanced non-small cell lung cancer: comparison of methodologies, J. Clin. Pathol. 66 (12) (2013) 1065–1069.

[9] C.D. Yeo, J.W. Kim, K.H. Kim, J.H. Ha, C.K. Rhee, S.J. Kim, Y.K. Kim, C.K. Park, S.H. Lee, M.S. Park, Detection and comparison of EGFR mutations in matched tumor tissues, cell blocks, pleural effusions, and sera from patients with NSCLC with

malignant pleural effusion, by PNA clamping and direct sequencing, Lung cancer 81 (2) (2013) 207–212.
[10] S.-G. Wu, C.-J.Y.u C-H Gow, Y.-L. Chang, C.-H. Yang, Y.-C. Hsu, J.-Y. Shih, Y.-C. Lee, P.-C. Yang, Frequent epidermal growth factor receptor gene mutations in malignant pleural effusion of lung adenocarcinoma, Eur. Respir. J. 32 (4) (2008) 924–930.
[11] G. Jian, Z.Z. Songwen, D. Qinfang, Z. Jie, T. Liang, Z. Caicun, Prediction of epidermal growth factor receptor mutations in the plasma/pleural effusion to efficacy of gefitinib treatment in advanced non-small cell lung cancer, J. Cancer Res. Clin. Oncol. 136 (9) (2010) 1341–1347.
[12] J. Soh, S. Toyooka, K. Aoe, H. Asano, S. Ichihara, H. Katayama, A. Hiraki, K. Kiura, M. Aoe, Y. Sano, Usefulness of EGFR mutation screening in pleural fluid to predict the clinical outcome of gefitinib treated patients with lung cancer, Int. J. Cancer 119 (10) (2010) 2353–2358.
[13] H. Kimura, Y. Fujiwara, T. Sone, H. Kunitoh, T. Tamura, K. Kasahara, K. Nishio, EGFR mutation status in tumour-derived DNA from pleural effusion fluid is a practical basis for predicting the response to gefitinib, Br. J. Cancer 95 (10) (2006) 1390–1395.
[14] M.-C. Wang, C.-L. Wang, T.-L. Chen, Chang JW-C, J.-J. Lu, P.-Y. Chang, C.-C. Chiou, Predicting outcomes of EGFR-targeted therapy in non-small cell lung cancer patients using pleural effusions samples and peptide nucleic acid probe assay, Clin. Chem. Lab. Med. (CCLM) 55 (12) (2017) 1979–1986.
[15] A.L. Volckmar, H. Sültmann, A. Riediger, T. Fioretos, P. Schirmacher, V. Endris, A. Stenzinger, S. Dietz, A field guide for cancer diagnostics using cell-free Dna: from principles to practice and clinical applications, Genes Chromosomes Cancer 57 (3) (2018) 123–139.
[16] R. Mead, M. Duku, P. Bhandari, I. Cree, Circulating tumour markers can define patients with normal colons, benign polyps, and cancers, Br. J. Cancer 105 (2) (2011) 239.
[17] T. Imamura, S. Komatsu, D. Ichikawa, T. Kawaguchi, M. Miyamae, W. Okajima, T. Ohashi, T. Arita, H. Konishi, A. Shiozaki, Liquid biopsy in patients with pancreatic cancer: circulating tumor cells and cell-free nucleic acids, World J. Gastroenterol. 22 (25) (2016) 5627.
[18] E. Crowley, Loupakis F. Di NF, A. Bardelli, Liquid biopsy: monitoring cancer-genetics in the blood. Nat Rev Clin Oncol, Nat. Rev. Clin. Oncol. 10 (8) (2013) 472.
[19] Z. Wang, Y. Cheng, T. An, H. Gao, K. Wang, Q. Zhou, Y. Hu, Y. Song, C. Ding, F. Peng, Detection of EGFR mutations in plasma circulating tumour DNA as a selection criterion for first-line gefitinib treatment in patients with advanced lung adenocarcinoma (BENEFIT): a phase 2, single-arm, multicentre clinical trial, Lancet Respir. Med. 6 (9) (2018) 681–690.
[20] L. De Mattos-Arruda, R. Mayor, C.K. Ng, B. Weigelt, F. Martínez-Ricarte, D. Torrejon, M. Oliveira, A. Arias, C. Raventos, J. Tang, Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma, Nat. Commun. 6 (2015) 8839.
[21] Y. Li, W. Pan, I.D. Connolly, S. Reddy, S. Nagpal, S. Quake, M.H. Gephart, Tumor DNA in cerebral spinal fluid reflects clinical course in a patient with melanoma leptomeningeal brain metastases, J. Neurooncol. 128 (1) (2016) 93–100.
[22] Y. Li, B. Jiang, J. Yang, X. Zhang, Z. Zhang, J. Ye, W. Zhong, H. Tu, H. Chen, Z. Wang, Unique genetic profiles from cerebrospinal fluid cell-free DNA in leptomeningeal metastases of EGFR-mutant non-small-cell lung cancer: a new medium of liquid biopsy, Ann. Oncol. 29 (4) (2018) 945–952.
[23] J. Lin, Y. Gu, R. Du, M. Deng, Y. Lu, Y. Ding, Detection of EGFR mutation in supernatant, cell pellets of pleural effusion and tumor tissues from non-small cell lung cancer patients by high resolution melting analysis and sequencing, Int. J. Clin. Exp. Pathol. 7 (12) (2014) 8813.
[24] A. Kawahara, C. Fukumitsu, K. Azuma, T. Taira, H. Abe, Y. Takase, K. Murata, E. Sadashima, S. Hattori, Y. Naito, A Combined test using both cell sediment and supernatant cell-free DNA in pleural effusion shows increased sensitivity in detecting activating EGFR mutation in lung cancer patients, Cytopathology 29 (2) (2018) 150–155.
[25] A. Kawahara, C. Fukumitsu, T. Taira, H. Abe, Y. Takase, K. Murata, T. Yamaguchi, K. Azuma, H. Ishii, S. Takamori, Epidermal growth factor receptor mutation status in cell-free DNA supernatant of bronchial washings and brushings, Cancer Cytopathol. 123 (10) (2015) 620–628.
[26] L. Tong, N. Ding, J. Li, X. Wang, Y. Zhang, X. Xu, M. Ye, C. Li, H. Zhang, X. Zhu, MA 20.13 etDNA: tumor-derived DNA from pleural effusion supernatant as a promising source for NGS-Based mutation profiling in lung Cancer, J. Thorac. Oncol. 12 (11) (2017) S1891.
[27] G. Antunes, E. Neville, J. Duffy, N. Ali, BTS guidelines for the management of malignant pleural effusions, Thorax 58 (Suppl. 2) (2003) ii29.
[28] K. Ong, V. Indumathi, W. Poh, Y. Ong, The diagnostic yield of pleural fluid cytology in malignant pleural effusions, Singapore Med. J. 41 (1) (2000) 19–23.
[29] A.A. Renshaw, B.R. Dean, E.S. Cibas, K.H. Antman, D.J. Sugarbaker, The role of cytologic evaluation of pleural fluid in the diagnosis of malignant mesothelioma, Chest 111 (1) (1997) 106–109.
[30] H. Li, R. Durbin, Fast and accurate long-read alignment with Burrows–Wheeler transform, Bioinformatics 26 (5) (2010) 589–595.
[31] E.A. Eisenhauer, P. Therasse, J. Bogaerts, L.H. Schwartz, D. Sargent, R. Ford, J. Dancey, S. Arbuck, S. Gwyther, M. Mooney, et al., New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1), Eur. J. Cancer (Oxford, England: 1990) 45 (2) (2009) 228–247.
[32] B.S. Sorensen, L. Wu, W. Wei, J. Tsai, B. Weber, E. Nexo, P. Meldgaard, Monitoring of epidermal growth factor receptor tyrosine kinase inhibitor-sensitizing and resistance mutations in the plasma DNA of patients with advanced non–small cell lung cancer during treatment with erlotinib, Cancer 120 (24) (2014) 3896–3901.
[33] H. Kimura, Y. Fujiwara, T. Sone, H. Kunitoh, T. Tamura, K. Kasahara, K. Nishio, EGFR mutation status in tumour-derived DNA from pleural effusion fluid is a practical basis for predicting the response to gefitinib, Br. J. Cancer 95 (10) (2006) 1390.
[34] J. Soh, S. Toyooka, K. Aoe, H. Asano, S. Ichihara, H. Katayama, A. Hiraki, K. Kiura, M. Aoe, Y. Sano, Usefulness of EGFR mutation screening in pleural fluid to predict the clinical outcome of gefitinib treated patients with lung cancer, Int. J. Cancer 119 (10) (2006) 2353–2358.
[35] G. Jian, Z. Songwen, Z. Ling, D. Qinfang, Z. Jie, T. Liang, Z. Caicun, Prediction of epidermal growth factor receptor mutations in the plasma/pleural effusion to efficacy of gefitinib treatment in advanced non-small cell lung cancer, J. Cancer Res. Clin. Oncol. 136 (9) (2010) 1341–1347.
[36] Y. Seki, Y. Fujiwara, T. Kohno, K. Yoshida, Y. Goto, H. Horinouchi, S. Kanda, H. Nokihara, N. Yamamoto, K. Kuwano, Circulating cell-free plasma tumour DNA shows a higher incidence of EGFR mutations in patients with extrathoracic disease progression, ESMO Open 3 (2) (2018) e000292.
[37] F.S. Togneri, D.G. Ward, J.M. Foster, A.J. Devall, P. Wojtowicz, S. Alyas, F.R. Vasques, A. Oumie, N.D. James, K.K. Cheng, Genomic complexity of urothelial bladder cancer revealed in urinary cfDNA, Eur. J. Hum. Genet. 24 (8) (2016) 1167–1174.
[38] N. Tchekmedyian, R. Mudad, F.F. Blanco, V.M. Raymond, J. Garst, M.G. Erlander, E. Haura, D. Berz, Longitudinal monitoring of ctDNA EGFR mutation burden from urine correlates with patient response to EGFR TKIs: a case series, Lung Cancer (2017) 22–28.
[39] D. Pu, H. Liang, F. Wei, D. Akin, Z. Feng, Q.X. Yan, Y. Li, Y. Zhen, L. Xu, G. Dong, Evaluation of a novel saliva-based epidermal growth factor receptor mutation detection for lung cancer: a pilot study, Thorac. Cancer 7 (4) (2016) 428–436.
[40] S. Ying, H. Ke, Y. Ding, Y. Liu, X. Tang, D. Yang, M. Li, J. Liu, B. Yu, J. Xiang, et al., Unique genomic profiles obtained from cerebrospinal fluid cell-free DNA of non-small cell lung cancer patients with leptomeningeal metastases, Cancer Biol. Ther. (2018) 1–9.
[41] J. Soh, S. Toyooka, H. Suehisa, N. Kobayashi, S. Ito, M. Yamane, M. Aoe, Y. Sano, K. Kiura, H. Date, EGFR mutation status in pleural fluid predicts tumor responsiveness and resistance to gefitinib, Lung Cancer 56 (3) (2007) 445–448.
[42] D. Liu, Y. Lu, Z. Hu, N. Wu, X. Nie, Y. Xia, Y. Han, Q. Li, G. Zhu, C. Bai, Malignant pleural effusion supernatants are substitutes for metastatic pleural tumor tissues in EGFR mutation test in patients with advanced lung adenocarcinoma, PLoS One 9 (2) (2014) e89946.
[43] C.W. Bedrossian, Diagnostic problems in serous effusions, Diagn. Cytopathol. 19 (2) (1998) 131–137.
[44] A. Kawahara, C. Fukumitsu, T. Taira, H. Abe, Y. Takase, K. Murata, T. Yamaguchi, K. Azuma, H. Ishii, S. Takamori, Epidermal Growth Factor Receptor Mutation Status in Cell-free DNA Supernatant of Bronchial Washings and Brushings, (2015).
[45] S. Shin, J. Kim, Y. Kim, S.M. Cho, K.A. Lee, Assessment of real-time PCR method for detection of EGFR mutation using both supernatant and cell pellet of malignant pleural effusion samples from non-small-cell lung cancer patients, Clin. Chem. Lab. Med. 55 (2017).
[46] H.-S. Han, D.-W. Eom, J.H. Kim, K.-H. Kim, H.-M. Shin, J.Y. An, K.M. Lee, K.H. Choe, K.H. Lee, S.T. Kim, EGFR mutation status in primary lung adenocarcinomas and corresponding metastatic lesions: discordance in pleural metastases, Clin. Lung Cancer 12 (6) (2011) 380–386.
[47] J. Zhang, J. Fujimoto, J. Zhang, D.C. Wedge, X. Song, J. Zhang, S. Seth, C.-W. Chow, Y. Cao, C. Gumbs, Intratumor heterogeneity in localized lung adenocarcinomas delineated by multiregion sequencing, Science 346 (6206) (2014) 256–259.