

# Triple Gene Analysis Using Samples Obtained by Endobronchial Ultrasound-guided Transbronchial Needle Aspiration

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## Abstract

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**Objective** A mutational analysis of tumor tissue samples is an important part of advanced lung cancer treatment strategies. This study evaluated the efficacy of a triple gene analysis using samples obtained via endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA).

**Methods** Either metastatic lymph nodes or primary lung mass samples obtained by EBUS-TBNA were collected between May 2011 and May 2013. We consecutively analyzed epidermal growth factor receptor (*EGFR*), V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and anaplastic lymphoma kinase (*ALK*) fusion genes using remnant tissue samples.

**Results** A total of 109 patients were diagnosed with non-small cell lung cancer (NSCLC). Of these, 70% were adenocarcinoma, 27% squamous cell carcinoma with NSCLC, and 3% were related to other types of lung cancer. *EGFR* mutations were detected in 23 cases (21.1%), *KRAS* mutations in 13 cases (11.9%), and *ALK* fusion genes in 5 cases (4.9%). The *ALK* fusion genes could not be analyzed in four cases because of insufficient tissue samples remaining after routine histochemistry and an *EGFR/KRAS* mutation analysis. We found that small biopsy samples from EBUS-TBNA were adequate for performing a triple gene analysis in 97 patients (96%). *ALK* fusion protein immunohistochemistry (IHC) was 100% consistent with fluorescence *in situ* hybridization (FISH).

**Conclusion** Small samples obtained by EBUS-TBNA were found to be sufficient for performing a triple gene analysis following routine histology and IHC. *ALK* IHC showed a very good concordance with FISH for detecting *ALK* fusion genes.

**Key words:** EBUS-TBNA, lung cancer, EGFR mutations, KRAS mutations, ALK fusion genes

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## Introduction

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An essential part of the treatment for advanced non-small cell lung cancer (NSCLC) is therapy tailored to the particular histology and mutation status of individual patients (1). Tumor cells with somatic mutations of the tyrosine kinase domain of the epidermal growth factor receptor (*EGFR*) respond well to tyrosine kinase inhibitors (TKI) (2). The RAS pathway, a major signaling pathway activated by *EGFR*, correlates with *EGFR* TKI resistance in the presence of gene mutations that encode the V-Ki-ras2 Kirsten rat sarcoma vi-

ral oncogene homolog (*KRAS*) domain (3). In addition, NSCLC incorporating a fusion gene located between the anaplastic lymphoma kinase (*ALK*) gene and the echinoderm microtubule-associated protein-like 4 (*EML4*) gene responds well to *ALK* inhibitors such as crizotinib (4).

Fewer than 30% of patients are able to undergo diagnostic surgery for lung cancer; therefore, most diagnoses arise from small biopsies acquired by bronchoscopy or percutaneous needle aspiration (PCNA). However, these are associated with some limitations regarding mediastinal staging (5). Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is a minimally-invasive alternative to

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mediastinoscopy for the diagnosis and staging of NSCLC (6). EBUS-TBNA can be initially performed to simultaneously diagnose and determine the mediastinal stage in cases of suspected mediastinal metastasis identified via integrated positron emission tomography/computed tomography (PET/CT). To provide treatment tailored to individual patients, an analysis of the lung cancer subtypes and genotypes, and in particular *EGFR/KRAS* mutations and *ALK* fusion genes, is critical (7). Several studies that used small NSCLC tissue samples obtained via EBUS-TBNA have proved that such pathologic classification is feasible (8, 9). As well as accurate cell typing, EBUS-TBNA can also be used to detect *EGFR* and *KRAS* mutations in cell blocks possessing tumor cells (10), as well as *ALK* fusion genes in metastatic lymph node samples (11). In this study, we performed simultaneous histologic classification and a mutation analysis (including *EGFR*, *KRAS*, and *ALK* gene rearrangement) using metastatic lymph node or lung tissue samples obtained by EBUS-TBNA. The efficacy of a triple gene analysis using these samples was investigated in patients with suspected metastatic cancer of the lymph nodes or a primary lung mass near the bronchus that made it possible to carry out EBUS-TBNA tissue sampling.

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## Materials and Methods

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### Patients

Between May 2011 and May 2013, patients at the Samsung Medical Center (Seoul, South Korea) with suspected primary lung cancer were retrospectively enrolled in this study. EBUS-TBNA was performed on suspected metastatic lymph nodes or lung masses to obtain tissue samples for diagnosis and a mutational analysis. If malignant cells were histologically detected, then mutation tests for *EGFR*, *KRAS*, and *EML4-ALK* were routinely performed at the discretion of the pathologist, except in cases of small cell lung cancer and extrathoracic malignancy. Most of the enrolled patients underwent chest CT and integrated PET/CT prior to EBUS-TBNA. The acquisition of the integrated PET/CT images was undertaken using the GE Discovery LS PET/CT system (GE Healthcare, Milwaukee, USA) consisting of a GE Advance NXi PET scanner and a GE LightSpeed Plus 8-slice CT scanner. We considered a lymph node or lung mass to be positive for malignancy if the maximum standardized uptake value ( $SUV_{max}$ ) was greater than 2.5 on  $^{18}F$ -fluorodeoxy glucose (FDG) PET/CT (12). The Institutional Review Board of the Samsung Medical Center approved this study and waived the requirement for informed consent from each patient. All medical records and personnel information was anonymized and assigned to subject identification code prior to data analysis.

### EBUS-TBNA

EBUS-TBNA was performed with a flexible convex probe ultrasonic puncture bronchoscope with a linear scanning

transducer set to a frequency of 7.5 MHz (CP-EBUS, BF-UC206F-OL8, Olympus, Tokyo, Japan) (6, 13). The procedure was carried out on an inpatient basis under local anesthesia and conscious sedation with midazolam. The indications for EBUS-TBNA in the mediastinal or hilar lymph nodes were the presence of either of these lymph nodes with a short axis diameter  $\geq 10$  mm on chest CT and a lymph node with a  $SUV_{max}$  greater than 2.5. EBUS-TBNA was also used to acquire lung mass tissue close to the bronchus, particularly when located in an area where PCNA would be difficult or dangerous to perform (14). All aspirate specimens were expelled onto glass slides, smeared, immediately fixed, and then sent for cytological and/or histological examination. Rapid on-site cytopathological evaluation was not carried out. Pathology with hematoxylin and eosin staining was recorded and classed as benign if it predominantly contained lymphocytes, but no evident malignant cells, or as malignant if it contained clearly malignant cells.

### Pathological and molecular processing of EBUS-TBNA samples

Core samples obtained from a 22-gauge needle were processed using standard histopathologic techniques. The fresh specimen was placed in a formaldehyde solution, which acted as a fixing agent. After wax infiltration and paraffin embedding, the specimen was mounted on a slide and diagnosed histologically using immunohistochemistry (IHC). To classify lung cancer, IHC was performed using monoclonal antibodies applied to CK, p53, p56, p63, TTF-1, and *ALK*. The Novocastra mouse monoclonal antibody p80 ALK (Clone 5A4, NCL-ALK, Leica, Wetzlar, Germany) was used for the immunostaining of ALK. If lung cancer was diagnosed, then the microdissection of tumor cells was done to extract DNA from the paraffin-embedded tissue specimens. A mutational analysis of *EGFR* exons 18-21 and *KRAS* codons 12, 13, and 61 was then undertaken. Direct sequencing was utilized for mutations of the *EGFR* exons 18-21. Some samples were analyzed with a peptide nucleic acid (PNA)-mediated real-time polymerase chain reaction (PCR) clamping method that enables the detection of *EGFR* mutants down to approximately 1% mutant-to-wild type, followed by direct sequencing to confirm the detection of *EGFR* mutations. A *KRAS* mutational analysis was simultaneously performed with the extracted DNA using direct sequencing or PNA-mediated real-time PCR clamping in codons 12 and 13 of exon 2. The rearrangement of the *ALK* locus on chromosome 2p23 was detected using the Vysis ALK Break-Apart fluorescence *in situ* hybridization (FISH) probe (Abbott Molecular, Des Plaines, USA). An expert pathologist (Choi YR) from the Department of Pathology selected samples containing sufficient tumor cells to conduct a qualified analysis. The positive criteria for *EML4-ALK* translocation was defined as a count of more than 50 cells and  $>15\%$  of the counted cells displaying green and red signals separated by at least two signal diameters and/or an isolated red signal. The translocation was considered to be negative if the sam-

**Table 1. Clinical Characteristics of the Patients with NSCLC.**

Characteristics	Patients (n=109)
Gender	
Male	77 (70.6)
Female	32 (29.4)
Age, y	63.3 ± 9.64
Histology of metastatic lymph node	
Adenocarcinoma	66 (60.6)
Favor adenocarcinoma	10 (9.2)
Squamous cell carcinoma	24 (22.0)
Favor squamous cell carcinoma	5 (4.6)
NSCLC-NOS	4 (3.7)
Clinical stage	
II	4 (3.7)
III	50 (45.9)
IV	51 (46.8)
Recurrence	4 (3.7)
Smoking	
Non-smoker	40 (36.7)
Smoker	69 (63.3)

Data are presented as the means ± SD or as *n* (%), unless otherwise indicated. NSCLC: non-small cell lung cancer, NOS: not otherwise specified

ples did not fulfil the above criteria.

### Statistical analysis

Clinical characteristics and the mutation status are presented as median (range) values for continuous variables and as numbers (percentage) for categorical variables. A comparison between the *EML4-ALK* fusion positive and negative groups was performed with Fisher's exact test. Concordance between the *ALK* IHC and *ALK* FISH methods was estimated with K-coefficients. A *p* value <0.05 was taken to indicate statistical significance based on a two-sided hypothesis. The IBM SPSS Statistics for Windows software package (ver. 22.0; IBM Corp., Armonk, USA) was used for the data analysis.

## Results

### Clinical characteristics of patients with NSCLC

Table 1 lists the baseline characteristics of the 109 patients included in this study. A total of 155 patients with suspected primary lung cancer underwent diagnostic EBUS-TBNA. Forty-six patients were excluded prior to analysis because they were diagnosed with small cell lung cancer, benign disease, or extrathoracic metastasis, or because the results were non-diagnostic. The median aspiration number of EBUS-TBNA per node and lung mass was 2.0 (range, 1-5) and 3.0 (range, 2-5), respectively. The samples diagnosed to be NSCLC were processed before undergoing IHC for histological classification and gene analysis (Fig. 1). The histology of the metastatic lymph nodes or lung tissue specimens in 109 patients revealed 66 adenocarcinoma (60.6%), 10 favor adenocarcinoma (9.2%), 24 squamous cell carcinoma (22.0%), 5 favor squamous cell carcinoma (4.6%), and 4 NSCLC-not otherwise specified (3.7%), respectively. In

total, 69 patients were smokers (63.3%), compared to 40 non-smokers (36.7%).

### Mutation status of samples obtained by EBUS-TBNA

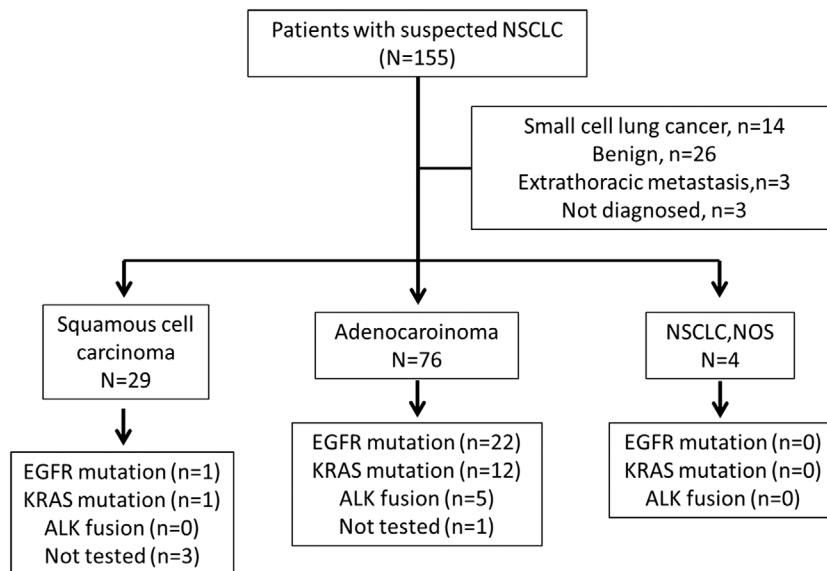
Table 2 summarizes the mutation status of *EGFR/KRAS* mutations, and *ALK* rearrangement. A total of 109 EBUS-TBNA samples underwent a mutation analysis. Among the 76 patients with adenocarcinoma, *EGFR* mutations were interpreted as positive in 22 samples (28.9%), *KRAS* mutations in 12 (12.8%) samples, and *ALK* rearrangement in 5 samples (7.1%). Fig. 2 shows representative images regarding the pathology and mutational tests including *EGFR*, *KRAS* and *ALK*. Among those patients with squamous cell carcinoma, *EGFR* mutations were interpreted as positive in one sample (3.4%); *KRAS* mutations were also interpreted as positive in one sample (3.4%). However, there was no positive interpretation regarding *ALK* rearrangements. Triple gene testing for *EGFR/KRAS* mutations and *ALK* rearrangement was simultaneously performed for 101 samples because the *ALK*-FISH test was not performed for 8 samples. The mean tumor cell percentage was 40% [interquartile range (IQR): 20-70] for adenocarcinoma, 30% (IQR: 10-60) for squamous cell carcinoma, and 30% (IQR: 20-70) for NSCLC-NOS. Sufficient samples in terms of both quantity and quality were obtained via EBUS-TBNA to enable the testing of 109 consecutive NSCLC tissue specimens. However, the *ALK* rearrangement test was not able to be carried out in 4 out of 101 consecutive samples. There were no residual tissue blocks containing tumor cells in order to carry out the *ALK* rearrangement test after the analysis of *EGFR* and *KRAS* mutation. The median duration of the entire process, from EBUS-TBNA to the final reports, was 14 days (range: 6-37 days).

### *EML4-ALK* fusion gene in NSCLC

The frequency of lung cancer incorporating the *EML4-ALK* fusion gene was 5 (4.9%) out of 101 patients, with a median age of 57.0 years. Comparison of the clinical characteristics between *EML4-ALK* fusion positive and negative patients is detailed in Table 3. The smoking status of the patients with lung cancer incorporating fusion genes was significantly lower (0.0 pack-year, IQR 0-0.75) compared to the patients with lung cancer and no *ALK* gene rearrangement (20.0 pack year, IQR 0-40). All lung cancer cases which included the *EML4-ALK* fusion gene had adenocarcinoma and no positive *EGFR/KRAS* mutations. On the other hand, lung cancer that was negative for fusion genes in 22.8% of the *EGFR* mutation positive cases and 11.9% of the *KRAS* mutations. The *ALK* IHC and *EML4-ALK* FISH methods closely correlated with the 5 *ALK* IHC positive and 92 *ALK* negative cases. All *ALK* IHC positive cases revealed *EML4-ALK* fusion at the *ALK* locus on chromosome 2p23.

## Discussion

This study showed that the EBUS-TBNA procedure could



**Figure 1.** Diagnostic algorithm of the study patients. *EGFR*: epidermal growth factor receptor, *KRAS*: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, *ALK*: anaplastic lymphoma kinase, NSCLC: non-small cell lung cancer

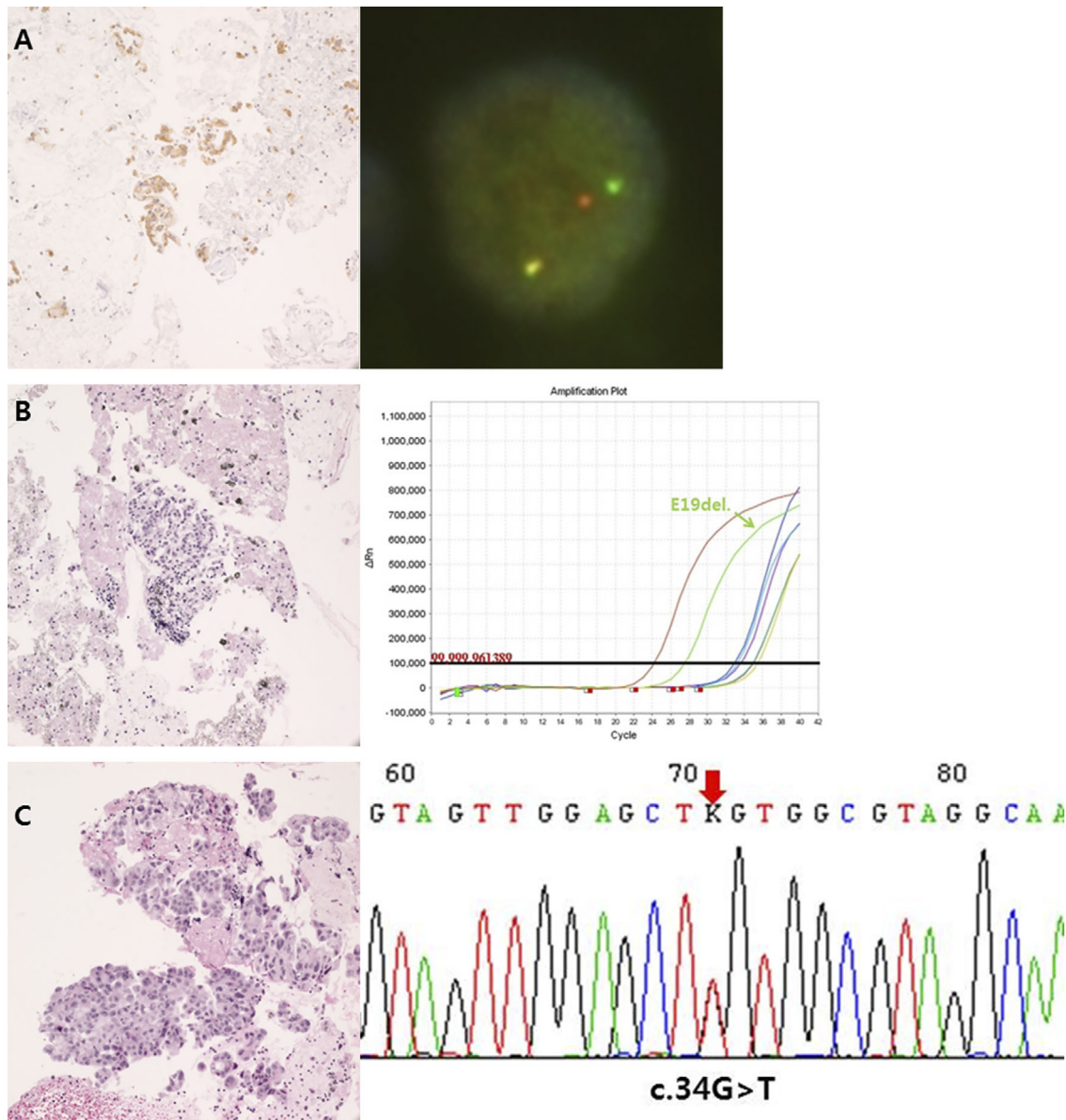
**Table 2.** Mutation Status According to the Histologic Subtyping.

	Adenocarcinoma	Squamous cell carcinoma	NSCLC-NOS
Subjects, <i>N</i>	76	29	4
Gender, M:F	50:26 (1.9:1)	25:4 (6.3:1)	2:2 (1:1)
Age, y	62.05 (53–70)	68.0 (61–71)	62.0 (56–74)
Tumor cell percentage	40% (20–70)	30% (10–60)	30% (20–70)
<i>EGFR</i> mutation positive			
Exon 19 deletion			
E746–A750del	5	0	0
L747–E749del	1	0	0
L747–P753del	2	0	0
E746–A750del	2	0	0
L747–S7521del	0	1	0
Exon 19 del and ins.			
T751–I759	1	0	0
Exon 19 + Exon 20			
L747–A750del+ T790M	1	0	0
Exon 20			
A770–V772 dup	1	0	0
Exon 21			
L858R	8	0	0
L861R	1	0	0
<i>KRAS</i> mutation positive			
12	10	1	0
13	1	0	0
61	1	0	0
<i>ALK</i> FISH			
Positive	5	0	0
Negative	64	24	4
NA	1	3	0

Data are presented as *n* (%) or as the median (IQR) values. NSCLC: non-small cell lung cancer, NOS: not otherwise specified, *EGFR*: epidermal growth factor receptor, *KRAS*: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, *ALK*: anaplastic lymphoma kinase

provide sufficient aspiration samples for 100 % (*EGFR* and *KRAS* mutations) and in 96% (*ALK* gene rearrangement) of the enrolled patients when they were processed consecutively. A routine triple gene analysis following pathologic subtyping was feasible in 96% of our study samples. The

*EGFR* gene is frequently expressed in NSCLC and somatic mutations in exons 18-21 have been shown to demonstrate a relationship with the treatment responses to *EGFR* TKI (2). *EML4-ALK* rearrangement should also be identified to predict the response from the *ALK* inhibitor (15). Such genetic



**Figure 2.** Representative images of the samples obtained by EBUS-TBNA. A, Carcinoma cells are positive for *ALK* with a membranous pattern of staining. The *ALK* fluorescence *in situ* hybridization test shows break apart rearrangement in 40 of 50 non-overlapping nuclei: one red, one green, and one fusion signal. B, Metastatic adenocarcinoma cells occupy about 5% of total specimen volume. Real-time polymerase chain reaction test detects a deletion mutation in exon 19: c.2235\_2,249 del 15 (p. E746\_A750 del); The standard cyclic threshold (Ct) is 34 and sample Ct is 27.5436. C, Metastatic adenocarcinoma cells occupy about 70% of the total specimen volume. *KRAS* sequencing shows a missense mutation in the 12th codon: c.34G>T (p.G12C).

information is a key to providing personalized treatments for patients with advanced NSCLC. Previous studies regarding gene analyses for *EGFR* and *KRAS* concluded that the use of EBUS-TBNA in advanced lung cancer allowed for the adequate interpretation of mutations (16). However, the quality of such interpretation using samples obtained by EBUS-TBNA has only rarely been investigated. Folch et al. described the success and failure rate of *EGFR* mutations and *ALK* translocations from nodal tissue specimens using EBUS-TBNA and other diagnostic methods. EBUS-TBNA was able to be performed in 42 out of 207 cases, resulting

in a success rate of 95.2% in *EGFR* mutations, 90.5% in *KRAS* mutations, and 90.5% in *ALK* FISH (17). In contrast, our study used samples obtained via EBUS-TBNA alone, and a total of 109 cases were enrolled providing a high success rate of 100% in *EGFR/KRAS* mutations and 96% in *ALK* translocations. The *ALK* translocation rate for adenocarcinoma was 7.1%. There was no overlap between *EGFR*, *KRAS*, and *ALK* mutations. Pathologist-interpreted *ALK* immunohistochemistry was performed in all NSCLC cases. The concordance rate of *ALK* IHC in the small samples obtained by EBUS-TBNA was perfectly matched in our study.

**Table 3. Comparison of the Patient Characteristics Between *EML4-ALK* Fusion Positive and Negative NSCLC.**

Characteristics	<i>EML4-ALK</i> fusion		p value
	Positive (n=5)	Negative (n=92)	
Age	57.0 (49-63)	64.0 (55-71)	0.096
Gender, Female	3 (60)	28 (30.4)	0.087
Smoking status, PY	0.0 (0-0.75)	20.0 (0-40)	0.023
Metastasis			
Brain	0 (0.0)	9 (9.8)	0.404
Bone	0 (0.0)	15 (16.3)	0.616
Pleura	1 (20.0)	20 (21.7)	0.962
Histology of LN			0.739
Adenocarcinoma	5 (100)	64 (69.6)	
SQC	0 (0.0)	24 (26.0)	
NSCLC-NOS	0 (0.0)	4 (4.3)	
<i>ALK</i> IHC			0.000
Positive	5 (100)	0 (0.0)	
Negative	0 (0.0)	92 (100)	
<i>EGFR</i> mutation status			0.440
Positive	0 (0.0)	21 (22.8)	
Negative	5 (100)	71 (77.2)	
<i>KRAS</i> mutation status			0.627
Positive	0 (0.0)	11 (12.0)	
Negative	5 (100)	81 (88.0)	

Data are presented as *n* (%) or as the median (IQR) values. PY: pack-year, LN: lymph node, SQC: squamous cell carcinoma, IHC: immunohistochemistry, *EML4-ALK*: echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase

This result shows that *ALK* translocation IHC could be employed as a screening method for *ALK* rearrangement prior to *ALK* FISH in EBUS-TBNA-sampled tissues.

EBUS-TBNA is a non-invasive procedure performed under conscious sedation to diagnose cancer of the mediastinal and hilar lymph nodes. Our institute has issued a protocol to reduce the time interval from the initial biopsy to the pathologic diagnosis and interpretation of gene mutations, since treatment delays affect the prognosis of advanced lung cancer (18). EBUS-TBNA can be performed as an initial procedure for simultaneous diagnosis and mediastinal staging if PET/CT reveals a significant mediastinal lymph node uptake in a patient with suspected lung cancer. In this study, we demonstrated that small samples obtained by EBUS-TBNA provided good quality subtyping for NSCLC with mediastinal metastasis. Of those patients diagnosed with NSCLC in our study, only 3.7% had NSCLC-NOS. The most common pathologic NSCLC classifications was adenocarcinoma (60.6%) and favor adenocarcinoma (9.2%), followed by squamous cell carcinoma (22%), and favor squamous cell carcinoma (4.6%). From the IHC results, 96.3% of the sampled tissues were classified by the time lung cancer was pathologically diagnosed. A multicenter study of 774 patients from the UK suggested that small samples obtained via EBUS-TBNA could be used to classify the NSCLC subtypes in 77% of cases (8). However, this study was limited in that it did not perform routine IHC. Our data indicate a higher rate of lung cancer subtypes because we routinely performed IHC in all tissues obtained by EBUS-TBNA. The median time lapse between EBUS-TBNA and the final reports, including the mediastinal stage, tumor diagnosis with pathologic subtype, and genetic infor-

mation (*EGFR/KRAS* mutations, and *ALK* rearrangement) was 14 days (range: 6-37 days). Past practice was to request a mutational analysis after the patient had been diagnosed with lung cancer via bronchoscopy or PCNA. Following diagnosis, EBUS-TBNA was then performed for mediastinal staging when the patient showed a significant mediastinal uptake on PET/CT. However, this led to delays in treatment because clinicians required information on the mediastinal stage, pathologic subtype, and mutation status before any decisions on treatment could be made. The protocol in place at our institute requires EBUS-TBNA to be performed initially to confirm the lung cancer staging or diagnosis, pathologic subtype, and genetic status of *EGFR*, *KRAS*, and *EML4-ALK* in patients suspected of having advanced lung cancer following PET/CT.

Our study is associated with some limitations. A recent analysis of the data from a large number of patients has reported that *ALK* rearrangements are mutually exclusive, with mutations in either *EGFR* or *KRAS* (19). Therefore, to be cost-effective, *EML4-ALK* fusion gene assessment should not be undertaken in samples harboring *EGFR* mutations. Second, the *ALK* IHC results were expressed as either positive or negative, and a scoring system according to IHC expression was not adopted. At the time we conducted our study there was little evidence that *ALK* IHC was a useful screening tool for *ALK* gene rearrangement. We also analyzed *ALK* gene arrangement despite the presence of positive *ALK* IHC cases. Third, we analyzed multiple genotypes using direct sequencing or a PNA-mediated real-time PCR clamping method. The sensitivity of these two methods in detecting *EGFR/KRAS* mutations may differ. Further studies using the PNA-mediated real-time PCR clamping method

may be required to validate this suggestion. In addition, the *ALK* FISH results using small biopsy samples from EBUS-TBNA may differ from those obtained with excision samples. Fourth, there was no information of the comparison data from samples obtained by transbronchial lung biopsy (TBLB) for the triple mutation analysis. The use of EBUS-TBNA samples may be further supported if we compared the detection rate of triple mutations from samples obtained between EBUS-TBNA and TBLB.

In conclusion, small tissue samples obtained via EBUS-TBNA were found to be sufficient to successfully detect triple mutations in advanced lung cancer. This demonstrates the usefulness of EBUS-TBNA as an initial modality with regard to the treatment of lung cancer.

**The authors state that they have no Conflict of Interest (COI).**

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