Article Title: Liquid biopsy assay for pulmonary adenocarcinoma using supernatants from core-needle biopsy specimens

Abstract:
Background: Genomic testing is the cornerstone of the treatment of patients with non-small-cell lung cancer. However, comprehensive molecular testing of small specimens may be inadequate due to limited tissue. Liquid biopsy has emerged as a new method of genotyping. In this study, we evaluate the feasibility of using supernatants from core needle biopsy samples of lung adenocarcinoma for genomic testing.

Methods: Core needle biopsy specimens and their supernatants were collected from patients (n = 48) with lung adenocarcinoma. Genomic testing results of the supernatant samples were compared with results derived from paired tissue samples from the same patient.

Result: All 48 supernatant samples yield adequate cell-free DNA, but the concentration of cell-free RNA did not meet the criteria for analysis. The concordance rate between the genomic testing results of supernatants and the corresponding tissue samples was 95.8% (kappa = 0.899). The coincidence rate of detectable mutations at the DNA level in the supernatants was up to 100%.

Conclusion: Core needle biopsy supernatants can provide a valuable specimen source for genotyping pulmonary adenocarcinoma. However, the method of preserving and extracting RNA from supernatant specimens needs further improvement.

Keywords: cell-free DNA, genomic testing, lung adenocarcinoma, percutaneous core-needle biopsy, supernatant

INTRODUCTION

According to global cancer statistics for 2020, lung cancer still has the highest mortality among all malignancies. Genomic testing plays an important role in the disease management of advanced non-small-cell lung carcinoma (NSCLC), especially for adenocarcinoma. Patients harboring actionable driver mutation can respond to targeted therapy and thus increase survival. The authoritative international guidelines, such as the National Comprehensive Cancer Network (NCCN), College of American Pathologists (CAP), International Association for the Study of Lung Cancer

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(IASLC), and Association for Molecular Pathology (AMP), suggest that molecular testing must be performed in advanced NSCLC patients.

Clinically, the most commonly used small specimens for molecular analysis are tissues or cells obtained by core needle biopsy (CNB) or fine-needle aspiration biopsy (FNAB). However, biopsy specimens may be biased because of temporal and spatial heterogeneity. Moreover, previous studies have shown that about 10–20% of small specimens remain inadequate for molecular testing due to poor sampling quality or sequencing failure. Liquid biopsy is an alternative approach for genomic testing in advanced NSCLC patients, especially in situation biopsy specimens that are inadequate for further molecular testing. Cell-free plasma DNA (cf-DNA) is the most commonly used liquid biopsy specimen in clinical practice, while its effectiveness is limited by the amount of tumor shedding and cf-DNA in plasma. Thus, multiple tumor-derived cf-DNA/RNA, including malignant pleural, ascites, sputum, and cerebrospinal fluid, are explored as potential alternative liquid biopsy specimens. Notably, a recent study has demonstrated that the supernatants from FNAB can be used for molecular testing. Because CNB is more widely used in the biopsy of lung mass due to its higher diagnostic accuracy than FNAB, this paper mainly studied the feasibility of using supernatants from CNB samples of lung adenocarcinoma for genomic analysis testing. As far as we know, there has been no report on the use of supernatants from CNB samples of lung cancer for genome detection.

PATIENTS AND METHODS

Patients

A total of 96 patients with highly suspected lung cancer who underwent a biopsy in our institution from September 2020 to September 2021 were included in this study. Tissue and corresponding supernatant specimens from CNB were collected. Excluding patients with benign pathologic findings, squamous-cell carcinoma, and those who did not undergo genetic testing, eventually 48 patients were included in statistical analysis. This protocol was in accordance with the Helsinki Declaration. Our institutional review board approved the study and waived the informed consent requirement.

Overall gene mutation status in supernatants from CNB specimens

The cf-DNA and cf-RNA were extracted from supernatant samples simultaneously using a QIAamp Circulating Nucleic Acid Kit (Qiagen) following the manufacturer’s instructions. The DNA and RNA of tumor tissue were extracted from formalin-fixed, paraffin-embedded samples using an FFPE DNA Extraction Kit (Amoy Diagnostics) according to the manufacturer’s protocol. The purified cf-DNA/RNA concentration was measured by the QuantiFluor dsDNA System (Promega).

Genomic testing

The concentration of cf-DNA and cf-RNA should be greater than 2 and 10 ng/μl, respectively. According to the manufacturer’s instructions, the genomic testing of all samples was carried out using an AmoyDx Multi-Gene Mutations Detection Kit (Amoy Diagnostics). The DNA-based mutation detection and RNA-based fusion detection real-time PCR assays were contained in the kit, which can detect 118 hotspot mutations/fusions in genes of EGFR, KRAS, BRAF, NRAS, HER2, PIK3CA, ALK, ROS1, MET, and RET simultaneously.

Statistical analysis

Categorical variables were described as frequencies and percentages, and continuous variables were described as the mean ± SD. Statistical analyses were performed using the Mann–Whitney U test for continuous variables or Fisher’s exact test for categorical variables in SPSS 23 (IBM). p < 0.05 was considered statistically significant.

RESULT

Patient characteristics

The baseline clinical and pathological characteristics of 48 patients with lung adenocarcinoma enrolled in this study are shown in Table 1. Except for smoking status, there was no significant statistical difference in most parameters between the positive and negative gene mutation groups. The mutation proportion was significantly higher in former smokers than those who had never smoked. Figure 1 shows the proportion of various genetic mutation statuses in the study patients enrolled.

Obtaining of tissue and corresponding supernatant specimens

Percutaneous puncture biopsy was performed for target lesions using a 16G or 18G CNB needle (Argon Medical Devices). The obtained tissue samples were placed in 20 ml of normal saline for temporary storage. After the operation, the tissue samples were transferred to formalin for further pathological diagnosis, and the remaining supernatants were collected and stored at −20°C until use.

DNA/RNA exaction

The cf-DNA and cf-RNA were extracted from supernatant samples simultaneously using a QIAamp Circulating Nucleic Acid Kit (Qiagen) following the manufacturer’s instructions. The DNA and RNA of tumor tissue were extracted from formalin-fixed, paraffin-embedded samples using an FFPE DNA Extraction Kit (Amoy Diagnostics) according to the manufacturer’s protocol. The purified cf-DNA/RNA concentration was measured by the QuantiFluor dsDNA System (Promega).
All 48 patients obtained tumor tissue samples and their corresponding supernatant samples via CNB. Except for two cases of MET, exon 14 skipping mutation were detected in tumor tissue specimens but not in supernatant specimens. All remaining tissue specimens and the corresponding supernatant specimens showed the same genomic testing results. The overall concordance rate was 95.8% (kappa = 0.899; see Table 2).

The effectiveness of genomic testing at the DNA level using supernatant samples

Excluded the two cases of MET 14 exon skip mutation, which should be detected at the RNA level, the remaining genomic testing results of the supernatant samples were 100% consistent with tissue samples at the DNA level. In the cases of MET 14 exon skip mutation, RNA levels detected a peak at the corresponding mutation site, although the peak was not high enough to diagnose the mutation.

**DISCUSSION**

Lung cancer is one of the cancers with the highest mortality and morbidity worldwide. In recent years, targeted therapy has gradually become a first-line treatment recommendation for unresectable NSCLC, especially for adenocarcinoma.

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**TABLE 1** Clinical and pathological characteristics of enrolled patients

| Mutation status | Overall N = 48 | Mutation positive N = 37 | Mutation negative N = 11 | p value |
|-----------------|---------------|-------------------------|-------------------------|---------|
| Age             |               |                         |                         |         |
| Mean ± SD      | 68.8 ± 10.8   | 68.7 ± 10.9             | 68.9 ± 10.8             | 0.902   |
| Sex            |               |                         |                         |         |
| Male           | 20 (41.7%)    | 13 (35.1%)              | 7 (63.6%)               | 0.162   |
| Female         | 28 (58.3%)    | 24 (64.9%)              | 4 (36.4%)               |         |
| Smoking status |               |                         |                         |         |
| Never          | 33 (68.8%)    | 8 (21.6%)               | 7 (63.6%)               | 0.022   |
| Former         | 15 (31.2%)    | 29 (78.4%)              | 4 (36.4%)               |         |
| Diameter of lesion |         |                         |                         |         |
| Mean ± SD (cm) | 3.0 ± 1.6     | 2.9 ± 1.1               | 3.3 ± 2.8               | 0.750   |
| Diameter of biopsy needle |       |                         |                         |         |
| 18G            | 12 (25.0%)    | 10 (27.0%)              | 2 (18.2%)               | 0.705   |
| 16G            | 36 (75.0%)    | 27 (73.0%)              | 9 (81.8%)               |         |
| Number of specimens |       |                         |                         | 0.500   |
| Mean ± SD      | 3.4 ± 1.4     | 3.4 ± 1.5               | 3.6 ± 1.1               |         |

**Abbreviations:** MWA, microwave ablation; SD, standard deviation.

**TABLE 2** Concordance analysis of overall status between tumor tissue samples and supernatant samples

| Overall mutation status in matched supernatant cf-DNA samples | Overall mutation status in tissues |
|--------------------------------------------------------------|-----------------------------------|
| Mutation positive                                           | Mutation positive                 |
| Mutation negative                                           | Mutation negative                 |
| Total                                                       | Total                             |
| Mutation positive                                           | 33                                |
| Mutation negative                                           | 2                                 |
| Total                                                       | 35                                |
| Concordance rate                                           | 95.8% (p = 0.5)                   |
| Kappa                                                      | 0.899 (p < 0.0001)                |
| Sensitivity                                               | 94.3%                             |
| Specificity                                               | 100%                              |
| PPV                                                        | 100%                              |
| NPV                                                        | 86.7%                             |

**Abbreviations:** NPV, negative predictive value; PPV, positive predictive value.

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**FIGURE 1** Scale maps of the gene mutation status of enrolled patients
Patients harboring actionable driver mutation will benefit from targeted therapy and have significantly longer survival. As outlined in the CAP/IASLC/AMP guidelines, evaluation of tissue specimens remains the cornerstone for NSCLC diagnosis and genomic testing. However, with the increasing number of molecular targets to be detected and the addition of immunotherapy-related indicators, the rational application of small specimens obtained from the biopsy is becoming more and more important. What is more, despite clinical efforts to improve the quality of small specimens, 10–20% of small specimens obtained by biopsy are insufficient for genomic testing. Thus, finding alternative specimen sources for genetic testing cannot be overemphasized. In this study, we demonstrated that supernatants from CNB samples of lung cancer samples contain a significant amount of DNA that can be used as an alternative specimen source for genomic testing. This substantial amount of DNA is often overlooked in clinical work. Furthermore, the result of mutation detection between tissue specimens obtained by CNB and corresponding supernatant specimens was highly consistent.

Although tissue specimens are recommended for genomic testing of NSCLC, some small specimens obtained by biopsy may not be sufficient for genomic testing because the biopsy target lesions are too small or located at high-risk biopsy sites, limiting the number of samples and the technical factors of physicians. Consequently, these patients may be treated without knowing the genotyping or may need to undergo a repeat biopsy. To solve this problem, liquid biopsy using cf-DNA/RNA for mutation detection has become the focus of research. Circulating cf-DNA in plasma is the most commonly used liquid biopsy specimen in the clinic and has been recommended in testing for EGFR T790M in patients who are suspected of relapse and do not have tissue samples available for mutation detection. Recently, the use of pleural effusion, cerebrospinal fluid, and sputum as liquid biopsy specimens for genotyping has also shown excellent prospects. However, liquid biopsy using fluids naturally present in the human body, including plasma, sputum, pleural effusion, and cerebrospinal fluid, is limited by the amount of cf-DNA/RNA in the tissue fluid, which is impacted by stage of disease, tumor burden, apoptosis rate, tumor vascularization, and the metastatic potential of the tumor. Thus, the positive rate of using these tissue fluids for genomic testing is not very high.

A study has shown that FNA supernatants are a valuable alternative source for genotyping lung adenocarcinoma, and the results are highly consistent with tissue specimens. Above all, the supernatants of biopsy specimens are not limited by tumor status, and the diagnostic efficiency is greatly improved. CNB is more widely used in clinical practice than FNA because of its ability to achieve higher diagnostic efficiency without on-site pathologist confirmation, and to provide a larger volume of tumor tissue for pathological typing and genomic testing. This study demonstrated that the overall concordance rate of genomic testing between tissue and its corresponding supernatants was 95.8%. The coincidence rate of detectable mutations at the DNA level in the supernatants was 100%. It is worth mentioning that in the process of patient collection, the puncture biopsy of a male patient only obtained a small number of tissue specimens due to the location of the target lesion adjacent to the large pulmonary vessels and the size of the lesion was small, so there were not enough samples for genomic testing while satisfying the diagnosis of the diseases. Because of advanced age, the patient could not tolerate surgery and chemotherapy. Based on the EGFR exon 21 L858R mutation result from the liquid biopsy of CNB supernatant, the patient received tyrosine kinase inhibitor (TKI) treatment and responded well. The ability to genotype the CNB supernatants has great significance for patients whose genomic testing results are unavailable because of limited tissue samples. Based on the mutation detected from CNB supernatants, these patients may qualify for targeted therapy or be eligible for enrollment into a clinical trial. The ability to genotype the CNB supernatant also allows tissue samples to be saved for other ancillary studies, including in situ hybridization assay (e.g. ALK and EGFR FISH) and immunohistochemistry (e.g. PD-L1).

A previous study showed that the concordance rate of genomic testing between tissues and CNB supernatants was slightly lower than that of FNA supernatants. The author attributes this to the fact that FNA supernatants are likely enriched in tumor DNA due to high tumor cell turnover and increased fragility of cells in the process of aspiration and centrifugation. In this study, the mean concentration of purified cf-DNA in the supernatant samples was 17.05 ng/μl (7.1–59.9 ng/μl), sufficient for genomic testing. We believe that the diagnostic efficiency of supernatants from CNB specimens is no less than that of supernatants from FNAB.

The method we proposed to obtain DNA from CNB supernatants for genotyping is simple and convenient, and can be easily incorporated into the clinical workflow. The CNB supernatants, which are rich in cf-DNA, can reduce the genomic testing time by eliminating the prior steps, such as extracting nucleic acid from tissue. For NSCLC patients, genomic testing results are critical to disease management, and reduced turnaround time can significantly improve patient care.

While our study demonstrated a very high concordance rate of genomic testing between tissues and their corresponding CNB supernatants, two cases of false-negative results were noted. Both were MET exon 14 skipping, which belongs to the mutation detected at the RNA level. The failure was due to poor RNA extraction. The reason may be that the RNA structure is unstable, and is rapidly destroyed in the supernatant of ordinary normal saline. It may be necessary to improve the components of the supernatant to protect RNA, therefore we should be vigilant against negative results, which can be further verified by tissue specimens. In addition, the mutation type of the gene fusions (e.g. ALK, RET, and ROS1) was not included in this study, and this also needs to be further explored.

In summary, this study demonstrated that CNB supernatant is a valuable specimen source for genotyping lung adenocarcinoma. However, the method of preserving and
extracting RNA from supernatant specimens needs further improvement. Further studies are needed to evaluate the practicability of incorporating CNB supernatant into the clinical workflow of genomic testing.

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CONFLICT OF INTEREST
The authors report no conflict of interest.

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