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Protocol to Improve Genotyping of Non-Small-Cell Lung Cancer Diagnosed Using EBUS-TBNA

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Abstract

Objectives: Targeted therapies for non-small-cell lung cancers (NSCLCs) are based on the presence of driver mutations such as epidermal growth factor receptor (EGFR) and the echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK) translocation. Endobronchial ultrasound-guided-transbronchial needle aspiration (EBUS-TBNA) is a first-line modality for diagnosing and staging NSCLC. A quality improvement protocol maximizing tissue acquisition for molecular analysis has not been previously described.

Methods: We instituted a standardized protocol designed from a multi-disciplinary meeting of the pulmonology, oncology, and pathology departments for the acquisition and on-site processing of samples obtained through EBUS-TBNA to improve the yield for genetic analysis of EGFR and ALK testing.

Results: Preprotocol there were 50 NSCLCs (29 adenocarcinomas) and postprotocol there were 109 NSCLCs (52 adenocarcinomas). A statistically significant increase in yield for molecular analysis was seen in both EGFR (36% preprotocol and 80% postprotocol, $P < 0.01$) and ALK (41% preprotocol and 80% postprotocol, $P < 0.01$). There was no difference in complications preprotocol and postprotocol.

Conclusions: Implementation of a standardized protocol with EBUS-TBNA was associated with an increase in adequacy for molecular genetic analysis in NSCLC.

Keywords

echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase; endobronchial ultrasound guided-transbronchial needle aspiration; epidermal growth factor receptor; lung cancer; non-small-cell lung cancer

Lung cancer is the leading cause of death from cancer and is the most commonly diagnosed cancer worldwide.¹ Approximately 75% to 80% of newly diagnosed lung cancers are non-small-cell lung cancers (NSCLCs); adenocarcinoma, large cell carcinoma, or squamous cell

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carcinoma make up the majority of cases.² Epidermal growth factor receptor (EGFR) and the echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK) translocation are two molecular subtypes for which targeted chemotherapy strategies have proven beneficial.^{3,4} Successfully identifying these driver mutations requires additional histologic material beyond what is needed for standard immunohistochemical stains used to differentiate NSCLC adenocarcinoma from squamous cell carcinoma.

Convex or linear probe endobronchial ultrasound-guided-transbronchial fine needle aspiration (EBUS-TBNA) is a safe and minimally invasive technique to diagnose and stage mediastinal and hilar lymph node involvement in lung cancer. EBUS-TBNA has been recommended as the initial and preferred modality to determine staging by the American College of Chest Physicians' lung cancer guidelines⁵ and has demonstrated superiority to mediastinoscopy for sampling mediastinal and hilar lymph nodes.⁶

Studies have shown that EBUS-TBNA can obtain sufficient material to test for genetic mutations, ranging from 77% to 98% for EGFR⁷⁻⁹ and 93% to 100% for ALK,^{10,11} but few have described how to achieve these yields in a manner that can be applied to centers with multiple performing physicians and trainees.^{12,13} A recent large cohort demonstrated that 22% of nonsquamous NSCLCs was never tested for EGFR and ALK,¹⁴ suggesting that centers need a standardized approach to increase the number of tested samples. No published investigations have reported a pre- and poststandardized specimen acquisition method for the purpose of optimizing identification of NSCLC histology and molecular genotyping for EGFR and ALK that may benefit centers that do not have high yields. We performed a quality improvement project, which standardizes specimen acquisition and processing during EBUS-TBNA with the purpose of increasing the number of cases with adequate histologic subtyping and molecular characterization of EGFR and ALK mutations.

Methods

We compared experience with EBUS-TBNA during a 2-year period (January 2012–December 2013) before implementing a standardization protocol (Fig. 1) with that during a 2-year period poststandardization (January 2014–December 2015). Institutional review board approval was obtained and informed consent was waived because this initiative was targeted for quality enhancement. EBUS-TBNA was performed using an Olympus BF-Convex Scope XBF-UC160 (Olympus, Tokyo, Japan) with a 22-gauge needle or a Pentax Convex Scope EB-1970UK (Pentax, Tokyo, Japan) with a 22-gauge cytology needle. The type of sedation was at the discretion of the attending physician. Moderate sedation involved titrated doses of fentanyl and midazolam. General anesthesia involved either a laryngeal airway or an endotracheal tube as determined by the bronchoscopist and anesthesiologist. Our bronchoscopists have established extensive experience with conventional and EBUS-TBNA.^{15,16} The standard nodal survey for known or suspected NSCLC is performed by ultrasound with aspirates obtained from N3 stations (contralateral paratracheal and contralateral hilar nodes) followed by N2 stations (ipsilateral mediastinal nodes and subcarinal nodes) followed by N1 stations (ipsilateral hilar nodes) or primary lesions. Our procedure standard is to use suction while aspirating the specimen.

Before the protocol, specimen acquisition was variable and the number of aspirates per node was operator dependent. The aspirates were rinsed or placed on slides or ejected as a clot block, or some combination of the three, depending on attending preference. Rapid on-site cytology (ROSE) was used both pre- and postprotocol. The new protocol (Fig. 1) was developed during coordinated discussions with participants from the pulmonology, pathology, and medical oncology departments in our center's thoracic oncology program. This approach was presented to Pulmonary Department faculty; circulated among bronchoscopy technicians, cytotechnicians, and trainees; and reinforced by means of posters in the bronchoscopy suite (both the moderate sedation and the general anesthesia procedure rooms).

Per protocol, two slides were made for each TBNA aspirate. One slide was stained for ROSE and the other was placed in ethanol. The remaining material in the needle was rinsed with saline into CytoLyt solution (Cytoc Corp, Marlborough, MA). Samples from each nodal station were grouped into separate ethanol and CytoLyt containers to avoid contamination and ensure appropriate staging. Once ROSE confirmed the presence of malignant cells, three dedicated passes were made and the full contents of the aspirate were rinsed with saline in the CytoLyt solution. If NSCLC was suspected, then a final pass was made and two unstained slides were reserved for EML4-ALK testing. At the completion of the case, the CytoLyt solutions were submitted to the pathology laboratory for histological processing to create a paraffin-embedded cell block. This specimen was used to create additional slides for immunohistochemical staining at the discretion of the performing pathologist, and this approach was not altered with the institution of the protocol.

In both pre- and postprotocol, if preliminary pathology was consistent with adenocarcinoma, not otherwise specified (NOS), or poorly differentiated, then the pathologist determined whether the sample was sufficient for EGFR and ALK testing. Postprotocol, if the sample was determined to be sufficient, then genetic testing for ALK and EGFR were performed without consulting the ordering physician or oncologist (reflex testing).

The following description for ALK and EGFR testing applies to both the preprotocol group and the postprotocol group. Unstained slides were tested for ALK gene rearrangement within our institution by fluorescent in situ hybridization (FISH). The remaining material from the cell block was sent for EGFR mutation testing via DNA polymerase chain reaction sequencing at an offsite Clinical Laboratory Improvement Amendments–certified laboratory. Neither the send-out laboratory nor the internal FISH testing differed before or after the protocol. Characterization of a sample as sufficient or insufficient was at the discretion of the attending pathologist who sent the specimen for genetic testing if he or she believed the cell block was sufficient. In general, for FISH, >100 tumor cells are needed. In contrast, for mutational analysis, a high tumor content of 50% to 70% is believed to be required.³

Statistical analyses were performed comparing results before and after the standardization. Adequate material was determined by the pathologist and specified in the report on a per-patient basis. Results are presented as percentages or means $\pm$ standard deviations. Continuous variables were compared between groups using Student *t* tests. Nominal

variables were analyzed using χ^2 tests for association followed by logistic regression. Results were considered significant when the *P* value was <0.05.

Results

A total of 499 patients were included in this quality improvement initiative: 148 before the implementation of the standardized protocol for specimen acquisition and 351 who had their procedure performed after the protocol was implemented. There were 50 (34%) NSCLCs preprotocol and 109 (31%) postprotocol. Patient demographics, indication for bronchoscopy, and staging are displayed in Table 1. There was no difference in age, sex, or cancer yield before and after protocol implementation.

There was no difference in overall complications (6.8% vs 4%, *P* = 0.19) nor complications requiring hospitalization following the procedure (3.4% [n = 5] preprotocol and 2.5% [n = 9] postprotocol [*P* = 0.61]). Complications requiring hospitalization included chest pain (2.0% preprotocol and 1.4% postprotocol), respiratory distress (0.7% preprotocol and 0.8% postprotocol), and arrhythmia (0.7% preprotocol and 0.3% postprotocol). Some of the other complications not including hospitalization were respiratory distress (0.7% preprotocol and 0.8% postprotocol), bleeding (1.3% preprotocol and 0.8% postprotocol), and bronchospasm (1.4% preprotocol and 0% postprotocol).

The average number of aspirates that were obtained per patient was 7 ± 3 in the preprotocol group and 10 ± 5 in the postprotocol group (*P* < 0.01) across all locations. In nodes containing metastatic NSCLC, the number of aspirates was different: 6 ± 3 preprotocol and 8 ± 3 postprotocol (*P* < 0.01). The distribution of lymph node stations or masses with NSCLC adenocarcinoma or NOS targeted for molecular genetic testing is shown in Table 2. The majority of cases were performed with general anesthesia, 97% preprotocol and 91% postprotocol, because we have anesthesia availability in the bronchoscopy suite. The procedure time in patients with NSCLC (adenocarcinoma, poorly differentiated, or NOS) was not different among groups (45 ± 15 minutes preprotocol vs 43 ± 20 postprotocol, *P* = 0.47).

Per patient, adequate samples (including cancer, lymphoid tissue, or granulomas and excluding unsatisfactory or suspicious) was 93% in the preprotocol and 94% in the poststandardization group (*P* = 0.17). Cancer yield per patient was 49% (n = 73) in the preprotocol group and 51% (n = 176) in the standardized group (*P* = 0.78). Adenocarcinoma was found preprotocol in 58% (n = 29) of NSCLCs and postprotocol in 46% (n = 52) of NSCLCs (*P* = 0.17). There was no difference in the number of samples that could not be definitively assigned as adenocarcinoma or squamous cell (NOS).

Preprotocol, 24% (n = 9) of nonsquamous NSCLC samples were not tested for EGFR or EML4-ALK compared with 6% (n = 4) EGFR and 8% (n = 5) ALK after the protocol (*P* = 0.03 and *P* = 0.05, respectively). Of the nonsquamous cell NSCLC, 13 samples (39%) in the preprotocol group and 41 (76%) in the postprotocol group were tested for EGFR or ALK (*P* < 0.01). More aspirates were performed after initiation of the protocol for both sufficient (EGFR *P* < 0.01, ALK *P* < 0.01) and insufficient samples (EGFR *P* < 0.01, ALK *P* < 0.01;

Fig. 2). The number of aspirates performed was not significantly different between sufficient and insufficient samples for either the preprotocol (EGFR $P=0.35$, ALK $P=0.24$) or postprotocol (EGFR $P=0.97$, ALK $P=0.78$) groups. The number of specimens that were sufficient for EGFR and ALK rose significantly after implementation of the standardized protocol: from 36% ($n=12$) to 80% ($n=42$) for EGFR ($P<0.01$) and from 41% ($n=13$) to 80% ($n=42$) for ALK ($P<0.01$; Fig. 3).

Discussion

The capability of EBUS to acquire sufficient material to reliably and routinely identify therapeutic biomarkers such as EGFR mutations and EML4-ALK translocations allows medical oncologists to effectively personalize treatment plans for patients with advanced NSCLCs. At our institution with an established EBUS-TBNA program and extensive operator experience with EBUS-TBNA, implementation of a protocol was associated with substantial improvement in molecular genetic testing. During the 2 years postprotocol, the number of EBUS procedures continued to rise, accounting for more cases.

Genotyping for EGFR mutations is central to standard care. Providing targeted therapy for patients who harbor EGFR actionable mutations can improve first-line responses compared with traditional cytotoxic chemotherapy.¹⁷ EGFR mutation testing has become the standard for all newly diagnosed nonsquamous lung cancers, regardless of age, smoking status, sex, or other clinical risk factors.¹⁸ Testing for EGFR mutations usually is done at the time of diagnosis, and collected tissue should be prioritized for molecular testing. According to published guidelines, tissue block is the preferred sample type, but cytospin cell block material is routinely used from bronchoscopy.³ The gold standard for EGFR mutation evaluation remains direct sequencing, for which submitted samples should have a high tumor cell content of at least 50% tumor cells to maximize available DNA for genetic evaluation.^{3,18} Some laboratories require a smaller amount. If an EGFR mutation is identified, then first-line treatments are adjusted as tyrosine kinase inhibitors such as erlotinib or gefitinib come to the forefront.

Another biomarker that has changed first-line treatment options for patients with lung cancer are the ALK translocations. Similar to what is noted in EGFR guidelines, EML4-ALK translocations should be tested for in all newly diagnosed advanced-stage adenocarcinomas regardless of the patient's age, smoking status, sex, or other clinical risk factors. Unlike EGFR mutations, which rely on direct sequencing as the gold standard for identification, ALK translocations are identified by a FISH assay that uses a dual-labeled break-apart probe. Identification of an ALK translocation in stage IV adenocarcinoma would warrant first-line therapy with crizotinib over standard platinum-based chemotherapy.¹⁸

Bronchoscopists who perform EBUS-TBNA must strive to maximize tissue acquisition for diagnosis, histological subtyping, and molecular analysis to obviate the need for more invasive procedures. The first objective is to test the samples. A retrospective review of >16,000 patients with NSCLCs demonstrated no testing on 22% of samples.¹⁴ With our protocol and reflex testing, we were able to double the number of samples tested for EGFR and ALK.

With the challenge of providing sufficient material for molecular analysis, optimum bedside specimen processing is imperative. We attribute the substantial increase in the quantity of material for ALK and EGFR testing in our improvement initiative to be multi-factorial. First, implementation of the protocol was associated with an increase in the number of nodal aspirates, with more consistent sampling among operators. Because there was no change in the number of aspirates in the sufficient compared with the insufficient samples, the increase in genetic yield cannot be attributed solely to more needle passes. Consistently rinsing the needle after each pass likely added material to the cell block that may have been lost by only smearing aspirates onto a slide, however. Second, additional dedicated needle rinses where no material was placed on a slide probably contributed to the cell block. Third, reflex testing may have reduced waste of material by precutting the cell block. When the cell block is accessed later, for additional slides, a fresh shave of the paraffin-embedded block must be wasted to realign the specimen. Although the precise impact of each of these factors was not measured, we believe that their cumulative effect was substantial.

There are some limitations to our study. First, reflex testing for actionable genetic mutations is controversial. There are cost concerns related to testing, especially if patients have early-stage disease and will undergo resection; however, the majority of patients undergoing EBUS had advanced stage disease IIIB or IV (62%–70%). Separate studies are needed to determine the cost-effectiveness of automatic testing for genetic mutations, taking into consideration the avoidance of repeat biopsies to obtain more material and whether the information gained by analyzing the markers was used in the treatment plan. Current guidelines encourage ALK and EGFR testing in patients with all stages of lung cancer.¹⁸ Second, some laboratories may require <50% for EGFR testing and that may necessitate a different standardized protocol than the one we used. Ongoing investigations of the role of microRNA may change the way we test for genetic mutations in the future.¹⁹

Procedural details represent other areas for consideration. We have anesthesiology support available in the bronchoscopy suite, and our preference is to perform EBUS under general anesthesia when multiple nodes are to be sampled or if the procedure is being conducted for suspected recurrence. In the latter setting, EBUS yields and negative predictive values have been shown to be lower.²⁰ Higher yields were demonstrated in the American College of Chest Physicians Quality Improvement Registry for general anesthesia EBUS cases,²¹ whereas other single-center studies demonstrated similar yields in the hands of experienced bronchoscopists.^{22,23}

There are limitations inherent in our study in potential variations among interpreting pathologists and performing bronchoscopists. Our EBUS-TBNA procedures averaged more passes than the four passes suggested by Yarmus et al,¹² and the yields were not as high as those achieved by Nakajima et al (93%).⁷ Hopkins et al²⁴ averaged 4.5 passes with 79% sufficiency for EGFR testing from the cell block, whereas Jeyabalan et al²⁵ averaged 3 punctures with 98% sufficiency for EGFR testing. Such variation confirms that as bronchoscopists, we can influence adequate material for genetic testing by standardizing bedside processing, but additional efforts need to be coordinated with pathologists in any effort to optimize processing technique and reduce unnecessary immunohistochemical stains to conserve material for genetic analysis. It remains possible that increased cumulative

experience and familiarity with the therapeutic importance of biomarkers influenced our bronchoscopists, but it is noteworthy that the overall diagnostic rates of NSCLC, rate of adenocarcinoma, and stage of disease were similar during both phases of this experience.

Because this comparison was made after establishing 4 years of institutional experience with EBUS-TBNA,¹⁶ we do not believe that a learning curve with EBUS was a factor. Although it is possible that other unidentified factors may have influenced our increasing yield, adherence to our protocol was associated with higher yields. It is interesting to note that despite additional needle punctures and nodal sampling, procedure duration was not longer in the postprotocol group and complications were not different among groups. This finding suggests that the protocol may have contributed to the efficiency and/or safety of the procedure. This observation has important implications in developing a standardized approach that can be used at centers at which multiple physicians perform bronchoscopy with an array of trainees of all levels of skill and experience.

Conclusions

Advances in therapeutic oncology have created an important role for personalized genomic medicine in treatment of NSCLCs. In this era of precision medicine, our study not only confirms the role of EBUS but also demonstrates the effectiveness of a standard protocol to increase the number of samples tested and obtain material sufficient for molecular genetic testing. Protocols in the bronchoscopy suite should be implemented to achieve higher yields with individualized modifications of this approach adapted for novel institutional variations and resources. As demonstrated during development and implementation of our multidisciplinary protocol, close collaboration and communication among all of the providers involved in the diagnosis, classification, and treatment of patients is essential to ensuring the retrieval of adequate tissue for the comprehensive diagnosis of NSCLC.

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Key Points

- Non-small-cell lung cancer therapies make testing for epidermal growth factor receptor and 4-anaplastic lymphoma kinase mutations central to patient care.
- Protocols to maximize tissue for diagnosis and molecular analysis are paramount.
- Our protocol doubled the yield of epidermal growth factor receptor and 4-anaplastic lymphoma kinase in specimens obtained by endobronchial ultrasound-guided-transbronchial needle aspiration lung cancer.

Fig. 1.

Intraprocedure protocol for specimen acquisition in the bronchoscopy suite. Step 1, obtain material on slides for on-site cytology with accompanying needle rinses for cell block contribution. Step 2, obtain additional needle rinses for cell block material if NSCLC is suspected. Step 3, performed if additional diagnoses on the differential warrant testing for lymphoma or microbiologic analysis. EGFR, epidermal growth factor receptor; H&E, hemotoxylin & eosin; IHC, immunohistochemical; NSCLC, non-small-cell lung cancer.

Fig. 2.

Pre- and postprotocol needle aspirates showing an increase in total number of aspirates, with no difference in material deemed sufficient or insufficient by pathology for molecular testing. ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor.

Fig. 3. Pre- and postprotocol sufficient material for EML4-ALK and EGFR testing. EGFR, epidermal growth factor receptor; EML4-ALK, echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase translocation.

Table 1.

Patient characteristics

	Preprotocol	Postprotocol	<i>P</i>
Total no.	148	351	
Age, y, mean $\pm$ SD	62.5 $\pm$ 12.5	62.1 $\pm$ 12.1	0.72
Female sex (%)	66 (45)	156 (45)	0.98
Cancer diagnosis on EBUS-TBNA (%)	73 (49)	178 (51)	0.78
NSCLC			0.21
Adenocarcinoma	29 (56)	52 (46)	
Squamous	15 (29)	50 (44)	
Poorly differentiated/NOS	8 (15)	11 (10)	
Indication for EBUS-TBNA in patients with sample positive for adenocarcinoma (%) ^a			0.74
Suspected lung cancer	25 (68)	44 (71)	
Newly diagnosed lung cancer for staging	3 (8)	5 (8)	
Suspected recurrence of lung cancer	9 (24)	12 (19)	
Suspected nonlung cancer	0	1 (2)	
Stage of adenocarcinoma (%) ^a			0.92
1a and 2a	2 (6)	2 (4)	
3a	12 (32)	17 (27)	
3b	7 (19)	11 (18)	
4	16 (43)	33 (52)	

^aNumbers include adenocarcinoma and poorly differentiated and NOS, all of which we reflexively tested for molecular genetic mutations.

EBUS-TBNA, endobronchial ultrasound-guided-transbronchial needle aspiration; NOS, not otherwise specified; NSCLC, non-small-cell lung cancer; SD, standard deviation.

Table 2.Characteristics of NSCLC adenocarcinoma^a sites sampled by EBUS-TBNA

	Preprotocol	Postprotocol	P
All node stations/masses			
N (%)	37	63	
Size ± SD, mm	18 ± 7	29 ± 16	<0.01
No. aspirates ± SD	6 ± 2	7 ± 4	<0.01
Node station (%)			
4R			
N (%)	13 (35)	14 (23)	
Size ± SD, mm	15 ± 4	19 ± 7	0.08
No. aspirates ± SD	5 ± 1	7 ± 2	0.07
4L			
N (%)	1 (3)	2 (3)	
Size ± SD, mm	12	15 ± 0	NA
No. aspirates ± SD	8	7 ± 1	0.33
7S			
N (%)	4 (11)	10 (16)	
Size ± SD, mm	19 ± 7	23 ± 7	0.41
No. aspirates ± SD	6 ± 2	7 ± 2	0.17
10R/11R nodes and right hilar masses			
N (%)	9 (24)	12 (19)	
Size ± SD, mm	19 ± 6	34 ± 18	0.02
No. aspirates ± SD	6 ± 2	8 ± 3	0.43
10L/11L nodes and left hilar masses			
N (%)	6 (14)	8 (13)	
Size ± SD, mm	20 ± 6	26 ± 12	0.25
No. aspirates ± SD	5 ± 1	9 ± 3	0.02
Paratracheal masses			
N (%)	1 (3)	10 (16)	
Size ± SD, mm	47	39 ± 12	0.55
No. aspirates ± SD	3	8 ± 1	0.02
Other masses or nodules			
N (%)	1 (3)	6 (10)	
Size ± SD, mm	14	42 ± 24	0.33
No. aspirates ± SD	8	6 ± 2	0.39
Combined sites ^b			
N (%)	1 (3)	1 (1)	
Size ± SD, mm	11R (8 mm), 7S (12 mm)	4R (18 mm), 4L (15 mm)	NA
No. aspirates ± SD	5 and 3, respectively	4 each	NA

^aIncludes NSCLC: adenocarcinoma, not otherwise specified, and poorly differentiated.

^bTwo patients had samples taken from two different sites and combined for immunohistochemical staining and molecular genetic testing.

EBUS-TBNA, endobronchial ultrasound-guided-transbronchial needle aspiration; NA, not applicable; NSCLC, non-small-cell lung cancer; SD, standard deviation.

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