Review article

The role of EBUS-TBNA in lung cancer restaging and mutation analysis

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Abstract: In recent years, several molecules targeting specific genetic aberrations were released for the treatment of patients affected by locally advanced and metastatic non-small cell lung cancer (NSCLC), leading to an improvement in survival. Moreover, inhibitors of PD-1 and PD-L1 immune checkpoints showed to improve survival, and they are now indicated as first-line treatment in selected patients. Hence, the collection of adequate samples for diagnosis, staging, genotyping and immunohistochemical analysis is a fundamental step in NSCLC treatment planning. When feasible, EBUS-TBNA is suggested as the first-choice diagnostic tool by most of the guidelines. Several studies demonstrated that mutation analysis is viable with high levels of accuracy on both cytological and histological samples obtained by EBUS-TBNA. No technical factor (type of needle, number of passes, use of rapid-on-site-examination, material processing, detection method) has been identified as uniquely influencing the diagnostic yield of molecular analysis. EBUS-TBNA demonstrated to be useful for the restaging of patients affected by locally advanced NSCLC who underwent induction chemotherapy or chemo-radiotherapy, as well as in those who show acquired resistance to targeted therapy and immunotherapy. Nevertheless, most authors agree that a high number of false negative results should be expected due to the likely presence of necrosis and fibrosis induced by neoadjuvant treatments. Therefore, in case of EBUS-TBNA negative sample, pathologic confirmation by surgical biopsy is recommended for the planning of definitive treatment. As suggested by a few preliminary experiences, a wide application of next-generation sequencing (NGS) on EBUS-TBNA specimens will lead to the development of better tailored treatments with simultaneous identification of a large number of gene alterations on a single sample at the time of diagnosis.

Keywords: EBUS-TBNA; non-small cell lung cancer (NSCLC); molecular analysis; PD-L1; restaging

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Introduction

Despite the advances of multimodality treatments, lung cancer is still one of the world-leading causes of death. It has been estimated that over 228,000 people will be diagnosed with lung cancer in 2020 in the USA, and up to 135,000 individuals will die of the disease (1). According to European data (2), pulmonary tumors represent the first cause of death for neoplasm in the male sex, and the second in women.

Non-small cell lung cancer (NSCLC) accounts for 85% of all cases (3) and up to 65% of the patients have locally advanced or metastatic disease at the time of diagnosis (4,5).

In recent years, molecular targeted treatments have progressively entered in standard therapeutic regimens for stage III–IV NSCLC (6,7). The latest update of NCCN guidelines (8) recommends osimertinib as first-line

treatment in patients with positive EGFR mutation, and alectinib in those with ALK rearrangement. Both NCCN and ASCO (9) guidelines strongly recommend the use of pembrolizumab, an immune checkpoint PD-1 inhibitor (ICI), as first-line treatment of patients with high PD-L1 expression (>50%), alone or in association with platinum-based regimens.

Molecular-based treatments are, however, not only reserved to patients with advanced, unresectable disease. There is indeed growing evidence that even subjects with limited disease may benefit from targeted therapies: several trials are investigating the use of tyrosine kinase inhibitors (TKIs) and ICIs as alternative neoadjuvant and adjuvant options for early stage NSCLC, or in case of recurrence after complete treatment (10,11).

At the present time, seven EGFR inhibitors, five drugs targeting ALK changes, four targeting abnormal ROS1, 2 for BRAF gene changes, two interfering with NTRK gene changes, and four PD-1/PD-L1 blocking agents are available, and some of them were approved for the treatment of mutation-bearing NSCLC in the clinical practice (12,13). This is the reason why the search of a broad molecular analysis, including targetable gene aberrations and immunohistochemistry for PD-L1 testing, is strongly recommended at the time of diagnosis of NSCLC, not only on surgical specimens but also on small biopsy samples (8,14).

Several guidelines suggest EBUS-TBNA as the procedure of choice for the diagnosis and staging of patients with suspected NSCLC. The American College of Chest Physicians (ACCP) guidelines for the diagnosis and staging of lung cancer (15) recommend EBUS-TBNA both in case of enlarged lymph nodes regardless of PET uptake, and in patients with normal-size lymph nodes at CT scan showing pathologic FDG uptake at whole body-PET scan. Moreover, ACCP guidelines and the combined guidelines of the European Society of Gastrointestinal Endoscopy (ESGE), the European Respiratory Society (ERS), and the European Society of Thoracic Surgeons (ESTS) (16) both advise that patients undergoing pulmonary resection for NSCLC should be preoperatively staged with EBUS-TBNA in case of tumors larger than 3 cm, centrally located lesions, or PET negative primary tumors.

In a recent prospective multicentric study (17), EBUS-TBNA showed a higher diagnostic yield when compared to any other bronchoscopic sampling technique and resulted to be independently associated with a higher probability of diagnosis at multivariate analysis. The combination (CUS) of EBUS-TBNA and endoscopic ultrasound-fine needle aspiration (EUS-FNA) allows complete staging of the mediastinum in patients with NSCLC, reaching a sensitivity value even superior to that of cervical mediastinoscopy (18), and should therefore be preferred whenever available (15,16).

With the discovery of the therapeutic value of targetable EGFR mutation in 2004, the availability of an adequate amount of tissue for histology subtyping and molecular analysis became a critical issue. Despite the uncertain consistency of the initial results (19), small samples and even cytological specimens proved to be appropriate for a full molecular assessment of NSCLC when properly handled (20,21). Moreover, in the studies by Heymann *et al*. (22) and Verocq *et al*. (23), the immunohistochemical analysis of PD-L1 expression on cytological and small biopsy samples from patients affected by NSCLC resulted comparable to the corresponding surgical samples.

Nowadays, EBUS-TBNA is a key diagnostic tool in patients with locally advanced or unresectable disease and for patients unfit for surgery because of comorbidities, reducing the need of invasive surgical diagnostic procedures. Considering these premises, EBUS-TBNA not only plays a key role in the diagnosis and staging of suspected lung cancer, but it also proved to allow accurate molecular characterization of the disease.

Adequacy of molecular genotyping and PD-L1 assessment on samples obtained by EBUS-TBNA: review of literature

In 2007, Nakajima *et al*. (24) first assessed the feasibility of EGFR mutation determination on samples obtained by EBUS-TBNA. In 43 out of 46 patients (93.5%) with newly diagnosed locally advanced or metastatic lung adenocarcinoma enrolled in the study, analysis of exons 19 and 21 of EGFR gene was possible after polymerase chain reaction (PCR) on histological core-biopsy tissue. The Authors concluded that EBUS-TBNA was an appropriate technique for EGFR mutation analysis; notably, specimens had a lower burden of contaminating cells with respect to those obtained with other non-surgical sampling techniques.

So far, a number of other studies investigated the adequacy of EBUS-TBNA samples for the search of several biomarkers (*Table 1*). Gefinitib and crizotinib were the first TKIs approved for the treatment of metastatic lung cancer patients, respectively expressing EGFR mutation and ALK translocation. Considering the higher rates of

Table 1 Results of molecular markers assessment on samples obtained by EBUS-TBNA from patients affected by NSCLC

Author, year	Country	Enrollment period	N° patients	Histotype	TNM stage	Type of sample	Markers assessed	Adequate samples for testing, %	Prevalence of positive samples†, %	Main findings
Nakajima, 2007 (24)	Japan	2003–2006	46	Adenocarcinoma	IIB–IV	H	EGFR	93.5	25.6	Histologic cores obtained by EBUS-TBNA contain a lower burden of contaminating cells compared to other non-surgical specimens
Garcia-Olivé, 2010 (25)	Spain	2006–2007	36	Adenocarcinoma, NOS NSCLC	IIA–IV	H	EGFR	72.2	7.7	EBUS-TBNA is useful to obtain samples suitable for EGFR mutation analysis. The occurrence of mutations is higher in patients with adenocarcinoma than NOS NSCLC
Sakairi, 2010 (26)	Japan	2008–2009	109	NSCLC	II–IV	H + C	EGFR	100	22.9	Cytological samples are suitable for ALK fusion genes analysis. Immunohistochemistry shows higher sensitivity than FISH and RT-PCR
							ALK	100	6.4	
Nakajima, 2011 (27)	Japan	2008–2009	156	NSCLC	II–IV	H + C	EGFR	98.7	26.9	Multigene mutation analysis is feasible on samples obtained by EBUS-TBNA
							KRAS	72.4	3.5	
							p53	72.4	41.6	
Santis, 2011 (28)	UK	2009–2011	132	NSCLC	NR	C	EGFR	95.5	10.5	EBUS-TBNA provides sufficient cytological material for EGFR and KRAS mutation analysis. COLD-PCR increases the sensitivity of detection of mutant sequences
							KRAS	98.4	17.5	
Navani, 2012 (29)	UK	2009–2011	119	NSCLC	NR	C	EGFR	90	6	EGFR mutation determination is feasible on cytological EBUS-TBNA samples
Okada, 2012 (30)	Japan	2006–2009	14	NSCLC	IIIA–IIIB	H + C	EGFR	100	14.3	EGFR mutation status on EBUS-TBNA samples may not reflect the status of primary tumors due to genetic heterogeneity
Esterbrook, 2013 (31)	UK	2009–2011	36	Non-squamous NSCLC	NR	C	EGFR	88.8	3.1	Cell blocks specimens are adequate for EGFR mutation testing
Neat, 2013 (32)	UK	NR	55	NSCLC	IIB–IV	C	EGFR	NR	NR	Evaluation for ALK rearrangement by FISH is possible in most cytological samples obtained by EBUS-TBNA
							ALK	94.5	5.7	
							KRAS	NR	23.6	
Jurado, 2013 (33)	USA	2010–2012	56	Adenocarcinoma, adenosquamous carcinoma	NR	C	EGFR	90	10	EBUS-TBNA under moderate sedation permits to obtain sufficient material for molecular analysis
							ALK	91	12	
							KRAS	75	25	

Table 1 (*continued*)

Table 1 (*continued*)

Author, year	Country	Enrollment period	N° patients	Histotype	TNM stage	Type of sample	Markers assessed	Adequate samples for testing, %	Prevalence of positive samples†, %	Main findings
Folch, 2013 (34)	USA	2007–2012	42	Adenocarcinoma, NOS NSCLC	II–IV	C	EGFR	95.2	11.9	Molecular genotyping with EBUS-TBNA is non-inferior to other minimally invasive and surgical techniques, and is superior to percutaneous needle biopsies. No factors responsible for failure of molecular testing have been identified
							ALK	90.5	2.4	
							KRAS	90.5	42.9	
Casadio, 2015 (35)	Italy	2012–2014	195	Adenocarcinoma, NOS NSCLC	IIIB–IV	C	EGFR	96.9	16.9	No statistical difference in mutational status defined by cytological EBUS-TBNA samples compared to a large surgical series
							ALK	98	3.9	
							KRAS	96.4	31.6	
Bravaccini, 2016 (36)	Italy	2012	115 (collectively with traditional TBNA)	Adenocarcinoma	NR	C	EGFR	100	14	Ineffective immediate fixation of cytological sample, subsequent incorrect handling, and low cellularity are causes of inadequacy for ALK evaluation
							ALK	76.5	10.2	
Guisier, 2016 (37)	France	2012–2014	111	Non-squamous NSCLC	NR	H + C	EGFR	79.3	11.4	The use of radial EBUS allows multi-gene molecular analysis in about 80% of patients with peripheral non-squamous NSCLC. Upper and middle lobe tumor location and >3 passes are independent predictors of increased molecular assessment feasibility
							ALK	79.3	5.7	
							KRAS	78.4	26.4	
							HER2	77.5	1.2	
							PI3K	77.5	0	
							BRAF	77.5	2.3	
							MET	93.3	28.6	
							ROS1	NR	0	
Lee, 2016 (38)	South Korea	2011–2013	109	NSCLC	II–IV	H	EGFR	100	21.1	Triple gene analysis was possible in 96% of patients with small biopsy samples obtained by EBUS-TBNA
							ALK	96.3	4.9	
							KRAS	100	11.9	
Jeyabalan, 2016 (39)	UK	2010–2014	80	Adenocarcinoma	NR	H	EGFR	98.8	6.3	Combined EGFR-ALK success rate was 99%. Needle size does not affect accuracy
							ALK	100	0	
Fernandez-Bussy, 2017 (40)	Chile	2014–2015	86	Adenocarcinoma, NOS NSCLC	NR	C	EGFR	97.7	25.6	Samples obtained by EBUS-TBNA are suitable for evaluation of acquired resistance to TKIs by ROS1 testing
							ALK	94.2	5.8	
							ROS1	83.3	8.3	

Table 1 (*continued*)

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Author, year	Country	Enrollment period	N° patients	Histotype	TNM stage	Type of sample	Markers assessed	Adequate samples for testing, %	Prevalence of positive samples†, %	Main findings
Rosso, 2017 (41)	Italy	2005–2016	54	NSCLC	NR	H	EGFR	98.1	7.5	EBUS-TBNA allows appropriate diagnosis, staging, and molecular characterization of NSCLC. ROSE does not improve the yield. Selection of needle can follow individual preference
							ALK	92.6	3.7	
							KRAS	100	11.9	
							BRAF	100	2.4	
							MET	100	50	
							ROS1	100	0	
Sakakibara, 2017 (42)	Japan	2013–2014	97	NSCLC	NR	H	PD-L1	100	NR	EBUS-TBNA enables better evaluation of PD-L1 expression than TBB, with results comparable to correspondent surgical samples, in particular in presence of high cellularity (>2,000)
Raad, 2018 (43)	USA	2012–2016	69	NSCLC	I–IV	H + C	EGFR	100	4.3	Availability of ROSE and >6 passes increase the yield of EBUS-TBNA for multiple molecular determinations, and possible for NGS
							ALK	90.5	4.8	
							KRAS	100	17.2	
							ROS1	94.1	7.8	
Bellinger, 2018 (44)	USA	2014–2015	109	NSCLC	I–IV	H + C	EGFR	80	NR	The application of a standardized protocol for specimen acquisition and processing improved the diagnostic yield for molecular genotyping with EBUS-TBNA
							ALK	80	NR	
Biswas, 2018 (45)	USA	2017	50	NSCLC	II–IV	C	ALK	88	NR	Cytology samples from EBUS-TBNA provide sufficient material for both ALK, PD-L1 and NGS testing
							PD-L1	86	32	
Fernandez-Bussy, 2018 (46)	Chile	2015–2017	23	NSCLC	NR	H	PD-L1	100	13	EBUS-TBNA provides adequate histological samples for PD-L1 analysis, but it is not clear if they are representative of primary tumor
Ghigna, 2018 (47)	France	2011–2017	398	NSCLC	NR	H	EGFR	79.4 (overall)	7	EBUS-TBNA and rapid molecular diagnostics consent molecular profiling along with pathologic definition at the time of diagnosis. ROSE reduces the number of needle passes and improves adequacy of molecular testing and NGS
							ALK		3	
							KRAS		23	

Table 1 (*continued*)

Table 1 (*continued*)

Author, year	Country	Enrollment period	N° patients	Histotype	TNM stage	Type of sample	Markers assessed	Adequate samples for testing, %	Prevalence of positive samples†, %	Main findings
Sakata, 2018 (48)	USA	2006–2016	61	NSCLC	I–IV	C	PD-L1	100	16.4	Increasing number of passes and large bore needles may reduce the number of false negative PD-L1 samples from EBUS-TBNA. Adequacy is influenced by the threshold of PD-L1 positive cells chosen as cutoff and cellularity of the sample
Cicek, 2019 (49)	Turkey	2013–2016	114	Adenocarcinoma, NOS NSCLC	IIIA–IV	C	EGFR	88.6	11.4	EBUS-TBNA provided adequate samples for ROS1 testing in a large population
							ALK	93.8	8	
							ROS1	91.8	1	
Smith, 2020 (50)	Canada	2016–2017	120	NSCLC	I–IV	C	PD-L1	91.6	48.2	No clinical or procedural factors are predictors of successful PD-L1 testing on EBUS-TBNA samples. Concordance with correspondent surgical tissue is 78%

†, PD-L1 samples were considered positive when TMB >50% (high expression). NSCLC, non-small cell lung cancer; C, cytological sample; H, histological sample; NOS, not otherwise specified; NR, not reported; TMB, tumor mutational burden; TBB, transbronchial biopsy; NGS, next-generation sequencing; TKI, tyrosine kinase inhibitor; FISH, fluorescence-in-situ- hybridization; RT-PCR, real-time polymerase chain reaction.

positive samples found in female, non-smoker patients with adenocarcinoma histology (51), molecular assessment was at first almost exclusively reserved to these cases. Moreover, as incidence of EGFR alterations is relatively higher in Asian race compared to Caucasians (ranging from 14% to 27%), Japanese groups were the first to report their experience on the topic (26,27,30).

Most Authors agree that the diagnostic yield of EBUS-TBNA for molecular genotyping is high. In some studies, EGFR and ALK determination was possible in the entire cohort of the patients enrolled (26,30,36,38,39,43), and in most of the experiences adequate specimens were available in over 90% of patients who underwent EBUS-TBNA. In 2018, Labarca *et al*. released a meta-analysis including 33 studies (almost 2,700 patients) evaluating the diagnostic power of EBUS-TBNA for NSCLC molecular characterization (52). The pooled diagnostic yield for EGFR and ALK determination reached 94.5% and 94.9%, respectively; combined EGFR and ALK analysis, reported by 9 of the trials analyzed, was successful in 94.2% of cases.

As a result of the introduction of new molecules, improved diagnostic and therapeutic pathway of lung cancer, and increased confidence with the technique, indication for molecular assessment on EBUS-TBNA samples has now been extended to patients with histotypes different from adenocarcinoma, as well as to those with limited disease. Guisier and colleagues (37) investigated the presence of multiple gene aberrations (including EGFR, ALK, KRAS, MET, and ROS1) in 111 patients with peripheral non-squamous NSCLC who underwent sampling with radial EBUS-TBNA. Biopsy tissue resulted adequate in about 80% of cases. Other trials confirmed the possibility to perform multiple molecular analyses on EBUS-TBNA samples, some reporting a percentage of sample adequacy even superior to 90% (38,41,43).

Patients showing with locally advanced or metastatic NSCLC, with wild-type EGFR and ALK and PD-L1 expression in over 50% of neoplastic cell population [i.e., tumor mutational burden (TMB)] at immunochemistry (IHC) are suitable for the treatment with PD-1 or PD-L1 ICIs (*Figure 1*). Significant results in terms of both local disease control and improvement of survival were demonstrated following treatment with these molecules (13). Considering that most patients with stage III–IV disease do not undergo surgical procedures, collection of adequate samples for IHC analysis by EBUS-TBNA has gained a prominent role.

Only few studies analyzed the feasibility of performance of PD-L1 testing by means of EBUS-TBNA, with diagnostic yield ranging from 86% and 100% of the patients tested (42,45,46,48,50). In the series by Sakakibara *et al*. (42), EBUS-TBNA samples showed a higher cellularity and contained better conserved tumoral cells with respect to those obtained with conventional transbronchial biopsy (TBB). Moreover, results of PD-L1 assessment were concordant to primary tumors and lymph node metastases with a good rate of correlation, as confirmed by another study (50).

As in case of other molecular biomarkers, cytological specimens demonstrated to be appropriate for a full analysis of PD-L1 with the currently available IHC platforms in the presence of adequate cellularity (45,48,50). Additional passes and large bore needles have been suggested to reduce confounding results due to possible tumor heterogeneity and choice of PD-L1 threshold; nevertheless, Smith and colleagues did not identify any significant procedural influencing factor (50).

Technical factors affecting accuracy of mutation analysis on EBUS-TBNA samples

It was demonstrated that accuracy of molecular analysis on lung cancer samples obtained by EBUS-TBNA is influenced by several intrinsic factors related to the tumor characteristics, such as histologic subtype, tumor location, target lymph node size, and grade of tumor heterogeneity between primary lesion and metastatic sites (30,37,42,46,53). Moreover, other factors potentially conditioning the rate of success are mutation prevalence in the examined population and ethnicity (52).

The role of technical features involved in EBUS-TBNA outcome for the search of molecular aberrations has been widely investigated. Several studies pointed out that the choice of needle, number of passes, use of rapid-on-site-evaluation (ROSE), sample cellularity and contamination by surrounding necrosis or blood elements, and sample processing are determinant factors to obtain suitable material (33). The CHEST guidelines for EBUS-TBNA released in 2016 recommend, regardless of ROSE availability, at least three passes for each sampled station, and possibly additional passes to increase effectiveness of mutation analysis, but with low level of evidence (54).

Others did not confirm these findings with contrasting results (34). The meta-analysis of Labarca *et al*. (52) failed to identify any procedural feature significantly correlated to provision of adequate material for molecular investigations.

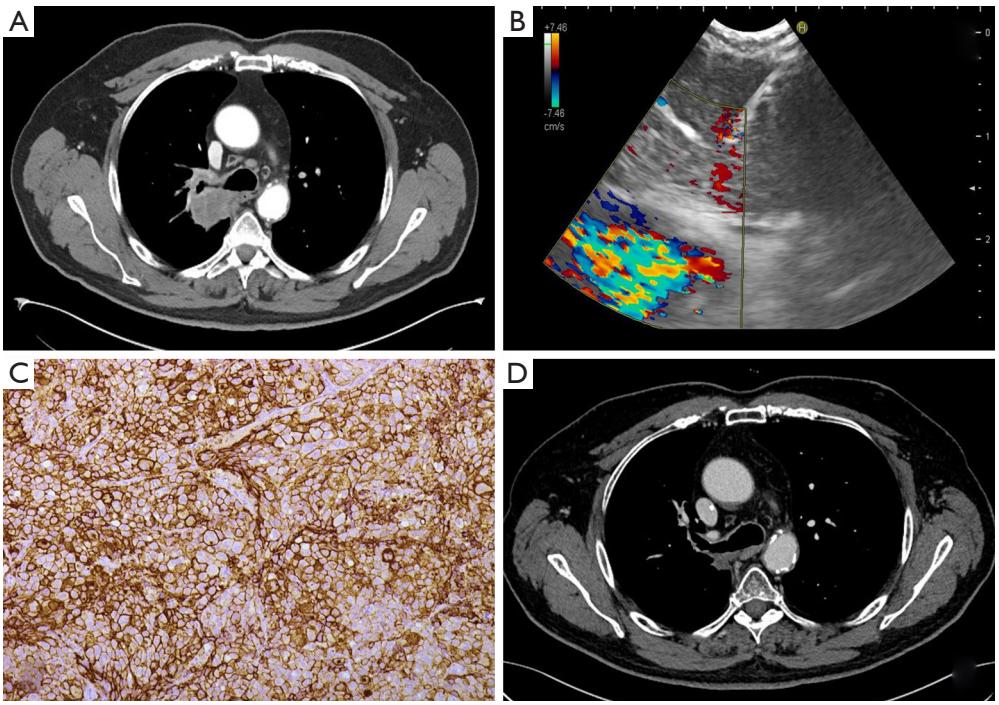

Figure 1 A 70-year old male patient was found to have a right solid pulmonary para-hilar mass invading the tracheobronchial angle (A); EBUS-TBNA resulted positive for adenocarcinoma G3 (TTF1 positive, p63 negative, synaptophysin negative, EGFR and ALK wild-type, KRAS positive) (B); at PD-L1 assessment (clone 22C3, Ventana Benchmark Ultra platform), 90% of neoplastic cells resulted positive (C); the patient underwent induction therapy with cisplatin-vinorelbine (four cycles) and concurrent radiotherapy. After restaging, the mass resulted resectable by means of pneumonectomy; yet, surgery was contraindicated due to poor respiratory function. Immunotherapy with durvalumab was started. Chest CT scan 7 months after diagnosis showed a significant reduction of the tumor (D).

Needle size and type of sample

In most of the published series, cytological and histological samples are obtained with the employment of 21- or 22-gauge needles. Although Authors supporting the use of larger bore needles confirm a similar diagnostic performance to 22-gauge needle, they report that samples obtained employing 21-gauge needles display conserved architecture, allowing better morphologic and genetic characterization of NSCLC (55). On the other side, 22-gauge needle has the advantage of being able to reach 'difficult' locations, such as 4L lymph node station, thanks to its flexibility.

Jeyabalan *et al*. (39) and Rosso and colleagues (41) analyzed the potential effect produced by the choice of needles of different size; both Authors, however, concluded that, given the comparable results, selection should follow the individual preference of the operator, as suggested by CHEST guidelines (54).

Number of passes and ROSE

In 2013, Yarmus and colleagues (56) analyzed the data of 85 patients affected by lung adenocarcinoma or not otherwise specified (NOS) NSCLC. Excellent results for mutation analysis including EGFR, ALK, and KRAS were obtained in patients submitted to at least 4 passes per sampled site and concurrent ROSE. Raad *et al*. stated that the rate of success could be increased by carrying out more than 6 passes in a Center with ROSE availability (43). In some cases, even higher number of biopsies (up to 20) have been reported (42).

The studies addressing the use of ROSE in patients undergoing EBUS-TBNA for genotyping of NSCLC gave discordant results. According to Ghigna *et al*. (47), fresh-frozen samples sent for on-site examination provide uncrushed genetic material for ancillary tests of higher quality than fixed samples. A randomized trial comparing two groups of NSCLC patients who underwent molecular analysis with or without ROSE found no significant

difference in terms of sensitivity and adequacy rate (57). Further investigations confirmed that, if an adequate number of passes per sampled station is performed (usually 3 to 4), it is possible to obtain a full molecular diagnosis of NSCLC regardless of the availability of ROSE (37,41,48,53). Hence, the use of ROSE is not mandatory, but it should be tailored on the basis of Center experience (54).

In the daily clinical practice, however, shortage of material for mutation analysis after routine processing for cytological and IHC analysis is common. Nevertheless, it has been showed that material obtained by even a single dedicated additional pass may provide sufficient material for a full molecular assessment (58), a factor that should always be considered to ensure adequate diagnosis, staging and molecular characterization of suspect lung cancer.

Sample management and detection method

Regardless of needle size, EBUS-TBNA sampled material can be processed in several ways both for histological and/or cytological examination. Cytological specimens may be smeared on glass slides or assembled as paraffin-embedded cell blocks following individual preferences.

In 2010, the conjunct consensus released by the International Association for the Study of Lung Cancer (IASLC) and the European Thoracic Oncology Platform (59) advised the use of core biopsies for EGFR characterization until better definition of the role of cytological specimens; similar conclusions are reported by IASLC for IHC analysis of PD-L1 (60).

Nevertheless, several studies analyzing the results of molecular determination and PD-L1 determination on EBUS-TBNA samples so far demonstrated that cytological specimens, in particular cell blocks, enable high quality processing for such purposes (26,28,29,31,32,35,45). Bravaccini *et al*. (36) reported that wrong specimen handling after withdrawal rather than the amount of tissue available for analysis is responsible for missing diagnosis and molecular characterization.

According to the guidelines of the World Association for Bronchology and Interventional Pulmonology (WABIP), none between smear glass cytology, cell block and tissue core biopsy is superior to the others to improve the likelihood to obtain adequate samples (61). In fact, cellularity of the sample, ratio between normal and tumoral cells, and performance of the adopted method of detection seem to be the factors mostly influencing the diagnostic yield. In most of the published series, PCR is the preferred method for amplification of target sequences; yet, the quantity of tumoral DNA necessary for completion of analysis may vary according to the used technique (28). With regard to ALK analysis, there are some evidences supporting superiority of IHC over fluorescence-in-situ-hybridization (FISH) and real-time PCR (RT-PCR) (26). However, no detection method demonstrated to be superior to others in the meta-analysis of Labarca *et al*. (52).

Lung cancer restaging and EBUS-TBNA

Stage III of tumor-node-metastasis (TNM) staging system for NSCLC includes a variety of clinical presentations ranging from large pulmonary masses invading neighboring structures to small primary lesions with mediastinal lymph node metastases. Despite upfront surgery may be an option in carefully selected patients (e.g., in case of single N2 station disease) (62), it is widely accepted that primary resection without a preliminary induction therapy is detrimental because of high risk of incomplete resection and later recurrence (63).

In recent years, 18-F-FDG-PET scan demonstrated to be a useful tool to ensure appropriate staging of both primary tumor and regional and distant metastases (8). Still, some questions were raised regarding the efficiency of imaging for disease restaging after induction treatments. A recent systematic review underlined that, even if SUVmax and other newly introduced metabolic parameters seem to be promising factors for the evaluation of response, further larger trials are required to confirm the results (64).

Therefore, pathologic assessment after neoadjuvant therapy should still be considered mandatory for an appropriate therapeutic planning. Repeated mediastinoscopy or more invasive surgical approaches have been for a long time the only available techniques for preoperative evaluation of patients undergoing radical treatment. However, many Authors reported non-negligible rates of morbidity and mortality, and inadequate sensitivity and accuracy as consequences of technical challenges caused by the presence of inflammatory fibrosis induced by primary staging mediastinoscopy and oncological treatment (65).

The advent of EBUS-TBNA offered the possibility of a minimally invasive staging, and an improvement of the results of imaging staging when used in association with PET-CT scan (66). A summary of studies that investigated the role of EBUS-TBNA in mediastinal restaging is reported in *Table 2*.

Mediastinal restaging with EBUS-TBNA after induction

Table 2 Performance of EBUS-TBNA for mediastinal restaging after neoadjuvant chemotherapy or chemo-radiotherapy

Author, year	N° patients	Sampling technique	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Accuracy, %
Herth, 2008 (67)	124	EBUS	76	100	100	20	77
Szlubowski, 2010 (68)	61	EBUS	67	86	91	78	80
Zielinski, 2013 (69)	88	CUS	64	100	100	82	NR
Szlubowski, 2014 (70)	106	CUSb	67	96	95	73	81
Nasir, 2014 (71)	32	EBUS	50	100	100	88	89
Genestreti, 2015 (66)	14	CUS	50	60	33	75	NR
Çetinkaya, 2017 (65)	44	EBUS	82	100	100	76	89
Muthu, 2018 (72)[†]	574	CUS	67	99	52	33	NR
Jiang, 2020 (73)[‡]	558	CUS	65	99	NR	35	NR

[†], Meta-analysis; pooled results with EUS-FNA; [‡], meta-analysis; results referred to EBUS-TBNA. CUS, combined EBUS and EUS; CUSb, combined EBUS and EUS using a single ultrasound bronchoscope; NPV, negative predictive value; NR, not reported; PPV, positive predictive value; EUS-FNA, endoscopic ultrasound-fine needle aspiration.

chemotherapy was first assessed in 2008 in a trial including 89 patients (67). Samples resulted positive in all patients showing stable disease at restaging CT scan and persistent metastases at subsequent intraoperative biopsy. However, 28 out of 35 EBUS-negative patients were found to have lymph node metastases at the time of surgery, resulting in suboptimal sensitivity and low (20%) negative predictive value (NPV). Nevertheless, the authors pointed out that these results were comparable to those obtained in patients who underwent induction therapy on the basis of non-invasive primary staging and later restaged with mediastinoscopy, and superior to repeated mediastinoscopy.

Several studies remarked the presence of a fair number of false negative patients influencing the NPV of EBUS for mediastinal restaging. Probably, residual cancer cells may be not detected in small EBUS samples, because of necrosis and fibrosis induced by neoadjuvant chemo- and radiotherapy (74). Nevertheless, some authors reported values of NPV superior to 80%, not as high as those obtained by surgical restaging, but with a significant lower rate of procedural morbidity (69,71).

The association of EBUS-TBNA and EUS-FNA may improve the performance of ultrasonographic restaging (69). Szlubowski and colleagues (70) showed that a full mediastinal restaging with combined EBUS and EUS is feasible with a single scope instrument (CUSb-NA). Both sensitivity and accuracy of CUSb-NA resulted significantly higher than EBUS-TBNA and EUS-FNA alone. However, these results have not been confirmed in another study with a smaller population (66).

Guidelines for the selection of endoscopic or surgical restaging of NSCLC are still lacking. However, considering the number of false negative patients found at restaging with EBUS-TBNA, pathologic surgical confirmation [either with mediastinoscopy, transcervical extended mediastinal lymphadenectomy (TEMLA), VATS, or thoracotomy] seems to be still advisable before considering definitive treatment, as confirmed by two recent meta-analyses (72,73).

Some patients treated with targeted therapy with TKIs or ICIs show incomplete response or tumor progression at follow up. In fact, the onset of new gene mutations inducing resistance to first-line treatments and tumor transformation into more aggressive, less differentiated histologic subtypes are well known phenomena (75-77). This is why disease restaging could help in the definition of the following treatment. Recovery times being notably shorter, restaging by means of EBUS-TBNA may reduce the time interval between diagnosis and therapy onset compared to surgical procedures. However, there are still few reports investigating the role of EBUS-TBNA for molecular restaging.

Kirita *et al*. (78) analyzed 70 patients with NSCLC who developed resistance to standard chemotherapy or targeted drugs. Eighteen patients were rebiopsied by means of EBUS-TBNA, and 52 with TBB. All EBUS-TBNA cases resulted diagnostic, compared to 83% of TBBs, even if the difference was not statistically significant. Genotyping was possible in all cases; one patient showed small-cell lung cancer (SCLC) transformation.

In another study by Izumo *et al.* (79), molecular analysis was required in 53 NSCLC patients previously treated with TKIs who developed resistance. The rate of adequate samples for mutation analysis was higher in the group of patients who underwent EBUS-TBNA compared to TBB under EBUS guidance (100% *vs.* 75%, respectively). In both Kirita and Izumo series no complications related to EBUS-TBNA procedure have been reported, confirming the safety of the technique. However, the number of patients enrolled in these trials was low, and larger studies are required to confirm these results.

Next-generation sequencing (NGS) and future perspectives

In most of the studies investigating the feasibility of molecular analysis on samples obtained by EBUS-TBNA, only one or two genes were tested for target aberrations search (*Table 1*). Some Authors demonstrated that both cytological and histological EBUS-TBNA specimens are suitable for multiple analyses (37,41,43). However, wasting of material is still a problem to be faced, and accurate selection of molecular tests is an essential step to achieve adequate characterization of the disease. Nevertheless, the number of available therapeutic molecules for mutated NSCLC is rapidly increasing (12), as well as the alterations to be analyzed on the available tissue.

NGS is a novel technique that enables the simultaneous identification of a large panel (from 50 to over 1,000) of gene alterations—including target driver mutations—assessed on a single platform (80). Hence, NGS is going to cover an important role in the therapeutic decision for patients undergoing targeted therapies, immunotherapy, and enrollment in clinical trials.

A few studies investigated the feasibility of NGS on samples obtained by EBUS-TBNA. One of the main limitations for its application is due to the necessity of increasing gradients of cellularity in the specimen according to the number of genes that have to be assessed. For this reason, the adequacy of small EBUS-TBNA samples for such purpose has been a note of concern.

Yet, it has been demonstrated that NGS analysis can be carried out not only on tissue core biopsies, but also on cell blocks, and even on cytology smears (81,82). Several studies reported a successful analysis in over 90% of EBUS-TBNA samples submitted for NGS (83-85).

In experienced centers, EBUS-TBNA has therefore emerged as a technique that enables provision of adequate specimens for a full molecular assessment in patients affected by NSCLC. Future research in the field of molecular analysis with EBUS-TBNA should be directed to the standardization of the sampling technique and tissue management, and the development of dedicated guidelines.

The low NPV, in particular in case of NSCLC restaging, is one of the main limitations of EBUS-TBNA, and studies are being carried out to overcome the high number of false negative cases with new more specific detection targets. Inage and colleagues (86) investigated the role of microRNAs assessment as tumor markers in patients undergoing NSCLC restaging after chemo-radiotherapy with encouraging results, as they were able to reach a NPV of 100%. However, further trials are needed to definitely improve the effectiveness of EBUS-TBNA in this field.

Conclusions

Samples obtained by EBUS-TBNA from patients affected by NSCLC are adequate for a full genetic profiling of alterations that can be targeted by tailored treatments. Despite the high number of technical variables involved (type of needle, number of passes, use of ROSE, sample management, detection method), none of these factors seems to sensitively affect the overall diagnostic yield of the technique.

The use of EBUS-TBNA should be encouraged in patients with NSCLC who need a restaging of disease after induction therapy, or progression in the course of therapy with TKIs or ICIs, to guide subsequent treatments. However, considering the relatively high number of false negative cases, it is still advisable to offer a surgical biopsy to patients without evidence of tumor cells on EBUS-TBNA specimen before definitive treatment.

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