—Original Article—

Determining factors of endobronchial ultrasound-guided transbronchial needle aspiration specimens for lung cancer subtyping and molecular testing

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ABSTRACT

Objective: This study is to explore the determining factors for testing epidermal growth factor receptor (EGFR) mutation and anaplastic lymphoma kinase (ALK) fusion after subtyping by immunohistochemistry (IHC) using samples obtained from endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA). **Materials and Methods:** Patients suspected with advanced lung cancer were performed EBUS-TBNA without rapid on-site evaluation(ROSE) from January 2015 to March 2016 in Shanghai Chest Hospital. All samples diagnosed as lung cancer by histopathology underwent IHC to identify subtypes. EGFR mutation and ALK fusion were tested in adenocarcinoma and non-small-cell lung cancer-not otherwise specified (NSCLC-NOS) using remnant tissue samples. **Results:** A total of 453 patients were diagnosed with lung cancer, including 44.15% (200/453) with adenocarcinoma and 11.04% (50/453) with NSCLC-NOS. With the average passes of 3.41 ± 0.68, samples obtained from EBUS-TBNA were adequate for performing EGFR mutation and ALK fusion gene analysis in 80.4% (201/250) of specimens after routine IHC. On univariate analysis, successful molecular testing was associated with passes per lesion (P = 3.80E-05), long-axis diameters (P = 6.00E-06) and short-axis diameters (P = 4.77E-04), and pathology subtypes of lesions (P = 3.00E-03). Multivariate logistic regression revealed that passes per lesion (P = 1.00E-03), long-axis diameters (P = 3.50E-02), and pathology subtypes (P = 8.00E-03) were independent risk factors associated with successful molecular testing. **Conclusions:** With at least three passes of per lesion, EBUS-TBNA is an efficient method to provide adequate samples for testing of EGFR mutation and ALK gene arrangement following routine histopathology and IHC subtyping. Determining factors associated with successful pathology subtyping and molecular testing using samples obtained by EBUS-TBNA are passes of per lesion, long-axis diameter, and pathology subtypes. During the process of EBUS-TBNA, selecting larger lymph nodes and the puncturing at least 3 passes per lesion may result in higher success rate in lung cancer subtyping and molecular testing.

Key words: Anaplastic lymphoma kinase fusion, endobronchial ultrasound-guided transbronchial needle aspiration, epidermal growth factor receptor mutation, immunohistochemistry, lung cancer

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INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality worldwide.[1] Totally, about 85% of lung cancer cases could be classified as non-small-cell lung cancer (NSCLC).[2] Currently, with the deepening of the pathogenesis to improve the prognosis of lung cancer, the personalized treatment has been a consensus in the management of lung cancer. In view of the importance of small biopsy specimens in the treatment of advanced NSCLC, International association for the study of lung cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) proposed that the terminology of histopathological diagnosis should be clearly minimized with adenocarcinoma, squamous cell carcinoma, small-cell lung cancer, and NSCLC-not otherwise specified (NSCLC-NOS) by IHC.[3] Moreover, the epidermal growth factor receptor (EGFR) mutation testing and anaplastic lymphoma kinase (ALK) fusion analysis were recommended in patients with the diagnosis of adenocarcinoma, NSCLC, favor adenocarcinoma, and NSCLC-NOS, who were more responsive to EGFR-TKI and ALK inhibitor. Furthermore, studies indicated that patients with adenocarcinoma could benefit from pemetrexed than those with squamous cell carcinoma,[4,5] while squamous cell carcinoma is associated with life-threatening hemorrhage in patients treated with bevacizumab.[3] Therefore, accurate subtyping and molecular analysis of lung cancer and molecular testing are crucial and indispensable.

However, due to the insidious clinical course of lung cancer, most patients are diagnosed at an advanced stage with a life expectancy of <1 year under palliative chemotherapy.[6] For such patients of Stage III/IV who cannot obtain tumor tissues by surgery, the emergence of endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA), a minimally invasive technique, can provide valuable information for a primary tumor diagnosis and mediastinal staging, which significantly changed the approach to diagnosing lung cancer and metastatic lymph nodes.[7-10] The fact that EBUS-TBNA could provide adequate samples for accurate subtyping and molecular analysis has been proved, while its determining factors remain unknown. Therefore, it is significant to explore the determining factors of EBUS-TBNA to obtain sufficient tissues for subtyping lung cancer and molecular mutation for individualized medicine.

MATERIALS AND METHODS

Patient selection

Between January 2015 and March 2016, inpatients suspected with advanced primary lung cancer in the department of pulmonary medicine who were primarily not considered to have surgical operations were prospectively enrolled and retrospectively analyzed in Shanghai Chest Hospital in this study. EBUS-TBNA was performed on suspected metastatic lymph nodes or tumors to obtain tissue samples for accurate subtyping and molecular analysis in adenocarcinoma and NSCLC-NOS. Patients meeting the following criteria underwent EBUS-TBNA: (1) Enlarged mediastinal/hilar lymph nodes and/or intrathoracic masses (at least 1 node >1 cm in the short axis) based on computed tomography or positive intrathoracic lymph nodes/lesions detected (defined as standardized uptake value >2.5) by positron emission tomography and (2) no contraindication to the procedure. All patients signed an informed consent form for EBUS-TBNA examination. The Ethics Committee of Shanghai Chest Hospital approved this study.

Endobronchial ultrasound-guided transbronchial needle aspiration specimen collection

EBUS-TBNA was performed with the patient under moderate sedation (midazolam) and local anesthesia (lidocaine), as described previously.[11,12] After white-light bronchoscopy was performed orally, the target lymph nodes and peripheral vessels were examined by EBUS, using a linear array ultrasonic bronchoscope (BF-UC260F-OL8; Olympus Ltd., Tokyo, Japan). Diameter of the target lymph nodes was measured and recorded under frozen ultrasound image. A dedicated 22G needle was used for aspiration (NA-201SX-4022; Olympus Ltd). Through our previous experiences and studies, we recommended that at least two but no more than five needle aspirations be performed for each target lesion without rapid on-site evaluation (ROSE), the number of moves of each pass was about twenty times with suction and stylet routinely.[12,13] All procedures were conducted by experienced bronchoscopists.

Pathological classification and subtyping

Samples considered as lung cancer by experienced pathologists were performed IHC of CK, TTF-1, P40, and CD56 according to the recommendation of the IASLC/ATS/ERS international multidisciplinary classification.[3] Other malignant tumors were

performed other immunohistochemical marker according to the judgment of pathologists. The positive expression of CK indicates that tumors are epithelial-derived tumors. Antibodies to TTF1 were used to identify primary lung adenocarcinomas, and antibodies to P40 were used to identify squamous cell lung cancer. Metastatic small-cell carcinoma is usually confirmed by CD56.[14] Subsequently, patients diagnosed with adenocarcinoma or NSCLC-NOS underwent molecular testing. Final diagnosis was confirmed by treatment and follow-up.

Molecular testing

EGFR mutations and ALK fusions were detected by conventional methods in the specimen diagnosed with adenocarcinoma and NSCLC-NOS. EGFR mutations were detected by amplification refractory mutation system–polymerase chain reaction using EGFR 21 Mutations Detection Kit (Amoy Diagnostics, Xiamen, China). DNA was extracted from 10 to 15 unstained formalin-fixed paraffin-embedded (FFPE) sections, each 5 μm thickness, using QIAamp DNA FFPE tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The concentration of DNA was measured by SMA4000 spectrophotometer (Merinton, Beijing, China). ALK fusion was tested by IHC using VENTANA ALK (D5F3) assay (F. Hoffmann-La Roche, Tucson, AZ, USA). ALK IHC weakly positive samples were confirmed by fluorescence *in situ* hybridization (FISH) using Vysis ALK Break Apart FISH Probe Kit (Abbott Molecular, Inc., IL, USA).[15]

Statistical analysis

Sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy rate of EBUS-TBNA for diagnosing lung cancer were calculated according to standard definitions. Univariate and multivariate analyses assessed the independent risk factors for the success of EGFR and ALK analyses. A *t*-test was used for the comparison of continuous variables, and the Chi-square test or Fisher's exact test, when appropriate, was used for categorical variables. Significance was considered for $P < 0.05$, and all analyses were two sided. Significant variables in univariate analysis or those deemed clinically important were then entered in a multivariable logistic regression model. The IBM SPSS Statistics for Windows software package (ver. 20.0; IBM Corp., Armonk, USA) was used for the data analysis.

RESULTS

A total of 513 patients with 582 lesions, including 521 lymph nodes and 61 masses, underwent diagnostic EBUS-TBNA with 1811 passes totally. The average passes of EBUS-TBNA were 3.11 ± 0.7 per lesion. Four hundred and fifty-three patients were diagnosed with lung cancer. Sixty patients were excluded from the analysis because they were diagnosed with inflammation, tuberculosis, and other malignancy diseases or because of the negative results. Flowchart is shown in Figure 1. No major procedure-related complications were observed.

Samples of 453 patients diagnosed with lung cancer were all adequate for IHC, including 200 with adenocarcinoma (44.15%), 50 with NSCLC-NOS (11.04%), 78 with squamous cell lung cancer (17.22%), and 125 with small-cell lung cancer (27.59%). Twenty-five patients were diagnosed with false-negative lung cancer [Figure 1]. The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of lung cancer diagnosed by EBUS-TBNA were 94.77% (453/478), 100% (3/3), 100% (453/453), 10.71% (3/28), and 94.80% (456/481), respectively.

A total of 250 EBUS-TBNA samples of 250 patients diagnosed with adenocarcinoma and NSCLC-NOS underwent molecular testing, including 201 samples that underwent both EGFR mutation and ALK fusion analyses successfully. EGFR mutations were interpreted as positive in 72 samples (35.82%) and ALK fusion in 12 samples (5.97%). However, the EGFR mutation and ALK fusion analyses were not able to be carried out in 49 out of the 250 samples (19.6%). There were no adequate residual tissue blocks containing tumor cells in order to carry out molecular analysis after hematoxylin and eosin (HE) staining and routine IHC. Table 1 summarizes all the mutation statuses detected in EBUS-TBNA samples. Factors including gender, pathology subtypes, region of the lesion, age, passes, and lesion size were analyzed [Table 2]. On univariate analysis, successful molecular testing was associated with passes per lesion (P = 3.80E-05), long-axis diameters (P = 6.00E-06) and short-axis diameters (P = 4.77E-04), and pathology subtypes of lesions (P = 3.00E-03). Multivariate logistic regression revealed that passes per lymph node (P = 1.00E-03), long-axis diameter (P = 3.50E-02), and pathology subtypes (P = 8.00E-03) were independent risk factors

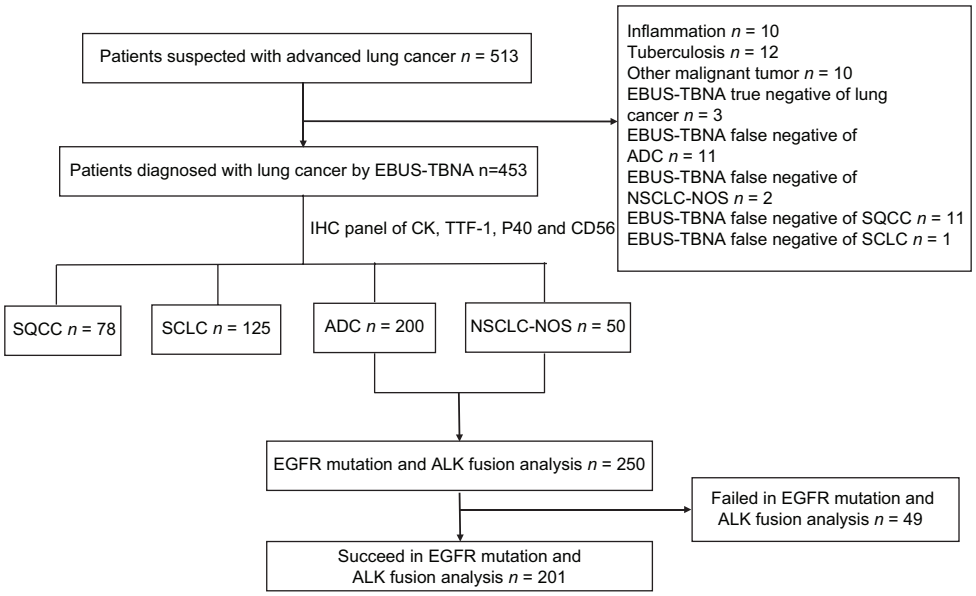

Figure 1. Flowchart of the eligible study population. Of 513 patients enrolled in the study, 453 were diagnosed with lung cancer. Of the 453 patients, 78 had SQCC, 125 had SCLC, 200 had adenocarcinoma, and 50 had NSCLC-NOS. Totally, 201 patients successfully underwent molecular analysis. ADC: Adenocarcinoma, ALK: Anaplastic lymphoma kinase, EBUS-TBNA: Endobronchial ultrasound-guided transbronchial needle aspiration, EGFR: Epidermal growth factor receptor, IHC: Immunohistochemistry, NSCLC-NOS: Non-small-cell lung cancer-not otherwise specified, SCLC: Small-cell lung cancer, SQCC: Squamous cell carcinoma

Table 1. Mutation status detected in endobronchial ultrasound guided-transbronchial needle aspiration samples

Mutation types	*n*
18exon G719X	1
19exon del	39
20exon ins	2
21exon L858R	28
21exon L861Q	1
19exon del + 21exon L858R	1
ALK fusion	12
Total	84

EGFR: Epidermal growth factor receptor, ALK: Anaplastic lymphoma kinase

associated with successful molecular testing [Table 3]. Figure 2 shows the relationship between passes per lesion and the successful rate of molecular testing.

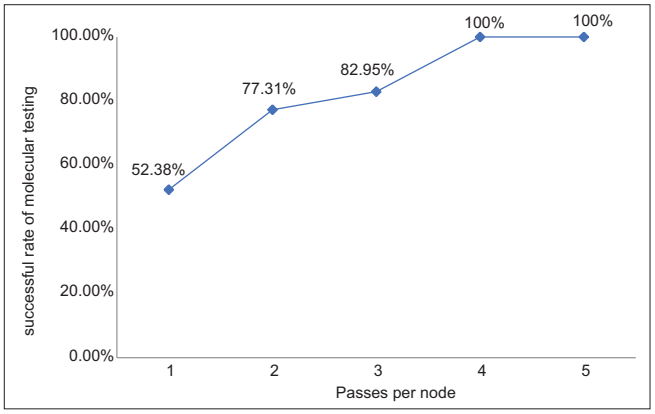

Figure 2. The relationship between passes per lesion and successful rate of molecular testing. As the number of passes increased, the success rate of molecular analysis increased gradually. When the number of passes reaches three, the successful rate reached 82.95%

DISCUSSION

The advent of targeted therapies has revolutionized the management of lung cancer.[16] Pathologists are now expected to subtype NSCLC whenever possible and order molecular testing in all cases of adenocarcinoma and NSCLC-NOS. However, for patients in advanced stage, only limited amount of tissue samples from a small biopsy specimen are available for molecular testing. EBUS-TBNA is a minimally invasive procedure that has shown considerable promise for subtyping NSCLC and collecting tissue for molecular diagnostics. However, only few studies have explored whether EBUS-TBNA samples collected in routine clinical practice can support IHC testing for accurate subtyping and molecular testing.[17-19] In this study, we explore the determining factors of molecular analysis after IHC to subtype lung cancer using samples obtained by EBUS-TBNA.

The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of lung cancer diagnosed by EBUS-TBNA were 94.77% (453/478), 100% (3/3), 100% (453/453), 10.71% (3/28), and 94.79% (455/480), respectively. The NSCLC-NOS rate

in our data was 11.04% (50/453). Some studies showed that small samples obtained from EBUS-TBNA could offer sufficient tissues for IHC after HE staining in 96%–98.7% of cases and to allow specific subtyping of NSCLC in 77%–79.5% of cases.[17-19] An outcome from Esterbrook *et al.* showed that the number of cases with insufficient tissue was only 1.3%, but 22.5% NSCLC-NOS did not have IHC performed, and it is not clear in all of these cases.[17] A multicenter study also investigated the suitability of EBUS-TBNA specimens for subtyping and genotyping of NSCLC. Twenty-three percent of patients had a final diagnosis of NSCLC-NOS.[18] However, these studies were limited in that they did not perform IHC routinely. All samples were sufficient for IHC in our study, and the data indicated a higher rate of 88.96% (403/453) for subtyping lung cancer because we performed IHC by TTF-1, P40, CK, and CD56 routinely in all samples diagnosed with lung cancer obtained by EBUS-TBNA. We considered that a combination of IHC markers could minimize the proportion of NSCLC-NOS. Moreover, there were no severe complications in all the samples.

The outcome of this study also indicated that the EBUS-TBNA procedure could provide feasible and sufficient samples for two molecular analysis including EGFR mutations and ALK gene fusion (80.40%) after a routine pathologic and immunohistochemical subtyping, which has a higher success rate compared with previous studies.[17,19-21] However, previous studies did not analyze the determining factors of specimens obtained by EBUS-TBNA for subtyping and genotyping. In contrast, our study used samples obtained via EBUS-TBNA alone and we recommended that every lesion should be punctured more than two passed, which achieve a high success rate. We suppose that the use of cell blocks in specimens is one of the reasons for the high success rate. Although it has been shown that cytology smears can be used for mutation assays, we preferred to use cell blocks according to the guidelines from the College of American Pathologists.[22] Cell blocks are recommended over smear preparations because of their ability to correlate with malignant cell content and the possible retention of more material for additional studies. Additionaly, all samples detected ALK(+) were validated by FISH, which is regarded as the gold standard in the diagnosis of ALK gene rearrangements, and the concordance rate was 100%. The results indicated that Ventana-D5F3 IHC is a valid alternative method to detect ALK(+) NSCLC. However, FISH has several disadvantages, such as the need of a fluorescence microscope for interpretation. Furthermore, the fluorescent signals can fade, and it is difficult to

Table 2. Chi-square test of predicting successful factors for epidermal growth factor receptor and anaplastic lymphoma kinase gene analyses in specimens undergoing endobronchial ultrasound guided-transbronchial needle aspiration

Characteristics	Successful (*n*=201)	Unsuccessful (*n*=62)	*P*
Gender			
Male	149	41	0.256
Female	52	21	
Pathology subtypes			
Adenocarcinoma	170	41	0.003
NSCLC-NOS	31	21	
Region			
Superior mediastinal nodes (2R, 4L, 4R)	96	27	0.255
Subcarinal nodes (7)	56	18	
N1 nodes (10L, 10R, 11L, 11Ri, 11Rs, 12L, 12R)	28	14	
Masses	21	3	

EBUS-TBNA: Endobronchial ultrasound guided-transbronchial needle aspiration, NSCLC-NOS: Non-small-cell lung cancer-not otherwise specified

Table 3. Univariate and multivariate analyses of factors predicting successful gene analysis in specimens diagnosed with adenocarcinoma and non-small-cell lung cancer-not otherwise specified undergoing endobronchial ultrasound guided-transbronchial needle aspiration

	Successful (*n*=201)	Unsuccessful (*n*=62)	Total (*n*=263)	Univariate (*P*)	Multivariate (*P*)	Adjusted OR (95% CI)
Age (range), years	59.39 (28-84)	61.21 (33-77)	59.82 (28-84)	0.19		
Passes	3.41 (2-6)	2.92 (2-4)	3.29 (1-6)	3.80E-05	0.001	0.47 (0.31-0.73)
Lesions size, mm						
Long-axis diameter	19.51 (15.20-24.50)	18.23 (14.30-23.10)	19.20 (14.30-24.50)	6.00E-06	0.035	0.80 (0.66-0.99)
Short-axis diameter	16.57 (12.20-27.90)	15.51 (11.20-25.70)	16.32 (11.20-27.90)	4.77E-04	0.16	0.87 (0.71-1.06)
Pathology subtypes (ADC/NSCLC-NOS)	170/31	41/21	211/52	3.00E-03	0.008	2.64 (1.29-5.41)

ADC: Adenocarcinoma, EBUS-TBNA: Endobronchial ultrasound guided-transbronchial needle aspiration, NSCLC-NOS: Non-small-cell lung cancer-not otherwise specified, OR: Odds ratio, CI: Confidence interval

detect morphologic details.[23] Chromogenic *in situ* hybridization allows a better morphologic evaluation of the tumors during the screening of gene rearrangement and could represent a reliable option to FISH.[24] Moreover, repeat testing of EGFR mutation and Kirsten rat sarcoma viral oncogene (KRAS) will only results in waste of specimens because the two genes are exclusive. Moreover, there were only gefitinib and crizotinib approved for EGFR mutation and ALK gene arrangement. Therefore, we only did the molecular testing of EGFR mutations and ALK rearrangements, but not KRAS mutations.[25] Therefore, other mutations such as KRAS mutation were not tested in this study, and a lower successful rate of molecular testing was shown in other studies, which needs further research and analysis.[26]

However, there were still 49 cases insufficient in EGFR mutation and ALK fusion testing for analysis and 25 cases were diagnosed with false-negative lung cancer. Results of Chi-square test indicated that the successful molecular testing was associated with pathology subtypes of the lymph nodes (P = 3.00E-03). On univariate analysis, successful molecular testing was associated with passes per lymph node (P = 3.80E-05), long-axis diameter (P = 6.00E-06), and short-axis diameter (P = 4.77E-04). Multivariate logistic regression revealed that passes per lymph node (P = 1.00E-03), long-axis diameter (P = 3.50E-02), and pathology subtypes (P = 8.00E-03) were independent risk factors associated with successful molecular testing [Table 3]. All samples obtained by puncturing four passes could have molecular testing with a successful rate of 100%. Therefore, we can conclude that, with more passes to puncture the lesion, the successful rate is higher. When a lesion was punctured with three passes, the successful rate for molecular testing could reach 82.95% [Figure 2]. Furthermore, when long-axis diameters of lymph nodes are longer, there would be longer pathways to obtain more tissues and tumor cells for molecular testing. In the factor of pathology subtypes, we found that the pathology subtypes which were diagnosed as NSCLC-NOS may have fewer malignant tumor cells than adenocarcinoma, so pathology subtypes cannot be determined and molecular testing is less likely to be successful.

A number of clinical trials demonstrated that the importance of EGFR gene mutation and ALK gene fusion in the treatment of advanced NSCLC is self-evident. The EGFR gene is frequently expressed in adenocarcinoma and somatic mutations in exons 18–21. Patients who were detected with EGFR gene mutation show a good response rate and progression-free survival, but not overall survival.[27,28] Similarly, ALK fusion should also be identified to predict the response from the ALK inhibitor.[29] Table 1 shows the types of EGFR and ALK mutations. It is obvious that the differences between EGFR mutation and clinical characteristics were statistically significant, including in gender, age, and the types of lung cancer, which was consistent with previous studies. Our study found that ALK fusion is not gender- and age-related. Similar results have been reported in some studies.[30] In this study, 12 patients with ALK gene fusion were all negative in EGFR gene analysis. Our study confirmed the conclusion that ALK gene fusion and EGFR mutation are exclusive.[31-34] However there are also special cases with double mutations.[35,36] However, the double mutation is still a rare event, which needs more studies to reveal the relevance between EGFR mutation and ALK fusion to guide the further treatment.

There are some limitations in our study. First, although the data we collected in this research were large, the single-center study possibly existed with some bias. Second, a previous study showed that, when ROSE was used, there was a 10% increase in the success of genotyping during EBUS-TBNA procedure.[37] However, based on the reality in our hospital, there were not enough cytologists or respiratory doctors to perform ROSE. Third, only pathological subtypes of adenocarcinoma and NSCLC-NOS were sent for molecular analysis; however, there are some studies which showed that there was a high rate of EGFR mutation in squamous cell lung cancer. Finally, we cannot disregard the potential effect of tumor heterogeneity that may lead to samples being unrepresentative of the whole tumor; however, this is an issue in the interpretation of all EBUS-TBNA studies.

CONCLUSIONS

Our study confirmed that EBUS-TBNA samples obtained without ROSE and subtyped by pathologists with HE and IHC markers can reach accurate classifications of lung cancer and reduce the proportion of NSCLC-NOS, and remnant specimens are sufficient for EGFR and ALK gene analyses.

Moreover, pathology subtypes, passes per lesion, and long-axis diameters are independent determining factors for successful molecular testing. It could achieve a higher successful rate of subtyping and molecular testing when selecting larger lesions and puncturing more than three passes per lesion. This demonstrates the usefulness of EBUS-TBNA as an initial modality with regard to the treatment of lung cancer. We anticipate that, as an increasing number of genetic variations and drug targets are verified, multigene mutation analysis will become more important in facilitating individualized treatment.

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Conflicts of interest

There are no conflicts of interest.

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