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## BRIEF REPORT: Adequacy of lymph node transbronchial needle aspirates using convex probe endobronchial ultrasound for multiple tumor genotyping techniques in non-small-cell lung cancer

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### Abstract

**Introduction**—Adequate tumor acquisition is essential to identify somatic molecular alterations in non-small-cell lung cancer (NSCLC), such as *EGFR* mutations and *ALK* translocations. The success and failure rates for tumor genotyping of tissue obtained from fine needle aspirates of nodal tissue using a convex probe endobronchial ultrasound (CP-EBUS) and other diagnostic modalities in routine NSCLC care have not been described.

**Methods**—Clinicopathologic data, tumor genotype success and failure rates were retrospectively compiled and analyzed from 207 patient-tumor samples sent for routine tumor genotype in clinical practice, including 42 patient-tumor samples obtained from hilar or mediastinal lymph nodes using CP-EBUS.

**Results**—The median age was 65 years, 62.3% were women, 77.8% were white, 26.6% were never smokers, 73.9% had advanced NSCLC and 84.1% had adenocarcinoma histology. Tumor tissue was obtained from CP-EBUS-derived hilar or mediastinal nodes in 42 cases (20.2% of total). In this latter cohort, the overall success rate for *EGFR* mutation analysis was 95.2%, for *KRAS* mutation 90.5%, and for *ALK* FISH 90.5%. In the complete 207 tumors, the success rate for *EGFR* was 92.3%, for *KRAS* 91.8%, and for *ALK* 89.9%. The failure rates were not significantly different when comparing CP-EBUS-derived nodal tissue versus all other samples or

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Conflict of interest:

DBC has received consulting fees from Pfizer, Roche and AstraZeneca. No other conflict of interest is stated.

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versus surgical biopsies of mediastinal nodes, but were significantly lower than image-guided percutaneous transthoracic core-needle biopsies.

**Conclusions**—The success rate of multiple tumor genomic analyses techniques for *EGFR*, *KRAS* and *ALK* gene abnormalities using routine lung cancer tissue samples obtained from hilar or mediastinal lymph nodes by means of CP-EBUS exceeds 90%, and this method of tissue acquisition is not inferior to other specimen types. Tumor genotype techniques are feasible in most CP-EBUS-derived samples and therefore further expansion of routine tumor genotype for the care of patients with NSCLC may be possible using targeted sample acquisition through CP-EBUS.

### Keywords

lung cancer; non-small-cell lung cancer; endobronchial ultrasound; EBUS; mediastinoscopy; never smokers; epidermal growth factor receptor; EGFR; anaplastic lymphoma kinase; ALK; KRAS; tumor genotype; failure; bone specimen; core biopsy; transbronchial needle aspiration; computed tomography; molecular testing

## INTRODUCTION

Non-small-cell lung cancers (NSCLCs) comprise a heterogeneous group of cancers characterized by different genomic abnormalities (1). In some NSCLCs, driver oncogenes have been identified; with the best studied including v-ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), epidermal growth factor receptor (*EGFR*) and anaplastic lymphoma kinase (*ALK*). Specifically, mutations in *EGFR* and rearrangements involving *ALK* are part of the pathogenesis of NSCLCs (predominantly in never and/or light smokers) and predict improved outcomes with therapy based on tyrosine kinase inhibitors (TKIs). Evidence-based practice guidelines now mandate adequate tissue acquisition for molecular studies aimed at identifying the aforementioned aberrations (2).

Over the last decade, the development of convex probe endobronchial ultrasound (CP-EBUS) with capability to obtain real-time visualization of mediastinal and hilar lymph nodes as well as guide transbronchial needle aspiration (TBNA) has revolutionized the diagnosis and staging of lung cancer. CP-EBUS offers a less invasive alternative for histologic sampling of these nodal stations when compared to mediastinoscopy, and evidence-based guidelines of diagnosis and staging of lung cancer now suggest the use of CP-EBUS-TBNA for the confirmation of suspected N2 and N3 disease. Several large prospective studies have demonstrated the sensitivity of CP-EBUS-TBNA to be equivalent to mediastinoscopy (3), the current gold standard, in indicating the true pathologic N stage. However, in the case of restaging, CP-EBUS-TBNA allows for multiple, repeat biopsy events in a minimally invasive way, and thus confers an advantage over mediastinoscopy (4). When compared to other clinical staging strategies, CP-EBUS has diagnostic accuracy of 98% versus 72.5% for PET and 60.8% for computed tomography (5). CP-EBUS-TBNA is an emerging method of tissue acquisition in NSCLC.

In the face of these two groundbreaking scientific advances – the characterization of the predictive value of driver oncogenes and the development of CP-EBUS as a method of tissue acquisition - it is evident that the combined use of CP-EBUS-TBNA and genotyping will likely impact the diagnosis, staging and treatment of non-small cell lung cancer. In this study, we aim to describe our experience in 207 patients with NSCLC who underwent diverse diagnostic procedures for genotypic analyses with a focus on the group that had tumor acquisition through CP-EBUS-TBNA.

## MATERIALS AND METHODS

### Patient selection

Patients with a diagnosis of lung cancer, who were seen by our providers and whose tumors were genotyped for at least *EGFR* mutations were retrospectively identified through an ongoing Institutional Review Board (IRB) approved protocol at Beth Israel Deaconess Medical Center (BIDMC2009-P-000182). Patients and tumor pairs were excluded if genotyping of at least *EGFR* mutation, *KRAS* mutation and *ALK* translocations were not performed. There were 207 patient-tumor specimens that were submitted for these multiple tumor genotype techniques between 2007 and 2012. The data cut off for analyses was December 19<sup>th</sup>, 2012. Study data were collected and managed using REDCap electronic data capture tools hosted at BIDMC.

### Tumor processing and genotype

Surgical (i.e., either incisional or excisional biopsies that required a surgical procedure) and core needle biopsies were processed using standard histopathologic techniques: 10% neutral buffered formalin fixation and paraffin-embedding. Cell aspirates or cell concentrates from fluid samples were collected into a methanol-water fixative (CytoLyt, Hologic Corp., Marlborough, MA), with residual material used to create a cell block using a plasma-thrombin method prior to formalin-fixation and paraffin embedding. Once a diagnosis was established on histologic and/or immunohistologic slides, the residual material in the formalin-fixed paraffin-embedded (FFPE) tissue blocks were submitted for molecular analysis. When multiple tissue blocks were available, the one with the highest tumor cellularity was chosen, without additional tumor microdissection or enrichment. Molecular analysis of tumor specimens was performed at a commercial vendor, Integrated Oncology (LabCorp, Esoterix Genetic Laboratories, LLC). DNA was isolated and *EGFR* exons 18 to 21 sequenced. For *KRAS* mutation analysis, exon 2 was amplified and subjected to single nucleotide primer extension to detect mutations at codons 12 and 13. *ALK* translocation status was analyzed using the Vysis *ALK* Break-Apart fluorescence in situ hybridization (FISH) probe (Abbott Molecular, Inc., Des Plaines, IL). Failure of the assays was defined as insufficient material to isolate DNA or inability to complete sequencing for *EGFR* and *KRAS* mutations, and lack of hybridization signals after two attempts for *ALK* FISH.

### Data collection

The site of biopsy (lung, lymph node, pleura, bone, brain, liver, pericardium, or adrenal) and the type of biopsy (surgical specimen [both excisional or incisional], core needle biopsy or cell block from aspirate/fluid) were extracted from the medical record. Slides from all specimens that failed molecular testing, as well as a subset of the successfully genotyped cases were re-reviewed by a pathologist (PAV), with data compiled on tissue preparation, tumor content, and other histological features annotated.

### EBUS technique and tumor collection with TBNA

The CP-EBUS bronchoscope used for tissue acquisition was a 7.5 MHz Olympus (BF-UC160F-OL8; Olympus America Inc., Center Valley, PA), fitted with color Doppler ultrasound capability. A 21 g needle was used to obtain TBNA samples. Two to eight passes (usually 3 passes) per lymph node were obtained. Lymph node stations reachable by CP-EBUS included paratracheal lymph nodes (2R, 2L, 4L, 4R), subcarinal (7), and the hilar, interlobar and lobar lymph nodes (10R, 10L, 11R, 11L, 12R, 12L). The lymph node aspirates were placed in methanol-water fixative and prepared as described above. At our institution, all CP-EBUS-TBNA cases were done by experienced interventional pulmonologists. As previously described, the learning curve on CP-EBUS-TBNA is likely to

require 50 or more supervised cases in order to accomplish a systematic mediastinal staging (8).

### Statistical methods

Fisher's exact test was performed to compare categorical variables. A  $p$ -value  $< 0.05$  was considered statistically significant. All  $p$ -values we reported were two-sided. We performed our statistical analyses with STATA version 12 (STATA Corp, College Station, TX).

## RESULTS

### Patient and tumor characteristics

Table 1 summarizes the clinical and pathological characteristics of the 207 patient-tumor pairs that were included in our cohort and highlights the characteristics of the 42 samples (42/207 [20.2%]) that were obtained from EBUS-derived mediastinal or hilar nodes (Table 1).

### Success and failure rates of tumor genotype techniques

In these 207 tumors, all three tests were ordered simultaneously. The success rate for *EGFR* mutation analysis was 92.3%, for *KRAS* mutation 91.8%, and for *ALK* FISH 89.9%.

In the 42 lymph node metastases obtained through EBUS-TBNA, the success rate for *EGFR* mutation analysis was 95.2%, for *KRAS* mutation 90.5%, and for *ALK* FISH 90.5% (Table 1).

### CP-EBUS-derived nodal tissue compared to other tissue acquisition techniques

To determine if CP-EBUS-derived nodal tissue had similar or different success rates when compared to other possible methods of tumor acquisition, we first compared CP-EBUS-derived nodal tissue to all remaining samples. Typical histologic examples of image-guided percutaneous transthoracic core-needle biopsies, bronchoscopic-guided lung biopsies, and cell blocks prepared from CP-EBUS-TBNAs of mediastinal lymph nodes are compared in Figure 1.

CP-EBUS-derived nodal tissues were obtained from N1, N2, and/or N3 nodes, the median number of FNA passes per node was 3.28 (range 2-8) and there were no reported complications. As indicated in Table 2, the failure rates were not significantly different between CP-EBUS-derived nodal tissue versus all other samples for *EGFR* mutation, for *KRAS* mutation, and for *ALK* FISH (Table 2).

To better match the tissue of origin (thoracic lymph nodes), we compared the success and failure rates of CP-EBUS-TBNA to those of mediastinal or hilar nodes acquired through surgical biopsies (either through mediastinoscopy or thoracic resections). There were no failures in the eight nodal tissues acquired through surgical technique. However, the success rates were not significantly different between CP-EBUS-derived nodal tissue versus these eight surgical nodal specimens for *EGFR* mutation; for *KRAS* mutation and for *ALK* FISH (Table 2).

We then compared CP-EBUS-derived nodal tissue to lung biopsies, either using image-guided percutaneous transthoracic core-needle biopsies or lung biopsies obtained through bronchoscopy (Table 2). The success rates were higher when CP-EBUS-derived nodal tissue was compared to image-guided percutaneous transthoracic core-needle biopsies for *EGFR* mutation, for *KRAS* mutation and for *ALK* FISH (Table 2). The success rates for CP-

EBUS-derived nodal tissue were similar to those of bronchoscopy-guided lung biopsies (Table 2).

We compared image-guided percutaneous transthoracic core-needle and bronchoscopy-guided lung biopsies in relation to estimated tumor cellularity, tumor area, use of touch preparation for rapid on-site evaluation, presence of extensive desmoplastic stromal response, number of slides cut from the paraffin block used for immunohistochemical/ ancillary studies, number of biopsy passes, location site within the lung and mass size. The only two major differences were in the use of a touch preparation (image-guided percutaneous transthoracic core-needle biopsies 8/8 cases versus 0/13 cases for bronchoscopy-guided lung biopsies,  $p<0.0001$ ) and the number of passes of needle for tissue acquisition (image-guided percutaneous transthoracic core-needle biopsies 8/11 cases with 2 passes versus 0/14 cases for bronchoscopy-guided lung transbronchial biopsies,  $p=0.072$ ). Complications, including pneumothorax, were also more common in image-guided percutaneous transthoracic core-needle biopsies (6/11 cases) when compared to bronchoscopy-guided lung biopsies (0/14 cases,  $p=0.009$ ).

### Failed specimens using CP-EBUS-derived nodal tissue

We attempted to determine factors specifically associated with molecular testing failure in specimens originating from CP-EBUS. In most of the samples that failed at least one of the tumor genotyping techniques used, we observed that there were insufficient tumor cells in the cell block specimen ( $<100$  tumor cells per slide and inferior dimension of tumor specimen). In CP-EBUS-derived nodal tissues that were successful 17/19 (89.4%) had  $\geq 100$  cells while in failure cases only 2/5 (40%) had  $\geq 100$  cells ( $p=0.042$ ). We also evaluated as other possible characteristics the size of the nodal tissue biopsied, the location of the node, the number of passes per lymph node, use of touch preparation for rapid on-site evaluation, presence of extensive desmoplastic stromal response, and number of slides cut from the paraffin block used for immunohistochemical and ancillary studies. None of these characteristics were significantly different between success and failure CP-EBUS-derived nodal samples.

## DISCUSSION

In this study, we provide evidence that CP-EBUS-TBNA is able to procure adequate lymph node tissue to carry out genetic analyses of *EGFR*, *KRAS*, and *ALK* using commercially-available techniques that are employed for everyday patient care. More than 20% of tumor samples submitted for tumor genotype at our academic medical center originated from CP-EBUS-TBNA of hilar or mediastinal nodes. In these metastases, the success rate for *EGFR* mutation analysis was 95.2%, for *KRAS* mutation 90.5%, and for *ALK* FISH 90.5%. More importantly, these CP-EBUS-TBNA samples were not inferior to surgical thoracic nodal samples and or to image-guided percutaneous transthoracic core-needle biopsies in our single institution series; results that may not reflect practice patterns at other centers.

The clinician (a medical oncologist, thoracic surgeon, or pulmonologist) is frequently faced with the choice of diagnostic sampling technique. Traditionally, image-guided percutaneous transthoracic core-needle biopsies have been used by most practices to obtain tissue from peripheral lung tumors even in the presence of hilar and/or mediastinal thoracic lymphadenopathy. However, our study shows that when compared to CP-EBUS-guided biopsies, image-guided percutaneous transthoracic core-needle biopsies of the lung had a significantly higher failure rate of tumor genotype techniques. The high failure rate of image-guided percutaneous transthoracic core-needle biopsies to yield adequate material for molecular studies has been reported by other academic medical centers (6). It is possible to speculate that tumor tissue loss during the aforementioned procedure and subsequent

preparation explains these results. Indeed in our series, image-guided percutaneous transthoracic core-needle biopsies more frequently had tissue loss (in the form of cytological adequacy evaluation of core biopsy touch-preparation slides at time of biopsy) than bronchoscopic-derived lung biopsies.

Interestingly, the absence of rapid on-site evaluation for CP-EBUS procedures did not seem to affect the success rate in our series and may have indeed led to a more thorough evaluation and repeated sampling of the lymph nodes. Also of note, in traditional image-guided percutaneous transthoracic core-needle biopsies of the lung there is an increased risk of complications (including pneumothorax) when doing multiple needle passes; a concern that is not significant with CP-EBUS-TBNA (7). However, the importance of extensive training in CP-EBUS-TBNA, particularly in complete and systematic staging for lung cancer, cannot be overemphasized (8).

The use of CP-EBUS-TBNA for tumor genotype in NSCLC has been reported by a few other groups, with successful analysis of at least one genotype (such as *EGFR* or *KRAS* mutations) in 72.2% to 98.4% of samples obtained (9-14). One of the largest published series described a cohort of 774 patients in 5 centers of the United Kingdom with known or suspected lung cancer who had CP-EBUS-TBNA done to determine if these samples were suitable for NSCLC subclassification and genotyping. The available tissue allowed subclassification of 77% of samples and genotyping for *EGFR* mutation in 90% of the cases (15). In our study, all samples were obtained using routine day-to-day procedures, underwent routine pathological subtyping with immunohistochemical markers, and then were subsequently sent for multiple tumor genotype analyses with a success rate that exceeded 90%. The aforementioned studies confirm that CP-EBUS-TBNA samples are adequate as a starting material for routine tumor genotype studies.

In summary, commercially-available tumor genotype techniques for *EGFR* mutation analysis, *KRAS* mutation analysis and *ALK* FISH are feasible in most EBUS-TBNA NSCLC samples. These encouraging findings may indicate that further expansion of routine tumor genotype into the care of patients with NSCLC may be possible using targeted sample acquisition through EBUS.

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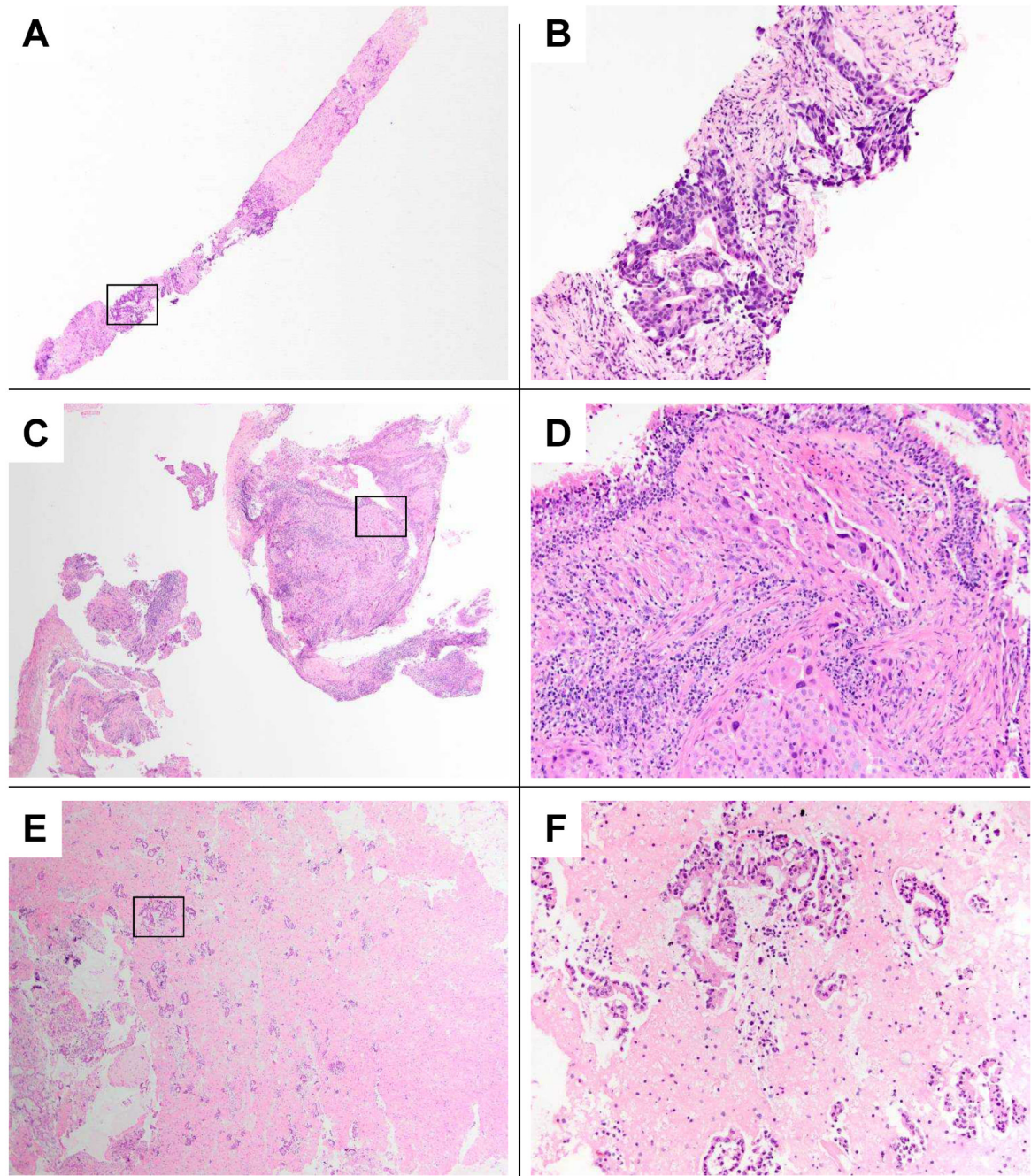
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**FIGURE 1. Histopathology of different minimally invasive methods for thoracic tissue sampling**

A-B: Image-guided percutaneous transthoracic core-needle biopsies from a left upper lobe lung mass. The low power field highlights one of two needle core biopsies obtained, both demonstrating significant fibrosis with intervening areas of invasive adenocarcinoma. On higher power, the tumor cells demonstrate a glandular morphology, with associated chronic inflammation. Cytologic rapid on-site evaluation of touch prep slides from the core biopsies confirmed adenocarcinoma, and subsequent tumor genotyping successfully identified a deletion mutation in exon 19 of the EGFR gene.

C-D: Bronchoscopy-guided lung biopsies of a right middle lobe lung mass. The low power field in panel C shows two of the six airway tissue biopsies obtained. The higher power view



demonstrates a poorly differentiated adenocarcinoma with cytoplasmic mucin vacuoles infiltrating submucosal lymphatic spaces, with surrounding bronchial associated lymphoid tissue and overlying respiratory epithelium. Tumor genotyping successfully identified a mutation in exon 20 of the EGFR gene.

E-F: Cell block preparation from an CP-EBUS-TBNA of mediastinal lymph nodes. The low power field view in panel E, covering approximately 25% of the cell block area, highlights the cellular specimen with numerous clusters of tumor cells in a background of eosinophilic fibrin clot. On higher power the background lymphocytes confirm lymph node sampling, and the tumor morphology is that of a moderately differentiated adenocarcinoma. Tumor genotyping was successful.

Panels A, C, and E 40× original magnification; panels B, D, and F 200× original magnification representing boxed area in prior panel; Hematoxylin and Eosin stains.

**Table 1**

Baseline patient and tumor characteristics of the overall cohort and of the CP-EBUS-TBNA cohort

	All patients (n=207)	CP-EBUS-TBNA lymph node cohort (n=42)
Age at the time of biopsy median(range)	65(29-89)	61.5(39-84)
Women n(%)	129(62.3)	28(66.7)
Race n(%)		
White	161(77.8)	34(81.0)
Asian	19(9.18)	2(4.76)
Black	17(8.21)	2(4.76)
Others	10(4.83)	4(9.52)
Smoking status n(%)		
Current smoker	42(20.3)	10(23.8)
Former smoker	110(53.1)	23(54.8)
Never smoker	55(26.6)	9(21.4)
Stage n(%)		
I	14(6.76)	0(0)
II	13(6.28)	1(2.38)
III	27(13.0)	7(16.7)
IV	153(73.9)	34(81.0)
Histology n(%)		
Adenocarcinoma	174(84.1)	36(85.7)
Squamous cell carcinoma	9(4.35)	0(0)
NSCLC(NOS)	22(10.6)	6(14.3)
Others	2(0.97)	0(0)
Anatomic site of biopsy n(%)		
Bone	13(6.28)	0(0)
Brain	17(8.21)	0(0)
Liver	3(1.45)	0(0)
Lung	98(47.3)	0(0)
Lymph node	51(24.6)	42(100)
Pleura	23(11.1)	0(0)
Others	2(0.97)	0(0)
Type of biopsy n(%)		
Core needle biopsy	39(18.8)	0(0)
Surgical biopsy	95(45.9)	0(0)
Cell block from FNA	61(29.5)	42(100)
Cell block from fluid	12(5.80)	0(0)

	All patients (n=207)	CP-EBUS-TBNA lymph node cohort (n=42)
<i>EGFR</i> mutation analysis		
Success n (% , [95% CI])	191(92.3 [87.5-95.4])	40(95.2 [82.6-99.2])
Positive/Mutated	32(15.5)	5(11.9)
Negative/Wild-type	159(76.8)	35(83.3)
Failure	16(7.73)	2(4.8)
<i>KRAS</i> mutation analysis		
Success n (% , [95% CI])	190(91.8 [87.0-95.0])	38(90.5 [76.5-96.9])
Positive/Mutated	65(31.4)	18(42.9)
Negative/Wild-type	125(60.4)	20(47.6)
Failure	17(8.21)	4(9.5)
<i>ALK</i> FISH analysis		
Success n (% , [95% CI])	186(89.9 [84.7-93.5])	38(90.5 [76.5-96.9])
Positive	11(5.31)	1(2.4)
Negative	175(84.5)	37(88.1)
Failure	21(10.1)	4(9.5)

CI, confidence interval

**Table 2**

Success and failure rates of genotype tests comparing CP-EBUS-derived lymph node cell blocks and other sources of tissue acquisition

<i>EGFR</i> mutation analysis					
	CP-EBUS-derived node cell blocks	All other methods of tissue acquisition	Mediastinal/hilar nodes from surgical biopsies	Lung core biopsies (bronchoscopy)	Lung core needle biopsies (image-guided)
Success n (%)	40 (95.2%)	151 (91.5%)	8 (100%)	12 (86%)	6(55%)
Failure n (%)	2 (4.8%)	14 (8.5%)	0 (0%)	2 (14%)	5(45%)
Total	42	165	8	14	11
p-value	ref	0.54	1	0.26	0.003
<i>ALK</i> FISH analysis					
	CP-EBUS-derived node cell blocks	All other methods of tissue acquisition	Mediastinal/hilar nodes from surgical biopsies	Lung core biopsies (bronchoscopy)	Lung core needle biopsies (image-guided)
Success n (%)	38 (90.5%)	148 (89.7%)	8 (100%)	12(86%)	6(55%)
Failure n (%)	4 (9.5%)	17 (10.3%)	0 (0%)	2 (14%)	5(45%)
Total	42	165	8	14	11
p-value	ref	1	1	0.63	0.01
<i>KRAS</i> mutation analysis					
	CP-EBUS-derived node cell blocks	All other methods of tissue acquisition	Mediastinal/hilar nodes from surgical biopsies	Lung core biopsies (bronchoscopy)	Lung core needle biopsies (image-guided)
Success n (%)	38 (90.5%)	152 (92.1%)	8 (100%)	13 (93%)	8(73%)
Failure n (%)	4 (9.5%)	13 (7.9%)	0 (0%)	1 (7%)	3(27%)
Total	42	165	8	14	11
p-value	ref	0.75	1	1	0.15