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Short communication

# Liquid biopsy of fine-needle aspiration supernatant for lung cancer genotyping

Nicolas Guibert[a,b], Hisashi Tsukada[c], David H. Hwang[d], Emily Chambers[b], Edmund S. Cibas[d], Tejus Bale[d], Julianna Supplee[a], Bryan Ulrich[a], Lynette M. Sholl[d], Cloud P. Paweletz[a], Geoffrey R. Oxnard[b,*]

[a] Translational Research Laboratory, Belfer Center for Applied Cancer Science, Dana Farber Cancer Institute, United States
[b] Lowe Center for Thoracic Oncology, Dana Farber Cancer Institute, United States
[c] Division of Thoracic Surgery, Brigham and Women's Hospital, United States
[d] Department of Pathology, Brigham and Women's Hospital, Boston, MA United States

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ABSTRACT

*Background:* Tumor genotyping is transforming lung cancer care but requires adequate tumor tissue. Advances in minimally invasive biopsy techniques have increased access to difficult-to-access lesions, but often result in smaller samples. With the advent of highly sensitive DNA genotyping methods used for plasma analysis, we hypothesized that these same methods might allow genotyping of free DNA derived from fine needle aspiration supernatant (FNA-S).

*Methods:* We studied patients with known or suspected lung cancer undergoing fine needle aspirate (FNA). After spinning the sample for cellblock, the FNA-S (usually discarded) was saved for genotyping. Supernatant cell-free DNA (SN-cfDNA) was extracted and tested by both droplet digital PCR (*EGFR*, *BRAF, KRAS* mutations) and highly sensitive amplicon-based next-generation sequencing (NGS).

*Results:* 17 samples were studied, including 11 FNAs from patients with suspected lung cancer and 6 FNAs from patients with lung cancer and acquired drug resistance. Of 6 newly diagnosed adenocarcinomas, 4 had a driver mutations (1 *EGFR*, 2 *KRAS*, 1 *HER2*) found on tissue; all of these could be detected in SN-cfDNA. The *EGFR* driver mutation was detected in all 5 adenocarcinomas with acquired *EGFR* resistance and the *EGFR* T790 M in three cases, in agreement with cellblock.

*Conclusions:* FNA-S is a rich source of fresh tumor DNA, potentially increasing the diagnostic yield from small FNAs. Through use of emerging techniques for highly sensitive genotyping, this widely available biospecimen has potential for facilitating rapid cancer genotyping at diagnosis and after drug resistance.

## 1. Introduction

Genotype-directed cancer care is transforming the management of non-small cell lung cancer (NSCLC) in patients harboring actionable molecular alterations [1]. However, there is a current paradox between the need to obtain adequate tumor biopsy samples for multiplexed analysis of a growing number of molecular biomarkers, and the development of minimally invasive biopsy techniques which can result in small tumor samples with limited amounts of DNA. Up to 15% of radiology-guided FNA and 10–20% of EBUS-TBNA are rejected from genotyping due to low tumor content [2,3]. In addition, results from these biopsies may be delayed because of the time required to complete the necessary tissue processing and diagnostic steps before genomic analysis. For all these reasons, up to 25% of patients receive treatment without knowledge of the mutational status [4].

Genotyping of plasma cell-free DNA (cfDNA) using highlight sensitive assays offers the potential for rapid and noninvasive characterization of advanced NSCLC [5]. However, genotyping of plasma cfDNA comes with well recognized limitations including variable tumor shed and imperfect assay sensitivity. Interestingly, free-floating fresh DNA is also available from some other body fluids, and could intuitively be studied using similar highly sensitive technologies. We hypothesized that supernatant leftover following centrifugation of cytology specimens, a widely available biospecimen which is usually discarded, could

* Corresponding author at: Lowe Center for Thoracic Oncology, Dana Farber Cancer Institute, 450 Brookline Ave, Boston, MA, United States.
*E-mail address:* geoffrey_oxnard@dfci.harvard.edu (G.R. Oxnard).

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be a rich source of fresh tumor DNA for genomic analysis.

## 2. Methods

### 2.1. Patients

Patients with either a suspected lung cancer (based on PET positive disease, *cohort 1*) or established NSCLC with acquired tyrosine kinase inhibitor (TKI) resistance (*cohort 2*) and planned FNA were prospectively included in the study, under IRB approval.

### 2.2. Sample handling

After collection, cytology samples were sent to the cytology laboratory (Brigham and Women's Hospital) for standard handling, which included processing of smear preparations or creation of liquid-based preparation slides, cytocentrifuge preparation (400G), creation of a cell block from the cell pellet (plasma-thrombin method, formalin fixation, paraffin embedding, cutting, staining) for diagnosis and genomics. The supernatant, usually discarded, was saved and refrigerated until being transferred to the Translational Research Laboratory (Dana-Farber Cancer Institute). The FNA-S (CytoLyt or physiologic saline) was frozen (−80 °C) the day of the procedure. The first FNA specimen analyzed was used to test 2 different processes (further hard spin at 1500G or no further spin).

### 2.3. Supernatant cell-free DNA (SN-cfDNA) genotyping

SN-cfDNA was extracted from 3 ml of FNA-S and eluted in 100 µl, using the QIAamp circulating nucleic-acid kit (Qiagen). Extracted DNA was quantified using fluorometric quantification (Qubit). 60uL of isolated DNA were tested for key mutations in *KRAS* (G12X) and *EGFR* (L858R, exon 19 deletion, or T790 M) by ddPCR, as described previously [6]. 20 µl (20–80 ng of DNA, with a minimal starting concentration of 4.6 ng/µl) were used for a tagged-amplicon plasma NGS covering hotspots and exons in 20 genes (QIAseq Targeted Actionable Solid Tumor Panel, Sup. Fig. 1) as per manufacture's protocol. Briefly, after enzyme-based DNA fragmentation, end repair and A-tailing, adapters, molecular barcodes and samples indexes are incorporated. Target enrichment is performed using gene-specific primers, and libraries are amplified using universal PCR. Libraries were then sequenced on an Illumina NextSeq, using 151 bp paired end reads. NGS analysis was performed using Qiagen's cloud based analysis portal: https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/(Fig. 1**A**).

## 3. Results

### 3.1. Specimen handling pilot

A further hard centrifuge (1500G) was performed in all cases. To confirm non-inferiority of this approach, we tested an alternative handling (no spin) in the first EBUS-TBNA specimen, a patient from *cohort 2,* with mutations detected at a relatively low allelic frequency (AF, Fig. 1**B**). Testing the sfDNA without first performing an additional hard spin, the *EGFR* driver exon 19 deletion was detected at 290 copies/mL and 1.2% AF, and the T790 M resistance mutation at 70 copies/ml and 0.4% AF. The further hard spin resulted in a slight decrease in the concentration of mutated *EGFR* (180 copies/ml for exon 19 deletion and 45 copies/ml for T790 M) but resulted in an increased AF (4% for exon 19 deletion; 0.8% for T790 M mutation). The increased AF is due to the considerable decrease in the contaminating, likely hematopoietic wild-type *EGFR* alleles (22900 copies/mL without the spin, 4320 copies/mL with the spin) permitting the reporting of higher AF variants. Because this extra spin enriched for tumor DNA, it was adopted for subsequent specimens studies.

### 3.2. Population

The population is summarized in Sup. Fig. 2 . 17 samples were studied: 11 EBUS-TBNAs (1 lung mass, 10 mediastinal nodes) from patients with suspected lung cancer (*cohort 1*), 5 EBUS-TBNAs (mediastinal nodes) and 1 CT-guided FNA (lung mass) from patients with acquired drug resistance (*cohort 2*). Median SN-cfDNA concentration was 4.8 ng/µl (range 0.3–79 ng/µl).

### 3.3. Genotyping of newly diagnosed lung cancer using FNA-S

Among the 11 patients with suspected lung cancer, the final diagnosis was non-malignant (n = 3), squamous cell carcinoma (n = 1), small cell carcinoma (n = 1) and adenocarcinoma (n = 6); these final 6 cases underwent further genotyping of SN-cfDNA. A *KRAS* G12C was detected in one specimen with both ddPCR (13% AF) and NGS (11% AF), and with NGS in another one (1.4% AF, no DNA left for ddPCR). A *BRAF* V600E mutation was detected at 40% AF using NGS and then cross-validated with ddPCR (46% AF). Finally, an *HER2* exon 20 insertion was detected in SN-cfDNA using NGS (36% AF); a ddPCR assay was not available for this variant. All of these results were in agreement with sequencing done on tissue (all 6 adenocarcinomas were tested using NGS [7], Table 1).

### 3.4. Genotyping of acquired resistance using FNA-S

6 patients with acquired drug resistance (5 EGFR-TKI, 1 ROS1 TKI) were included. The driver could be detected in SN-cfDNA in all 5 patients with acquired resistance to EGFR-TKIs by ddPCR but was missed in one patient using NGS (Table 1). Three patients were found to also be positive for *EGFR* T790 M. A *PIK3CA* E726 K mutation, not present pretreatment, was detected in a *ROS1* rearranged patient at the time of resistance to lorlatinib by NGS in both tumor and FNA-S.

### 3.5. Accuracy of FNA-S genotyping

Results for all patients, comparing tissue genotyping, SN-cfDNA ddPCR and SN-cfDNA NGS are summarized in Table 1. No *KRAS* or *EGFR* ddPCR false positives were detected in 6 adenocarcinomas negative in tumor (specificity 100%). Studying 9 samples with matched tumor and SN-cfDNA NGS, and limiting our analysis to genes covered by both panels, no false positives were found.

## 4. Discussion

The use of cytology specimen supernatant for cancer genotyping has previously been reported using pleural fluid, [8,9] CSF [10,11] and bronchoscopic brushings/washings [12], these. prior studies being focused on *EGFR* genotyping only. More recently, Wei et al. reported very appealing data using the residual supernatant from a more heterogeneous population including FNAs but also fluids derived from different solid tumors, without further centrifuge. The high DNA concentration (176 ng/µl) suggests these specimens were still of high cellular content [13]. This latter study didn't include cases with acquired resistance. The number of druggable genotypes is increasing and the pressure of corresponding targeted therapies leads to the emergence of different subclonal competing cell populations, [14] creating a need to cover a wider range of alterations at resistance.

The proof of concept study we report here suggests a clear value for genomic analysis of FNA-S, a widely available and commonly discarded biospecimen. While tissue analysis remains mandatory for diagnosis, our results illustrate a complementary approach: the genotyping of SN-cfDNA derived from FNA supernatant may save tumor material for diagnosis, immunohistochemistry and in situ assays and thus improve the overall yield of these small size specimens. FNA-S offers an immediately available additional source of fresh DNA, potentially increasing