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**DOTTORATO DI RICERCA IN
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**THE ROLE OF EBUS TBNA IN DIAGNOSIS OF LUNG TUMOR –
FROM MORPHOLOGY TO MOLECULAR TESTING**

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INTRODUCTION

Lung cancer remains one of the leading causes of cancer-related mortality worldwide, accounting for a significant proportion of oncologic deaths. In the United States alone, more than 225,000 new cases are diagnosed annually, with approximately 160,000 fatalities attributed to the disease (1). The early and accurate diagnosis, along with precise staging, is critical for establishing an optimal treatment strategy tailored to the patient's specific disease profile.

In recent years, the therapeutic landscape for both small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) has undergone remarkable transformation. Advances in molecular oncology have facilitated the identification of genetic mutations that drive tumorigenesis, leading to the development of targeted therapies that significantly improve patient outcomes. Additionally, the exploration of tumor-immune system interactions has paved the way for the introduction of immunotherapeutic agents, which have revolutionized the management of lung cancer.

Given these advancements, there is an increasing necessity to obtain high-quality tissue samples that enable comprehensive histopathological and molecular characterization of lung tumors. Endoscopic procedures, particularly endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA), have emerged as minimally invasive, highly effective techniques for obtaining diagnostic material. The ability to procure adequate specimens for histological examination, immunohistochemical profiling, and molecular testing is paramount in facilitating personalized treatment approaches.

EBUS-TBNA has become a cornerstone in the diagnostic and staging workflow of lung cancer, allowing for the acquisition of cytological and histological samples with minimal patient morbidity. The application of this technique extends beyond conventional morphological assessment to include next-generation sequencing (NGS), fluorescence in situ hybridization (FISH), and polymerase chain reaction (PCR)-based assays. These methodologies enable the identification of actionable genetic alterations, such as EGFR mutations, ALK rearrangements, and PD-L1 expression levels, which are crucial in guiding therapeutic decisions.

As precision medicine continues to shape modern oncology, the role of EBUS-TBNA in lung cancer diagnosis and management is expected to expand further, ensuring that patients receive the most effective, individualized treatments based on their tumor's molecular profile. Thus, ongoing research and technological advancements in endoscopic tissue acquisition and processing will remain fundamental in optimizing patient outcomes in lung cancer care.

Several works have demonstrated the diagnostic power of the material taken via EBUS TBNA, while very few studies have been conducted evaluating the possibility of molecular characterization through material taken with needle aspiration. The primary objective of this study is to evaluate the diagnostic accuracy of samples obtained through EBUS-TBNA for immunomolecular analysis of centrally located lung tumor. Additionally, this study investigates the sampling techniques and specimen preservation methods employed to determine the most effective approach for obtaining adequate material, thereby ensuring the optimal therapeutic pathway for cancer patients.

LUNG CANCER OVERVIEW

Lung cancer remains the leading cause of cancer-related mortality in men worldwide, with late diagnosis representing a major challenge in improving patient outcomes. Lung cancer screening has been explored as a strategic approach, given that early detection and timely intervention have been shown to reduce mortality in other solid tumors, such as cervical and colorectal cancer. WHO divides lung cancer into two major classes based on its biology, therapy, and prognosis: non-small cell lung cancer (NSCLC), and small cell lung cancer (SCLC). NSCLC accounts for more than 80% of all lung cancer cases, and it includes two major types: 1) nonsquamous, including adenocarcinoma, large-cell carcinoma, and other subtypes; and 2) squamous cell (epidermoid) carcinoma.

Among NSCLC subtypes, adenocarcinoma is the most prevalent, particularly among individuals who have never smoked.

Recent advancements in screening, minimally invasive diagnostic and therapeutic techniques, stereotactic ablative radiotherapy (SABR), targeted therapies, and immunotherapies have significantly improved survival outcomes. Between 2009–2010 and 2015–2016, the 2-year relative survival rate for NSCLC increased from 34% to 42%, with 5-year survival rates ranging from 15% to 62.5% depending on biomarker status. Consequently, molecular testing plays a crucial role in guiding treatment selection, particularly in patients with advanced or metastatic disease. The advent of precision medicine has revolutionized lung cancer management by identifying clinically relevant genetic alterations that inform targeted therapy selection while minimizing exposure to ineffective treatments. Given that 1–3% of NSCLC tumors may harbor concurrent genetic alterations, obtaining an adequate tissue sample is essential to enable

comprehensive mutational analysis and maximize therapeutic opportunities.

IMMUNOMOLECULAR TARGETS

Epidermal Growth Factor Receptor (EGFR) gene mutations represent critical molecular targets in the diagnostic and therapeutic landscape of non-small cell lung cancer (NSCLC). EGFR, a receptor tyrosine kinase expressed on epithelial cells, is frequently overexpressed in various malignancies. The most common activating mutations, including exon 19 deletions and the p.L858R point mutation in exon 21, are strongly associated with sensitivity to tyrosine kinase inhibitor (TKI) therapy. Consequently, molecular testing for EGFR mutations is recommended in NSCLC patients (stage IB–IIIC) when TKI treatment is considered. In addition to these prevalent mutations, rarer EGFR alterations—such as exon 21 p.L861Q, exon 18 p.G719X, and exon 20 p.S768I—constitute approximately 10% of EGFR-mutant NSCLC cases and may also confer responsiveness to specific TKIs like osimertinib and afatinib. Therefore, individualized mutation-specific treatment approaches should be considered whenever feasible. The assessment of EGFR mutation status is primarily conducted using real-time polymerase chain reaction (PCR) and next-generation sequencing (NGS), which are the most widely employed molecular testing methodologies.

Anaplastic lymphoma kinase (ALK) gene rearrangements play a crucial role in the pathogenesis of non-small cell lung cancer (NSCLC), leading to aberrant activation of the ALK kinase domain and dysregulated downstream signaling. The most prevalent ALK fusion partner is echinoderm microtubule-associated protein-like 4 (EML4), though multiple

other fusion variants have been identified. Molecular testing for ALK rearrangements is essential when tyrosine kinase inhibitor (TKI) therapy is being considered, particularly in patients with NSCLC stages IB–IIIB. While resection specimens may provide technically more straightforward testing conditions, initial diagnostic biopsy samples are also suitable for analysis. The presence of ALK rearrangements is a predictive biomarker for sensitivity to targeted oral ALK TKI therapies, underscoring the necessity of accurate and timely molecular testing. Among available methodologies, fluorescence in situ hybridization (FISH) using a break-apart probe assay was the first widely implemented approach for detecting ALK rearrangements and remains a key technique in clinical practice.

ROS1 (ROS proto-oncogene 1) gene rearrangements represent a critical molecular alteration in non-small cell lung cancer (NSCLC), leading to aberrant activation of the ROS1 kinase domain and subsequent dysregulated signaling. These rearrangements result from chromosomal translocations that generate ROS1 fusion proteins, with commonly identified fusion partners. The presence of a ROS1 rearrangement is clinically significant, as it predicts sensitivity to targeted therapy with oral ROS1 tyrosine kinase inhibitors (TKIs), which have demonstrated substantial efficacy in ROS1-rearranged NSCLC. Detection of ROS1 rearrangements relies on molecular diagnostic techniques, with fluorescence in situ hybridization (FISH) using a break-apart probe methodology serving as the gold standard for identification. Given its therapeutic implications, routine testing for ROS1 rearrangements is recommended in patients with NSCLC to guide precision oncology approaches.

BRAF (B-Raf proto-oncogene) point mutations play a critical role in the pathogenesis of non-small cell lung cancer (NSCLC) by driving

constitutive activation of the MAP/ERK signaling pathway. BRAF encodes a serine/threonine kinase involved in cellular proliferation and survival and activating mutations (V600E) result in aberrant pathway signaling. The presence of this specific mutation has been associated with sensitivity to targeted therapy using combined BRAF and MEK inhibitors, offering a personalized therapeutic approach for affected patients. Molecular assessment of BRAF mutation status is typically performed using real-time polymerase chain reaction (PCR) and next-generation sequencing (NGS), both of which provide high sensitivity and specificity for mutation detection.

KRAS (KRAS proto-oncogene) point mutations play a critical role in the oncogenesis of non-small cell lung cancer (NSCLC) by driving constitutive activation of the MAP/ERK signaling pathway. KRAS encodes a G-protein with intrinsic GTPase activity and activating mutations lead to dysregulated downstream signaling. The presence of KRAS mutations has significant clinical implications, as it is associated with poorer overall survival compared to KRAS wild-type tumors. Moreover, KRAS-mutated tumors exhibit a low likelihood of harboring additional targetable alterations, making further molecular testing less beneficial in many cases. However, a notable exception is the KRAS p.G12C mutation, which confers sensitivity to specific oral KRAS G12C inhibitors, offering a targeted therapeutic approach in affected patients. The identification of KRAS mutations is primarily achieved through next-generation sequencing (NGS) and real-time PCR, which are the most widely utilized methodologies for molecular testing in NSCLC.

The MET (mesenchymal-epithelial transition) receptor tyrosine kinase plays a crucial role in cellular signaling pathways regulating proliferation,

survival, and motility. In non-small cell lung cancer (NSCLC), mutations leading to the loss of exon 14 (METex14 skipping) result in dysregulated receptor degradation and prolonged signaling, promoting oncogenesis. A broad spectrum of molecular alterations can lead to METex14 skipping, including point mutations, insertions, and deletions affecting splice sites. Importantly, the presence of this alteration is associated with a significant response to oral MET tyrosine kinase inhibitors (TKIs), making it a critical predictive biomarker. For the accurate detection of METex14 skipping events, next-generation sequencing (NGS)-based testing is the preferred methodology, offering high sensitivity and specificity compared to other molecular techniques.

RET (*rearranged during transfection*) gene rearrangements involve structural alterations in the RET receptor tyrosine kinase, leading to constitutive activation of downstream signaling pathways in non-small cell lung cancer (NSCLC). These rearrangements result in oncogenic driver events, promoting tumorigenesis through dysregulated RET kinase domain signaling. Importantly, the presence of a RET fusion is predictive of sensitivity to selective RET tyrosine kinase inhibitors (TKIs), irrespective of the specific fusion partner. For diagnostic detection, fluorescence in situ hybridization (FISH) using break-apart probes can be employed; however, this approach may fail to identify certain fusion events. Next-generation sequencing (NGS) offers a more comprehensive and sensitive alternative, with RNA-based NGS being preferable to DNA-based NGS, as it enhances the detection of RET rearrangements by directly analyzing fusion transcripts.

Programmed death ligand 1 (PD-L1) is a co-regulatory molecule that can be expressed on tumor cells, contributing to immune evasion by inhibiting T-cell-mediated cytotoxicity. Checkpoint inhibitor antibodies disrupt the interaction between PD-1 and PD-L1, thereby enhancing the antitumor activity of endogenous T cells. Immunohistochemical (IHC) analysis of PD-L1 expression serves as a predictive biomarker for selecting patients most likely to benefit from first-line anti-PD-1/PD-L1 therapies. Various antibody clones have been developed for PD-L1 IHC, and while some exhibit comparable staining intensity and distribution, others differ significantly. The definition of PD-L1 positivity or negativity is contingent upon the specific antibody clone and platform used, which may be unique to each checkpoint inhibitor therapy. The availability of multiple assays for PD-L1 has raised concerns among pathologists and oncologists regarding standardization and comparability. In non-small cell lung cancer (NSCLC), PD-L1 IHC interpretation primarily focuses on the proportion of tumor cells exhibiting membranous staining, rendering it a continuous variable, whereas scoring criteria may differ across other malignancies. Notably, while PD-L1 expression can be elevated in tumors harboring oncogenic drivers, targeted therapy should take precedence over immune checkpoint blockade in these cases to optimize treatment efficacy.

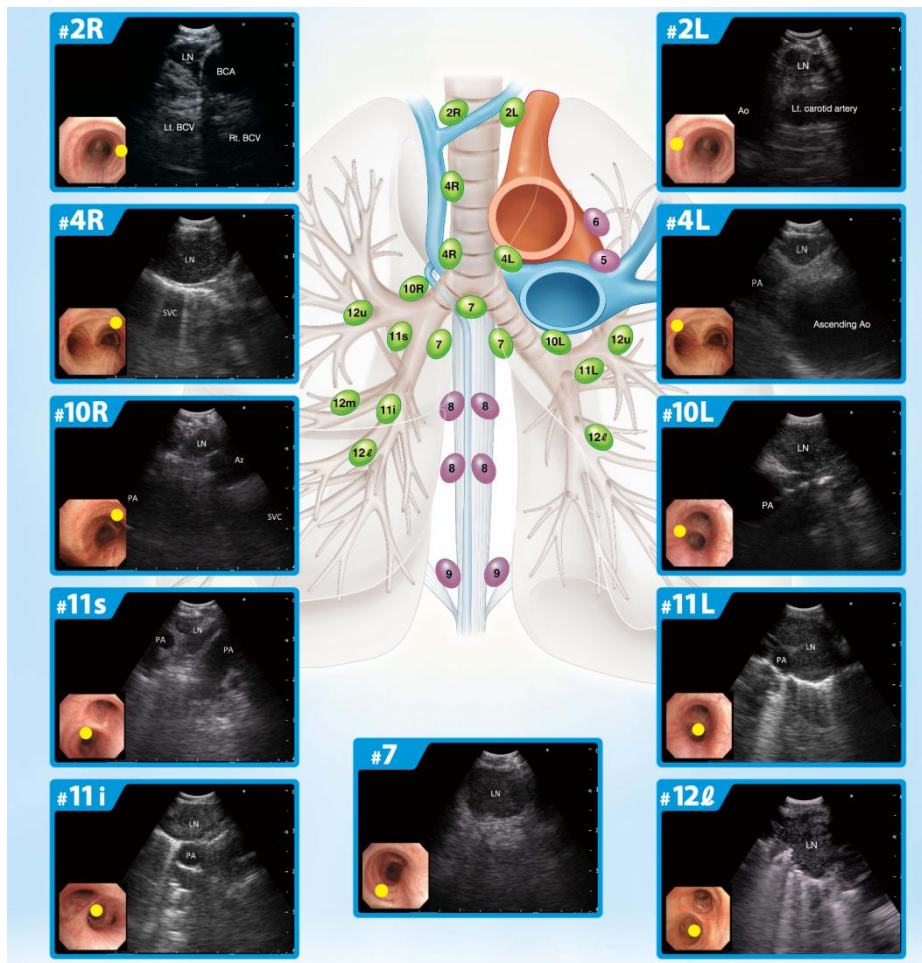
Immunotherapy, particularly immune checkpoint inhibitors (ICIs) targeting the PD-1/PD-L1 and CTLA-4 pathways, has transformed the management of non-small cell lung cancer (NSCLC). Agents such as pembrolizumab, nivolumab, atezolizumab, and durvalumab have demonstrated substantial improvements in overall survival, especially in tumors with high PD-L1 expression. In metastatic NSCLC without actionable mutations, PD-1/PD-L1 inhibitors are administered either as monotherapy or in combination with chemotherapy to enhance immune activation.

Molecularly targeted therapies have also advanced, driven by the identification of oncogenic driver mutations through next-generation sequencing (NGS). Tyrosine kinase inhibitors (TKIs) directed against mutations in EGFR, ALK, ROS1, BRAF, MET, RET, and KRAS have become standard treatments for genetically defined subsets of patients, often providing prolonged disease control with manageable toxicity profiles. For instance, osimertinib has become the first-line choice for EGFR-mutated NSCLC, while alectinib and lorlatinib are preferred for ALK-rearranged tumors.

A crucial step in guiding these personalized therapies is obtaining adequate tissue samples for histopathological and molecular characterization. Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) has become a cornerstone in the diagnostic work-up of lung cancer, particularly for mediastinal and hilar lymph node staging.

EBUS TBNA (Endobronchial Ultrasound Transbronchial Needle Aspiration)

EBUS-TBNA is a minimally invasive diagnostic procedure that allows for the sampling of mediastinal and hilar lymph nodes through the airways under real-time ultrasound guidance. The technique is performed using a bronchoscope equipped with a linear ultrasound probe, which provides high-resolution imaging of lymphatic structures and adjacent vascular anatomy. Once the target lymph node is identified, a fine needle is advanced through the tracheobronchial wall to obtain cellular material. The indications for the method include the diagnosis and staging of lung and



mediastinum lesions adjacent to the central airways by sampling suitable biological material. EBUS-guided biopsies can be performed on the

upper and inferior paratracheal stations (2 and 4), subcarinal (7), hilar (10) and interbronchial (11)(Figure1), with a median of sensitivity in hilar-mediastinal lymph node staging of 89%, and a negative predictive value equal to 91%.

Figure 1: regional lymph node mapping by EBUS

Lymph node station 5 sampling is a subject of debate. We believe that it can be considered in case of large lymphadenopathies that expose the station to biopsy without the obstruction of adjacent vascular structures.

TECHNICAL ASPECTS

Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is typically performed under general anesthesia due to the technical complexity of the procedure, which is more demanding than conventional bronchoscopy because of the larger scope diameter and prolonged procedure time. However, it can also be conducted under moderate sedation using narcotics, short-acting benzodiazepines, and local anesthesia.

The EBUS bronchoscope provides a white-light image with an approximately 30-degree forward oblique view, making its handling technically more challenging compared to a standard bronchoscope. The thickness and reduced flexibility of the EBUS bronchoscope limit its accessibility to the bronchial tree, particularly when a needle set is inserted into the working channel. Full flexion of the bronchoscope with the needle set in place may risk damaging the instrument. While EBUS procedures are relatively straightforward within the trachea, mainstem bronchi, and bronchus intermedius, maneuvering the bronchoscope into the right and left upper lobes and the narrow right middle lobe can be challenging. However, EBUS-TBNA can be performed even at the segmental bronchial level within the lower lobes.(Figure2)

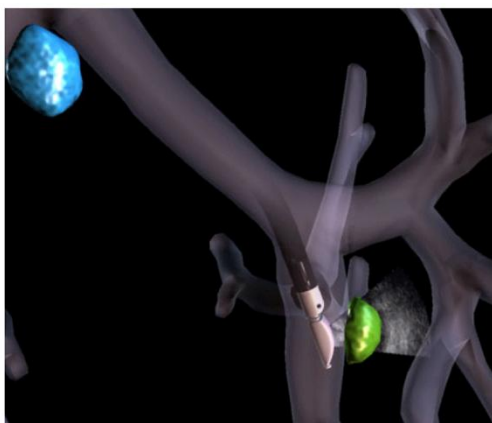


Figure 2: EBUS position for lymph node station 11L

A balloon can be placed over the ultrasonic probe and inflated with normal saline during EBUS-TBNA to enhance ultrasound image quality when direct probe contact with the bronchial wall is compromised by cartilage or bronchial angulation. However, balloon inflation is less beneficial in narrower bronchi where probe contact is already optimal.

Needle insertion into the intercartilaginous space can be difficult. Identifying tracheal cartilage on ultrasound images facilitates accurate needle placement. When targeting is challenging, an initial puncture into

the mediastinal fat space followed by redirection within the mediastinum may be employed. Adjusting needle depth before insertion is critical to prevent puncturing vascular structures or cystic lesions. Cases of pericarditis and post-procedural infections associated with EBUS-TBNA have been reported, potentially linked to full needle extension.

Needle movement within the target lesion has not been extensively studied. Generally, a back-and-forth motion is performed 10–20 times, with directional adjustments to sample a larger area. Even within the same lymph node, targeting regions with higher malignancy suspicion based on ultrasound imaging can improve yield.

Negative pressure may be applied, although its impact on diagnostic yield remains unclear. A recent randomized study found no significant difference in sample adequacy and diagnostic yield between aspirations with and without negative pressure. However, further studies are needed to evaluate its role in sample volume and diagnostic accuracy for small lymph nodes. Negative pressure should be released before withdrawing the needle from the lesion to minimize contamination with bronchial epithelial cells. Additionally, the needle must be fully retracted into the sheath before removal from the working channel to prevent bronchoscope damage.

A thorough understanding of mediastinal anatomy through ultrasound imaging is essential for EBUS-TBNA. However, identifying targets based solely on ultrasound may be difficult. Correlating target locations on computed tomography (CT) with bronchial anatomy enhances localization during ultrasound-guided aspiration. Initial identification of the bronchial segment adjacent to the target under white-light imaging can facilitate navigation. Recognizing major anatomical landmarks such as the superior

vena cava, aorta, azygos vein, heart, and thyroid is critical for procedural success.

Understanding the characteristic echogenic features of benign and malignant lymph nodes is essential for selecting target nodes. Typical benign lymph nodes exhibit an oval shape, a size of <1 cm, indistinct margins, a central hilar structure, relatively high and homogeneous echogenicity. In contrast, malignant nodes commonly appear round, >1 cm in size, with distinct margins, absence of a central hilum, eccentric cortical thickening, relatively low echogenicity, heterogeneous echotexture, and areas of necrosis. While ultrasound features alone cannot confirm malignancy, they aid in selecting target nodes and regions within a node for sampling.

Doppler mode can be employed to assess vascularity and avoid puncturing major vascular structures. During lung cancer staging, multiple lymph node stations are typically sampled. Selection criteria include nodal size, positron emission tomography (PET) findings, lymphatic metastatic pathways, ultrasound features, and the impact on treatment decisions. EBUS-TBNA provides an advantage over mediastinoscopy by allowing node selection based on real-time ultrasound assessment. The number of nodal stations sampled should be tailored to each patient based on these factors.

The number of aspirations per lesion varies based on procedural objectives. When EBUS-TBNA is performed for lung cancer diagnosis, obtaining an adequate tissue sample is crucial for molecular testing, including epidermal growth factor receptor (EGFR) mutation analysis and anaplastic lymphoma kinase (ALK) gene rearrangement detection. However, the optimal number of aspirations for molecular testing has not been established.

A 22-gauge (22G) needle is commonly used for EBUS-TBNA, although alternative sizes (19G, 21G, 25G) are available. However, their advantages remain unproven.

Sample processing depends on procedural intent. Smearing samples onto glass slides minimizes sample loss but is technically more challenging for molecular analyses than tissue core or cell-block specimens. Histological tissue cores obtained via EBUS-TBNA can be processed similarly to biopsy samples and are suitable for molecular studies such as EGFR and ALK mutation analysis. Cell-block specimens processed into paraffin blocks are also used for molecular testing.

Rapid on-site evaluation (ROSE) can be employed during EBUS-TBNA to assess sample adequacy.

EBUS-TBNA is a safe procedure with a low complication rate. A meta-analysis reported a complication rate of 0.15%. In a registry study of 1,317 cases, complications occurred in 1.44% of cases, including bleeding requiring intervention (n=3), pneumothorax (n=7), sustained hypoxia (n=4), respiratory failure within 24 hours (n=3), clinically significant airway injury (n=1), and hypotension (n=1). Transbronchial lung biopsy performed during the same session was identified as a risk factor for complications.

Infectious complications are uncommon. However, cases of mediastinitis, pericarditis, and primary tumor infections have been reported, particularly following EBUS-TBNA of cystic or necrotic lesions. There is no consensus on prophylactic antibiotic use for EBUS-TBNA. Careful procedural technique is necessary to avoid puncturing the pericardial space or cystic structures.

The risk of significant bleeding is low. However, medications that increase bleeding risk should be discontinued when possible.

The learning curve for EBUS-TBNA varies among operators. One study reported a sensitivity of 96% after 10 procedures, while another observed substantial improvements in diagnostic accuracy after 25–50 cases. A study by Kemp et al. comparing five bronchoscopists demonstrated a wide range in the time required to achieve procedural competency. Computer-based EBUS-TBNA simulators are available for training, and skills acquired through simulation are transferable to clinical practice.

EBUS-TBNA has become the standard technique for sampling mediastinal lesions. A thorough understanding of its technical aspects is crucial to optimizing diagnostic yield and ensuring procedural safety. EBUS-TBNA can be safely performed under general anesthesia, local anesthesia, or sedation. Proficiency in equipment handling and ultrasound interpretation is essential. While the risk of complications is low, awareness of potential adverse events and preventive measures is necessary to minimize risks.

MATERIAL AND METHODS

The study was conducted on patients who underwent EBUS-TBNA procedures as a part of the diagnostic process for suspected or confirmed lung cancer between SEPTEMBER 1, 2022 and January 31, 2025, at ClinicaPierangeli - Gruppo Synergo-Pescara. All patients had been

previously examined with computer tomography of the chest (chest CT) (figure 1), with additional positron emission tomography-computed tomography (PET-CT) when clinically indicated. Throughout the study period, close collaboration was maintained among the pathologist, anesthesiologist, and surgeon to optimize the collection and processing of tissue samples. This multidisciplinary approach aimed to ensure that the obtained specimens were adequate for the required immunohistochemical and molecular analyses. By refining sampling techniques and procedural parameters, efforts were made to maximize sample adequacy for comprehensive molecular profiling, ultimately improving the accuracy of diagnostic and therapeutic decision-making.

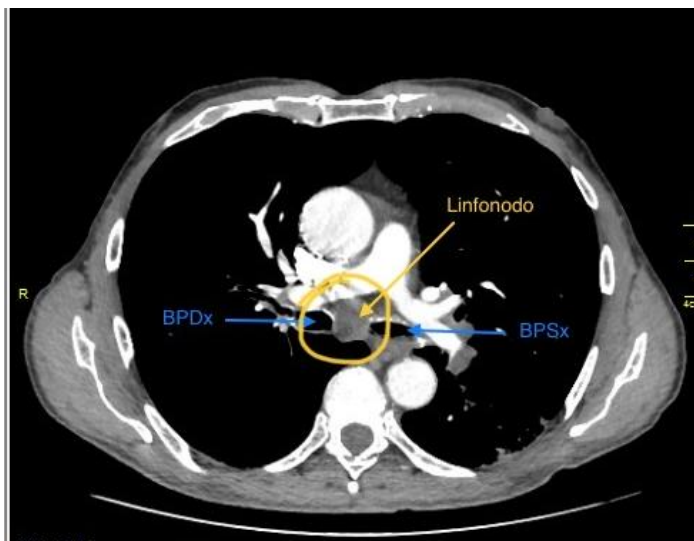


Figure 3: Chest CT scan showing an increase in the size of the subcarinal lymph node.

TECHNICAL DESCRIPTION OF EBUS-TBNA

EBUS-TBNA procedures were performed in our operating room under general anesthesia using a combination of propofol and remifentanyl administered intravenously. Airway management was achieved with a laryngeal mask to facilitate the procedure (Figure 4-5).

Figure 4-5: Anesthetic setting for EBUS-TBNA: laryngeal mask airway, syringe pumps for intravenous infusion.



All procedures were conducted with a linear ultrasound bronchoscope (Olympus BF-UC190F; Olympus Corp., Tokyo, Japan), ensuring high-resolution imaging of the mediastinal and hilar lymph nodes (Figure 6).



Figure 6: Olympus BF-UC190F echoendoscope

Fine-needle aspiration was primarily performed using a 19-gauge EBUS-TBNA needle (Figure 7), with occasional use of 22- or 25-gauge needles when indicated (EGNA-U403SX4019, N5782330, EGNA-U401SX4022).

The procedures were consistently performed by the same surgeon to maintain technique consistency and optimize sample quality.

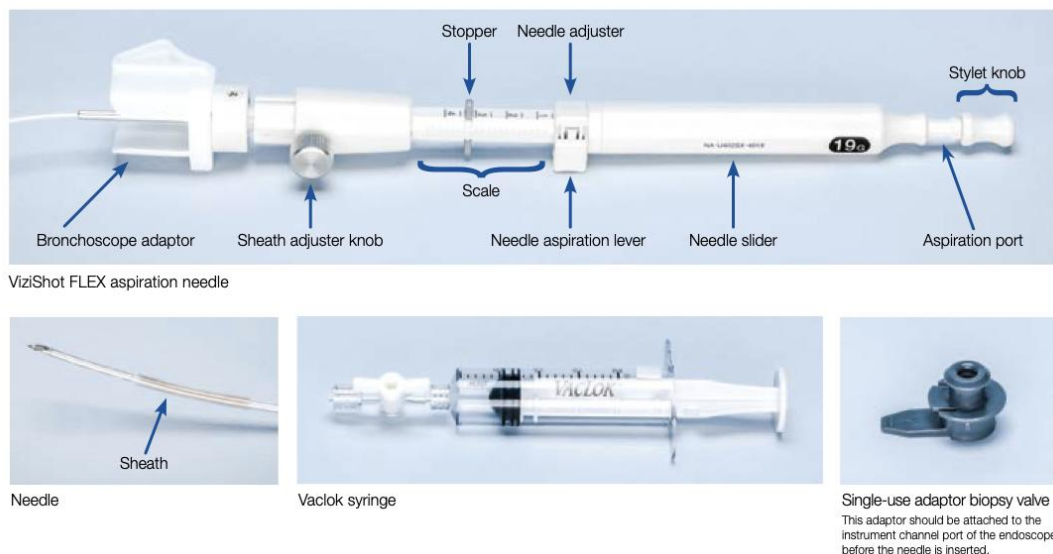


Figure 7: ViziShot FLEX 19G EBUS-TBNA Needle

Systematic ultrasound evaluation of the thoracic lymph nodes was conducted prior to biopsy, assessing lymph node morphology, size, echogenicity, and the presence of necrosis. Hilar and mediastinal lesions, along with pathologically enlarged lymph nodes displaying a short axis greater than 10 mm on CT scan or increased metabolic activity on PET-CT, were identified and targeted for sampling (Figure 8).



Figure 8: Ultrasonographic image of fine-needle aspiration of the 4R lymph node

Following established guidelines for EBUS-TBNA specimen acquisition and preparation for diagnostic and molecular testing, 3 to 5 passes were performed per lymph node station. Each pass involved 10 to 15 needle insertions within the lymph node to maximize sample adequacy (Figure 9).



Figure 9: Thoracic surgeon performing EBUS-TBNA.

cases, led to an increased quantity of collected material. However, with higher aspiration pressures, the risk of contamination and cellular alteration also increased (Fig. 11-12). Therefore, since 2024, we have routinely employed low-intensity aspiration (5–10 cc).

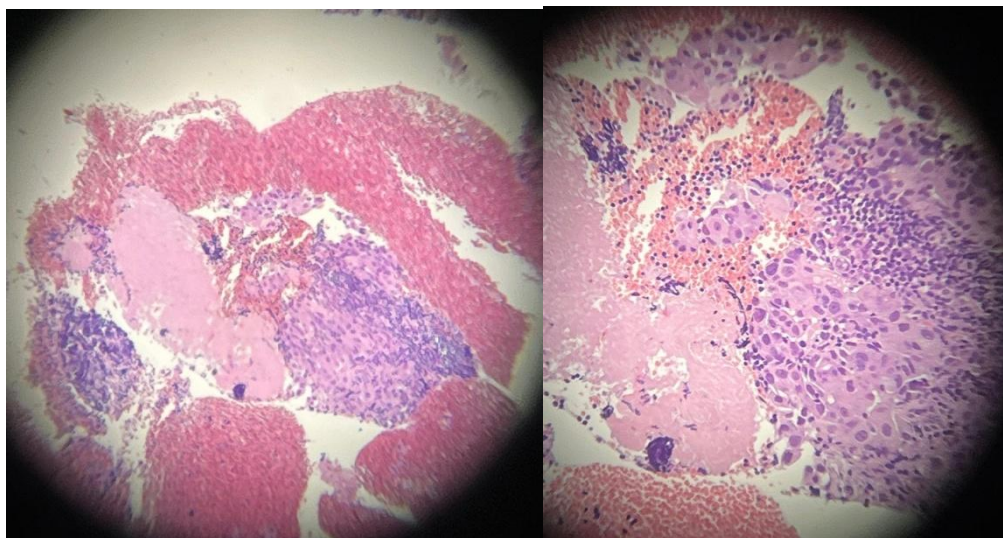


Fig 11-12: Microscopic image of adenocarcinoma obtained via EBUS-TBNA (magnification 4x and 10x, respectively), showing blood contamination

Rapid on-site evaluation (ROSE) was not available at our center.

Collected samples were fixed in formalin for 12 hours before being transported to the pathology laboratory. In cases with limited sample quantity or suboptimal quality, the slide smear technique was preferred for cytological examination.

Under a laminar flow hood, the samples were placed into cassettes and subjected to dehydration processes within a tissue processor. Subsequently, the material was embedded in paraffin, frozen, and sectioned using a microtome. Hematoxylin and eosin staining was applied for diagnostic evaluation (Figure 13-14).

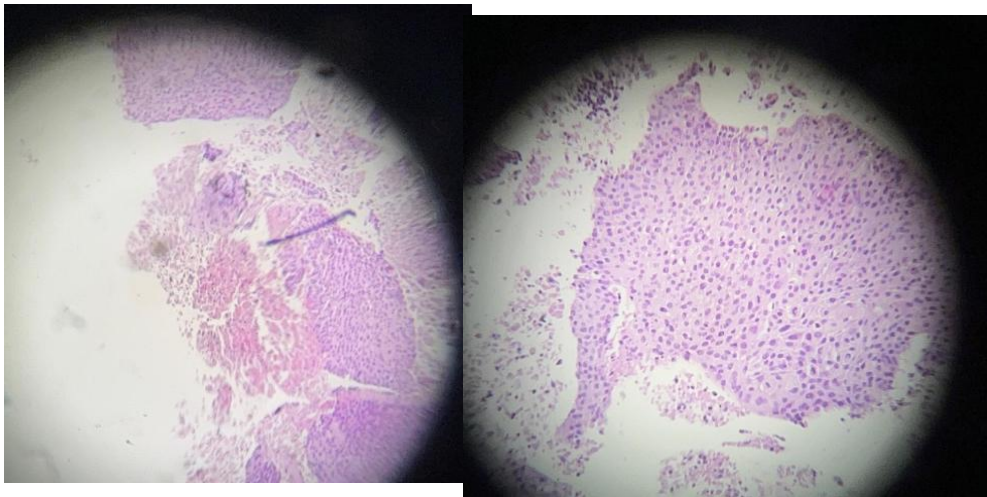


Figure 13-14: Microscopic image of adenocarcinoma obtained via EBUS-TBNA (magnification 4x and 10x, respectively)

In instances of limited sample availability, the pathology team prioritized preservation for immunological and molecular analysis. Immunohistochemical assessments were conducted on the remaining tissue prior to dispatching the samples to a specialized reference center for comprehensive immunomolecular analysis.

Following tissue processing, immunomolecular analyses were conducted to determine key oncogenic drivers and immune-related biomarkers essential

for targeted therapy selection. Extracted DNA and RNA underwent quality assessment using spectrophotometric and fluorometric quantification methods to ensure sample integrity. Next-generation sequencing (NGS) was performed to detect clinically relevant mutations in driver genes such as EGFR, ALK, ROS1, BRAF, MET, RET, and KRAS. In addition, fluorescence in situ hybridization (FISH) was utilized for chromosomal rearrangements, particularly in ALK and ROS1-positive cases.

Immunohistochemical (IHC) staining was applied to assess PD-L1 expression levels, which play a critical role in selecting patients for immune checkpoint inhibitor therapy.

Samples with inadequate tumor cellularity underwent additional enrichment steps, including cytological smear assessment and microdissection, to optimize molecular testing performance.

It is not possible to perform immunomolecular investigations at our center, which is why data collection presents gaps in our study.

RESULTS

During the study period, a total of 32 EBUS-TBNA procedures were performed by a single operator for diagnostic purposes in patients with known or suspected lung cancer. Two patients requested that their samples be analyzed at external centers and were therefore excluded from the study.

The final study cohort included 30 patients, with a mean age of 68.7 years (range: 52–86 years). The majority were male (23/30), while 7 were female. Smoking history was available for 10 patients, of whom 5 were former smokers, 3 were current smokers, and 2 had never smoked.

Biopsy samples were obtained from mediastinal lymph node stations 2R, 2L, 4R, 4L, 5, 7, and 11. In cases of hilar masses, the tumor itself was directly sampled. (Tab.1)

Patient	Age	Smoking Status	Sex	Sample Date	Biopsy Site	Histology	TTF1	P40
VVP	65	YES	M	2023	hilar neoplasm	Adenocarcinoma	+	-
DSG	66	FORMER	M	2024		7 Adenocarcinoma	+	-
LRR	57	NO	F	2023	11R	Adenocarcinoma	+	-
DAC	65	FORMER	M	2023	hilar neoplasm, 4R	Adenocarcinoma	+	-
CM	80	FORMER	M	2024	2-4R	Adenocarcinoma	+	-
PG	66	NO	M	2023		7 Adenocarcinoma	+	-
MP	63	YES	M	2023		7 Adenocarcinoma	+	-
CG	71	YES	M	2023		5 CA EPIDERMIDALE	-	+
PR	74	YES	F	2025	4R	Adenocarcinoma	+	-
DTR	68		F	2023	4R	Adenocarcinoma	+	-
DGG	79	FORMER	M	2025	4R	Large Cell Carcinoma		-
BAF	52		M	2024		7 Lymphoma	NV	NV
SE	68		M	2023		7 Carcinoma NOS	-	-
DAG	69		M	2022		7 False Negative	NV	NV
CM	73		M	2022	hilar neoplasm	CA EPIDERMIDALE	-	+
FAMF	86		M	2024	4R	Squamous Cell Carcinoma	-	+
MM	66		F	2023	4R	Adenocarcinoma	+	-
CM	70		M	2025		7 Adenocarcinoma	+	-
LN	70		M	2024	4L	Squamous Cell Carcinoma	-	+
VG	62		F	2024	hilar neoplasm	Squamous Cell Carcinoma	-	+
AA	77		M	2024		7 Small Cell Lung Carcinoma	NV	NV
PA	74	FORMER	M	2023	4R	Adenocarcinoma	+	-
DL	74		M	2024		7 Squamous Cell Carcinoma	-	+
RG	75	YES	F	2022	4R	Small Cell Lung Carcinoma	NV	NV
DDF	65		M	2022		7 False Negative	NV	NV
IM	75		M	2022		7 Non-Diagnostic	NV	NV
VD	54		M	2023		11 False Negative	NV	NV
FG	72		F	2023	4R	Lymphoma	NV	NV
DPP	73		M	2023	4R	Negative	NV	NV
PB	54		M	2023	4L	Negative	NV	NV

Tab. 1: Summary table of the characteristics of the study patients and the obtained results.

Lung cancer was diagnosed in 22 patients. Among them, 14 (73.33%) had non-squamous cell lung cancer, including 12 cases of adenocarcinoma (40%), 1 case of large cell carcinoma (3.33%), and 1 case of carcinoma not otherwise specified (NOS) (3.33%). Squamous cell carcinoma was

identified in 6 patients (18.18%), while small cell lung carcinoma (SCLC) was diagnosed in 2 patients (6.6%).

Mediastinal lymphoma was diagnosed in 2 patients (6.6%). Additionally, there were true negative cases (inflammatory lesions confirmed by alternative diagnostic methods) and 3 false-negative cases (10%), in which lung cancer was later diagnosed through mediastinoscopy.

One sample (3.33%) was deemed non-diagnostic. (Tab2)

Tab 2: EBUS TBNA diagnosis data

Diagnosis	Number of Cases	Percentage (%)
Non-squamous cell lung cancer	14	73.33%
Adenocarcinoma	12	40%
Large cell carcinoma	1	3.33%
Carcinoma NOS	1	3.33%
Squamous cell carcinoma	6	18.18%
Small cell carcinoma	2	6.6%
Mediastinal lymphoma	2	6.6%
True negatives	Confirmed with other analyses	-
False negatives (confirmed via mediastinoscopy)	3	10%
Non-diagnostic samples	1	3.33%

Due to the inability to perform immunomolecular analysis at our center, we experienced a significant data loss that impacted the study.

The data were collected through direct phone calls to the patients or their family members, anonymized, and subsequently analyzed. We decided to include only data from patients who were able to provide a report of the performed analyses or documentation of an oncological consultation. A

total of 10 patients were considered. In 9 cases, immunomolecular analyses were successfully performed on the samples we obtained, while in one case, the limited amount of material did not allow for further analysis.

Therefore, we will present the characteristics of the 9 patients for whom immunomolecular data are available.

These included 1 patient diagnosed with small cell lung cancer (SCLC) and 8 patients diagnosed with adenocarcinoma (ADK). The data, provided by either the patient or their family members, were not always complete.

In all 9 patients, the material obtained through EBUS-TBNA was sufficient for immunohistochemical analysis of PD-L1. Immunohistochemical analysis was performed using the PD-L1 antibody (SP263) on the Ventana Benchmark ULTRA platform, with an internal laboratory control included.

Among the 8 patients with non-small cell lung cancer (NSCLC), molecular testing for EGFR mutations was feasible in 7 cases, with 1 patient testing positive (non smoker, female patient).

EGFR mutation analysis was performed using real-time PCR (cobas® EGFR Mutation Test v2, Roche) on the Cobas Z480 platform.

Immunohistochemical analysis of the ALK gene was conducted in 4 patients using a specific anti-ALK antibody (Clone D5F3, Ventana) on the Ventana Benchmark ULTRA platform. No positive immunoreactivity was observed in any of the examined cases.

Mutation analysis of the BRAF gene (exon 15, codon 600) was performed using real-time PCR on the Cobas Z480 platform. The test was conducted in 3 patients, all of whom tested negative.

KRAS mutation analysis in codons 12, 13, and 61 was performed using real-time PCR (cobas® EGFR Mutation Test v2, Roche) on the Cobas Z480 platform in 1 patient, with no detectable mutations. (tab3)

Patient	Age	Smoking Status	Sex	Sample Date	Biopsy Site	Histology	TTF1	P40	Staging	PD-L1	EGFR	ALK	ROS1	BRAF	KRAS
VVP	65	YES	M	2023	hilar neoplasm	Adenocarcinoma	+	-	IIB	+	-	-	N/A	-	N/A
DSG	66	FORMER	M	2024		7 Adenocarcinoma	+	-	IIIA	-	-	N/A	N/A	N/A	N/A
LRR	57	NO	F	2023	11R	Adenocarcinoma	+	-	IIB	-	+	N/A	N/A	-	N/A
DAC	65	FORMER	M	2023	hilar neoplasm, 4R	Adenocarcinoma	+	-	IVA	+	-	-	N/A	N/A	N/A
CM	80	FORMER	M	2024	2-4R	Adenocarcinoma	+	-	IIIA	+	-	N/A	N/A	N/A	N/A
PG	66	NO	M	2023		7 Adenocarcinoma	+	-	IVB	-	-	-	N/A	-	-
MP	63	YES	M	2023		7 Adenocarcinoma	+	-	IVB	-	N/A	-	N/A	N/A	N/A
CG	71	YES	M	2023		5 CA EPIDERMIOIDALE	-	+	IIIC	+	-	N/A	N/A	N/A	N/A
RG	75	SI	F	2022	4R	SCLC	N/A	N/A	IIIA	+	N/A	N/A	N/A	N/A	N/A

Table 3: Results of Immunomolecular Analysis

DISCUSSION

The limited sample size in this study is attributable to the availability of multiple diagnostic sampling techniques at our Institution, including mediastinoscopy, CT-guided biopsy, EBUS-TBNA and uniportal thoracoscopy. Consequently, whenever feasible, we prioritized the use of well-established techniques capable of providing a larger amount of diagnostic material, such as mediastinoscopy over EBUS-TBNA.

Nevertheless, statistical data analysis shows results consistent with the literature, indicating EBUS-TBNA as a reliable procedure with good sensitivity and specificity, capable of providing adequate material for both diagnosis and the immunomolecular evaluation of pulmonary tumor pathologies.

Specifically, among the 30 cases analyzed, approximately 87% of the samples (26 patients) were diagnostic. Analysis of the period during which the biopsies were performed revealed that all three false-negative samples and the single non-diagnostic sample were obtained in 2022. This suggests that the learning curve of both the operating surgeon and, potentially, the pathologist handling microbiopsies had not yet been fully completed. Notably, no further non-diagnostic samples have been recorded since then.

The primary objective of our study was to demonstrate the effectiveness of the EBUS-TBNA procedure in providing diagnostic and sufficient material for both diagnosis and immunomolecular evaluation in patients with lung cancer. Unfortunately, the inability to perform certain analyses at our center prevented the complete collection of data. Nevertheless, among the 10 patients for whom information was available, the collected samples

were suitable for the detection of immunomolecular alterations in 90% of cases (9 patients).

Furthermore, the study results are likely influenced by the unavailability of immunomolecular analysis reports, as these tests are not performed at our institution. However, in the cases analyzed, the collected samples were deemed sufficient for the planned investigations.

It would be desirable to continue the study through active collaboration between our center and institutions performing immunomolecular testing, ideally with large-scale data sharing.

Particular attention should be given to the learning curve, which applies both to the thoracic surgeon performing the biopsy and to the pathologist handling microbiopsies.

Multidisciplinary collaboration between the surgeon and pathologist is crucial in the applied technique, especially in the absence of rapid on-site evaluation (ROSE) of the collected material.

Through discussions with the pathology colleague, the need to establish an optimized protocol for sample collection and preservation emerged.

In our experience, after an initial learning period, we opted to send all collected material fixed in formalin, as smear samples on slides often did not allow for additional immunohistochemical or molecular investigations beyond direct observation with hematoxylin-eosin staining.

Over the years, we have refined the technique by using, whenever possible, larger gauge needles (19G instead of 22-25G). The use of aspiration with a syringe during sampling is undoubtedly beneficial; however, it must be carefully weighed against the high risk of contamination or cellular alteration (Fig. 11-12). Therefore, since 2024, we have routinely employed low-intensity aspiration (5–10 cc).

CONCLUSIONS

Endobronchial ultrasound Transbronchial Needle Aspiration (EBUS TBNA) is a reliable, safe, rapid, and cost-effective technique with good specificity and sensitivity following an appropriate learning curve. It allows for the collection of sufficient material for both diagnostic purposes and the immunomolecular study of pulmonary neoplastic diseases. Although this study has evident limitations, it can be considered a valuable starting point for further investigations. Moreover, it highlights the need for a structured patient management pathway, emphasizing the importance of collaboration among different centers to ensure the most efficient and comprehensive care through a multidisciplinary approach.

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