



Prospective evaluation of EBUS-TBNA specimens for programmed death-ligand 1 expression in non-small cell lung cancer patients: a pilot study

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ABSTRACT

Objective: EBUS-TBNA cytological sampling is routinely performed for pathological diagnosis, mediastinal staging, and molecular testing in lung cancer patients. EBUS-TBNA samples are not formally accepted for testing programmed death-ligand 1 (PD-L1) expression. The objective of the study was to compare the feasibility, reproducibility, and accuracy of PD-L1 expression assessment in cytological specimens and histological samples. **Methods:** We prospectively collected histological (transbronchial forceps biopsy) and cytological (EBUS-TBNA) samples from peribronchial neoplastic lesions during an endoscopic procedure at the same target lesion for the pathological diagnosis and molecular assessment of stage IV non-small cell lung cancer (NSCLC). **Results:** Fifteen patients underwent the procedure. Adequate cytological samples (at least 100 neoplastic cells) were obtained in 12 cases (92.3%). Assessment of PD-L1 expression was similar between histological and cytological samples (agreement rate = 92%). Sensitivity and diagnostic accuracy of EBUS-TBNA cytological specimens were 88.9% and 100%, respectively. **Conclusions:** The evaluation of PD-L1 expression in EBUS-TBNA cytological specimens is feasible and presents good reproducibility when compared with routine histological samples. EBUS-TBNA cytological samples could be used for the assessment of PD-L1 expression in patients with NSCLC as a minimally invasive approach in stage IV NSCLC cancer patients.

Keywords: Ultrasonography; Biopsy, needle; Lung neoplasms; Molecular targeted therapy.

INTRODUCTION

Despite the advances in diagnostic modalities and imaging methods, lung cancer remains a leading cause of death worldwide.⁽¹⁾ Up to 80% of patients present with advanced disease at the time of diagnosis, and systemic therapy may represent the only treatment option.^(2,3)

Novel therapeutic strategies using molecular targeted drugs focused on genetic alterations have demonstrated to be the best treatment option in various clinical scenarios.⁽⁴⁾ Molecular targeted therapies improve survival in metastatic adenocarcinoma with genetic mutations such as epidermal growth factor receptor (EGFR), ALK, ROS proto-oncogene 1 tyrosine kinase (ROS1), and v-Raf murine sarcoma viral oncogene homolog B (BRAF) rearrangement.⁽⁵⁾

More recently, immunotherapy with monoclonal antibodies blocking the programmed death-ligand 1 (PD-L1) has shown to be a promising treatment option in patients with advanced non-small cell lung cancer (NSCLC) in terms of overall survival when compared with standard

chemotherapy regimens.⁽⁶⁾ Thus, the evaluation of PD-L1 protein expression is essential in identifying patients that may benefit from immunotherapy the most.^(7,8)

In the last several years, minimally invasive procedures have become the standard of care for the diagnosis and staging of NSCLC patients. Procedures such as EBUS-TBNA often provide all the necessary information, from tissue sampling to molecular evaluation, and cause few complications.⁽⁹⁻¹²⁾ Cytological specimens by EBUS-TBNA have successfully been used for the assessment of various molecular targets, such as EGFR, ALK, and ROS-1,⁽⁸⁾ and have proven to be adequate and comparable to histological samples for the evaluation of target markers.⁽¹³⁾

Diagnostic immunohistochemical assays to evaluate PD-L1 expression in tumor cells were officially developed to test tumor tissue samples.⁽¹⁴⁾ However, their use for cytological evaluation of cell-block specimens is not accepted "worldwide," especially for patients included in clinical studies. Therefore, the objective of this pilot study was to evaluate and compare the feasibility and

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reproducibility of PD-L1 expression assessment in EBUS-TBNA specimens and in histological specimens.

METHODS

The study was approved by the research ethics committee of the institution, and all participants gave written informed consent.

Patients with suspected advanced (stage IV) NSCLC underwent bronchoscopy for pathological definition, and molecular assessment of pulmonary lesions was carried out. Patients were selected on the basis of the identification of lesions on CT that showed a high probability to be sampled by both EBUS-TBNA and transbronchial biopsy. During the procedure, both histological (transbronchial biopsy) and cytological (EBUS-TBNA) samples were collected from the same peribronchial neoplastic lesion. A flow chart of the procedure is shown in Figure 1.

Fifteen consecutive patients who underwent bronchoscopy for the pathological diagnosis and evaluation of molecular PD-L1 expression were included in the analysis. Specimens were considered adequate if a minimum of 100 viable tumor cells were present. Samples with fewer than 100 viable tumor cells were considered inadequate and were excluded from the analysis.⁽¹⁵⁾

EBUS-TBNA samples

EBUS-TBNA samples were collected from peribronchial lesions adjacent to the airways. The procedure was performed under local anesthesia (1% lidocaine) and moderate sedation provided by an anesthesiologist. Ventilation was spontaneous. All procedures were performed by the same team of interventional pulmonologists using a convex probe (EBUS Convex Probe BF-UC180F; Olympus Europa SE & Co. KG, Hamburg, Germany) and a dedicated ultrasound processor (EU-ME2; Olympus). EBUS-TBNA specimens were collected with a 22G dedicated needle (Vizishot NA-201SX-4022; Olympus).

A very small amount of the aspirated material was pushed out by the internal stylet and smeared onto glass slides, air dried, and stained with modified May-Grünwald-Giemsa (Diff-Quik) stain for rapid on-site evaluation (ROSE). The remaining aspirate and other needle passes—minimum of 3 needle passes, ranging from 3 to 5 according to the percentage of tumor cells present on the smear (ROSE)—were placed in saline solution for cell-block processing and further cytological evaluation.⁽¹⁰⁾

Histological samples

A transbronchial biopsy was performed inserting a large (2.8 mm) endoscopic forceps into the pulmonary lesion. Neoplastic pulmonary lesions were previously confirmed with radial EBUS probe and fluoroscopy, and a guide sheath kit (SG-201-C; Olympus) was used in order to maintain the correct position of the forceps. No endobronchial visible lesions were biopsied.

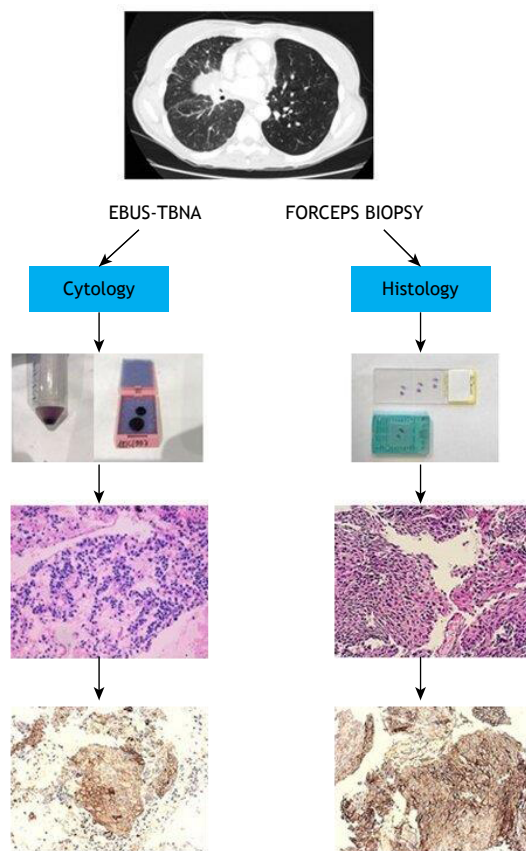


Figure 1. Flow chart of collection and analysis of cytological and histological specimens. The same peribronchial lesion was biopsied by forceps biopsy (right side) and EBUS-TBNA (left side).

The first biopsy sample obtained was rolled onto a glass slide for “biopsy imprinting” and immediate cytological evaluation (ROSE) for the adequacy of the specimen. Once adequacy was confirmed, further biopsies were performed and immediately fixed in formalin for histological evaluation, as previously described.⁽¹⁶⁾

PD-L1 and immunohistochemistry technical aspects

Cell blocks from EBUS-TBNA specimens were prepared with no methanol-based fixative. The cytological material was centrifuged, stained with H&E, coated with fluid agarose to form a firm cell block, and finally processed in accordance with standard histopathological methods used for formalin-fixed paraffin-embedded samples.⁽¹⁷⁾

Ten consecutive 2-to-3-mm thick sections were obtained from each cell block; the first and the last sections were stained with H&E to make sure that diagnostic tumor cells were present in all of the slides. In selected cases, in order to differentiate between adenocarcinoma and squamous cell carcinoma, we performed immunocytochemical stains for thyroid transcription factor-1 and p40 (an antibody that recognizes Δ Np63, a p63 isoform suggested to be highly specific for squamous/basal cells).⁽¹⁸⁾

PD-L1 expression was evaluated with the PD-L1 IHC 22C3 pharmDx kit (Agilent Technologies, Santa Clara, CA, USA), a qualitative immunohistochemical assay that uses monoclonal mouse anti-PD-L1 antibody, clone 22C3, using the EnVision FLEX visualization system on Autostainer Link 48 (Agilent).⁽¹⁵⁾

Specimens were considered adequate if a minimum of 100 viable tumor cells were present. In each case, a tumor proportion score (TPS) was calculated. TPS is the proportion of viable tumor cells showing partial or complete membrane staining. TPS was considered negative if the proportion of stained cells was < 1%; weakly positive, if it ranged from 1% to 49%; and strongly positive, if it was ≥ 50%.

Two experienced pathologists independently examined all samples. Disagreements were discussed and resolved by consensus. Forceps biopsy samples were processed in accordance with standard histopathological methods.

Statistical analysis

Continuous data were reported as means and standard deviations. Categorical and numerical data were presented as absolute and relative frequencies. Inadequate samples (adequate biopsy samples presenting > 100 viable cells but inadequate EBUS-TBNA samples) were excluded from the accuracy analysis because the objective of the study was to show concordance between samples. To test the correlation between risk classes, Spearman’s rank correlation test was used. A ROC curve was generated to determine the best threshold. Significance was set at $p < 0.05$. Statistical analysis was performed using RStudio, version 3.6.1 “Action of the Toes” (RStudio Inc., Boston, MA, USA), with the packages *standard*, *rcmdr*, and *irr*.^(19,20)

RESULTS

Fifteen patients were included in the study. Adequate samples (at least 100 viable neoplastic cells) were

obtained from both cytological and histological specimens in 12 patients (80%). Demographic characteristics of patients were the following: 13 male patients (83.3%); and median age = 66 years (range: 54-78 years). Regarding tumor cell types, adenocarcinoma and squamous cell carcinoma were identified in 11 and 4 patients, respectively. Histological and cytological PD-L1 expression results are shown in Table 1.

Three patients with inadequate samples were included in the aggregate analysis but excluded from the accuracy analysis. In 11 patients, there was complete agreement between cytological and histological PD-L1 expression results regardless of the subtypes: adenocarcinoma, in 9 patients (Figure 2); and squamous cell carcinoma, in 2 (Figure 3). In one case (adenocarcinoma), there were discordant results (negative cytology and weakly positive histology). The results of PD-L1 expression in EBUS-TBNA cytological samples showed an area under the ROC curve of 0.79 (Figure 4A). Sensitivity, diagnostic accuracy, and negative predictive value were 88.9%, 91.7%, and 75.0%, respectively. PD-L1 staining showed a negative reaction, a weakly positive reaction, and a strongly positive reaction in 16.7%, 16.7%, and 66.7% of the histological samples, respectively, and in 25.0%, 8.3%, and 66.7% of the cytological samples.

Considering the different cutoffs for PD-L1 expression, the agreements between histological and cytological specimens considered negative, weakly positive, and strongly positive were 80%, 67%, and 100%, respectively. The Spearman’s rank correlation test showed a highly significant correlation between the TPS of histological and cytological samples ($\rho = 0.836$; $p = 0.0060$; Figure 4B).

DISCUSSION

Nowadays, EBUS-TBNA is part of the daily routine clinical practice in various thoracic diseases.⁽¹⁰⁾ Due to its low invasiveness and the possibility of

Table 1. Histological subtypes and programmed death-ligand 1 (PD-L1) expression results.

Patient	Histological subtype	PD-L1 histology (TPS%)	PD-L1 cytology (TPS%)
1	Adenocarcinoma	5	2
2	Adenocarcinoma	2	< 1
3	Adenocarcinoma	90	90
4	Squamous cell carcinoma	60	60
5	Adenocarcinoma	< 1	< 1
6	Adenocarcinoma	60	55
7	Adenocarcinoma	80	75
8	Adenocarcinoma	80	80
9	Adenocarcinoma	80	70
10	Adenocarcinoma	70	70
11	Squamous cell carcinoma	< 1	< 1
12	Adenocarcinoma	70	80
13	Squamous cell carcinoma	80	-
14	Adenocarcinoma	2	-
15	Squamous cell carcinoma	2	-

TPS%: tumor proportion score in %.

obtaining repeated samples, EBUS-TBNA is often the procedure of choice for the pathological diagnosis and molecular assessment in patients with advanced stage NSCLC.⁽²¹⁾ Up to 80% of NSCLC patients present with advanced disease at the time of diagnosis and could be potential candidates for targeted drug therapy.⁽²⁾ In our experience, up to 98.5% of the patients are diagnosed with a minimally invasive procedure that provides cytological and cell-block specimens.⁽¹⁰⁾

The modern oncological approach associates minimally invasive procedures with less invasive oncological treatments for better survival and lower complication rates. The development of an optimal modality that enables the acquisition of sufficient amounts of high-quality tissue without surgery is essential in the molecular targeted therapy era. Molecular testing is crucial in the management of patients with NSCLC lung cancer, especially in directing targeted therapy with EGFR tyrosine kinase inhibitors and other molecular markers.⁽⁸⁾ Molecular targetable mutations such as *EGFR* were initially evaluated in histological specimens until it was demonstrated that they could also be assessed in EBUS-TBNA specimens with equivalent sensitivity.⁽¹³⁾

In the last years, immunotherapy for the treatment of lung cancer with immunotherapeutic agents targeting the immune checkpoint pathways, such as PD-L1, has shown promising results, with prolonged clinical responses and tolerable toxicity.⁽⁶⁾

Selection of patients that could benefit from immunotherapy is mandatory in advanced NSCLC,⁽²²⁾

and PD-L1 is the only biomarker validated and approved as a companion diagnostic tool prior to immunotherapy in clinical practice. PD-L1, together with EGFR, BRAF, ALK, and ROS-1, represents a mandatory biomarker to be evaluated in samples used for the pathological diagnosis of NSCLC so that the best treatment strategy can be offered. Other markers, such as HER2, KRAS, RET, and *MET* 14 exon skipping mutation, are also recommended.⁽²³⁾

To date, the gold standard for the assessment of PD-L1 expression is immunohistochemistry performed in formalin-fixed paraffin-embedded histological specimens,⁽²⁴⁾ and there is limited evidence that PD-L1 expression could be reliably assessed in EBUS-TBNA cytological specimens in daily clinical practice. A previous study⁽²⁵⁾ reported the feasibility of cytological evaluation of PD-L1 in a variety of 30 cytological preparations from samples of patients with NSCLC. The authors concluded that cell-block preparations could replace histological tissue for determining PD-L1 status in NSCLC patients. However, that study evaluated different types of cytological specimens involving different types of tumors, different collection sites, and different analytical laboratory processes, causing several biases.⁽²⁵⁾

Another study⁽²⁶⁾ reported a comparison between cytological and histological samples. That study presented with a considerable bias related to the lack of standardization of cytological and histological samples. Cytological samples were obtained from different sites

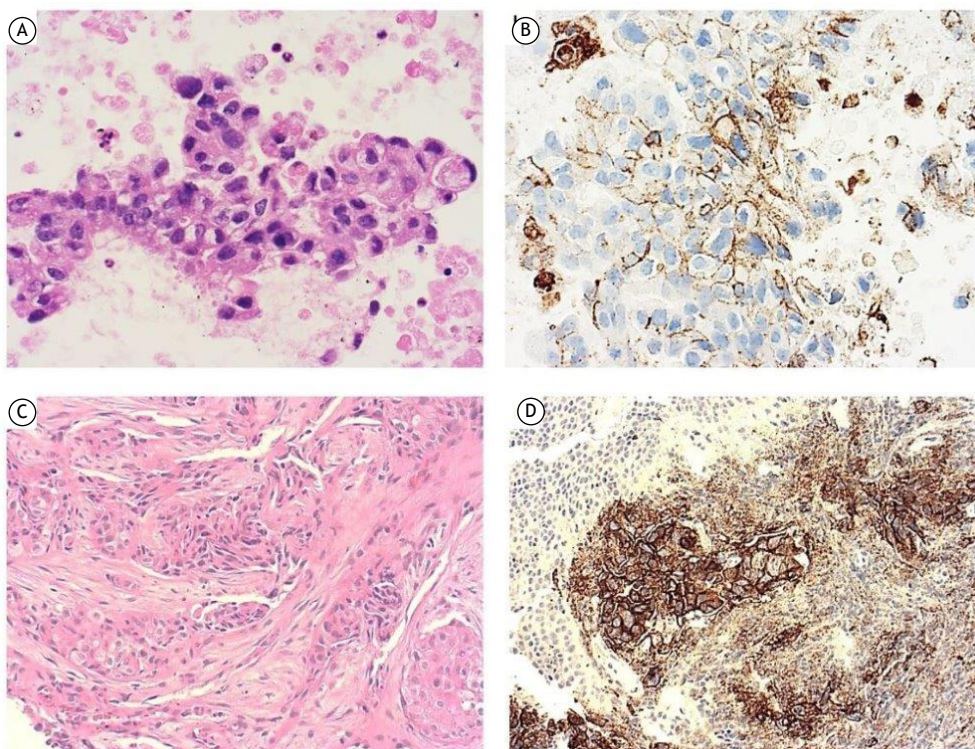


Figure 2. Photomicrographs of EBUS-TBNA (A and B; formalin-fixed paraffin-embedded cell-block sections) and forceps biopsy samples (C and D) of a patient diagnosed with adenocarcinoma (solid type), using H&E (A and C) and PD-L1 staining (B and D). The percentage of PD-L1 positive cells is higher than 50% in both samples.

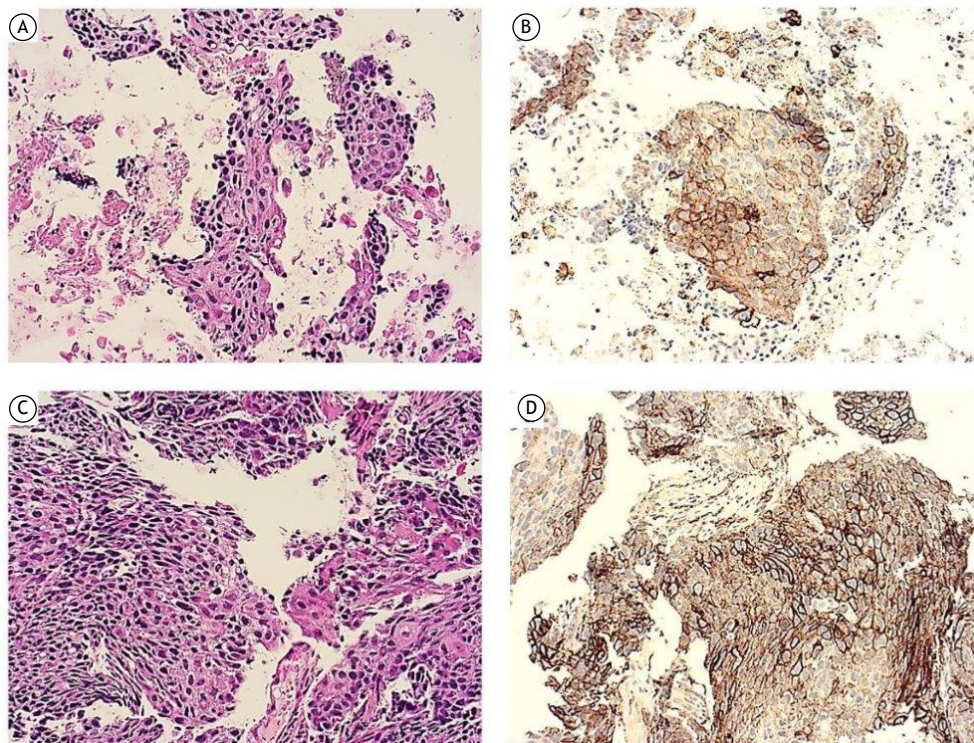


Figure 3. Photomicrographs of EBUS-TBNA (A and B; formalin-fixed paraffin-embedded cell-block sections) and forceps biopsy samples (C and D) of a patient diagnosed with nonkeratinizing squamous cell carcinoma, using H&E (A and C) and PD-L1 staining (B and D). The percentage of PD-L1 positive cells is higher than 50% in both samples.

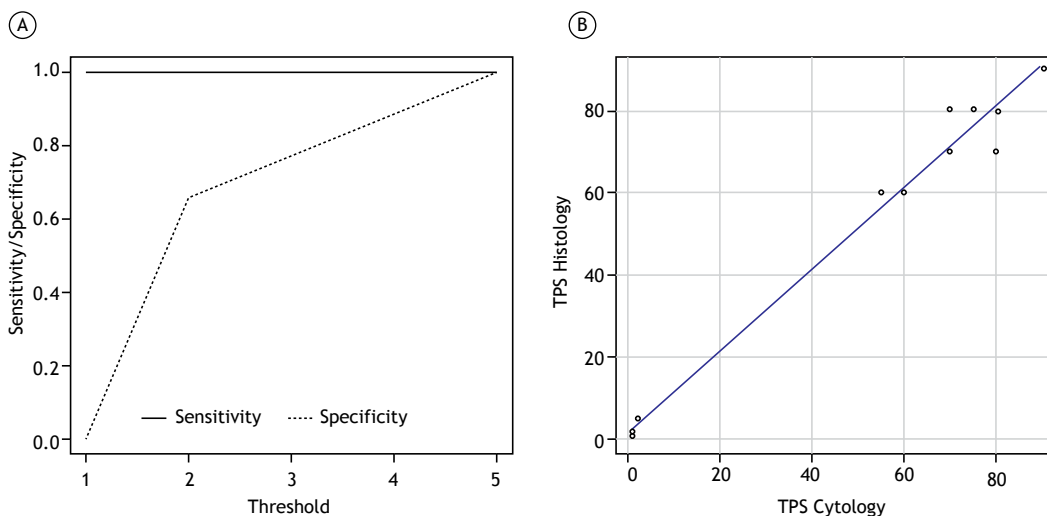


Figure 4. In A, ROC curve of programmed death-ligand 1 expression results (AUC = 0.79; sensitivity = 88.9%; and specificity = 91.7%). In B, scatter plot of tumor proportion score (TPS) for cytology and histology ($\rho = 0.836$; $p = 0.006$).

with different needle types, and histological samples were obtained from many different sites, including needle biopsies. In addition, cytological and histological samples were not collected at the same time. Different pathological subtypes, including malignant mesothelioma and metastasis other than lung cancer, were included in the analysis. As it is known, PD-L1 expression in

tumors is dynamic and can change over time and according to different tumor sites; therefore, collecting samples at different times can generate a bias in the analysis of PD-L1 expression itself.⁽⁷⁾ In the present study, we prospectively evaluated the feasibility of PD-L1 expression in EBUS-TBNA samples comparing them with histological specimens of the same lesion

that were collected at the same time, thereby avoiding any collection or selection bias that could change the PD-L1 expression profile.

Our results showed an excellent agreement between cytological and histological specimens in the evaluation of PD-L1 expression in NSCLC specimens. The agreement between histological and cytological specimens regarding PD-L1 expression was 80%, 67%, and 100%, respectively, for negative, weakly positive, and strongly positive results. In one case, there were discordant results: negative cytology (< 1%) and focal, weakly positive histology (2%). We excluded inadequate samples (adequate biopsy presenting > 100 viable tumor cells but EBUS-TBNA samples < 100 viable cells). Inadequate samples were related to the presence of blood in excess or necrosis in the cell block.

This pilot study has limitations. The major limitation was the small number of patients included in the analysis. Selecting patients with peribronchial lesions that are able to be biopsied with a forceps and EBUS-TBNA is quite infrequent, but it was mandatory to exclude any possible sample bias. Another limitation of the study is related to the reproducibility of PD-L1 expression results in EBUS-TBNA lymph node specimens. Although PD-L1 expression may be different between the primary tumor and lymph node metastasis, a good concordance (70–90%) has been reported at clinically relevant cutoffs.⁽²⁴⁾

In the present study, despite the limited sample size, the feasibility and reproducibility of PD-L1 expression results in EBUS-TBNA specimens have demonstrated that it is possible to obtain sufficient tissue sample from one single procedure for pathological diagnosis, staging, and complete molecular assessment, underpinning the personalized therapy era that combines minimally invasive procedures with biological agents for the best oncological results. The good concordance between histological and cytological samples shows promising results for the evaluation of PD-L1 expression in EBUS-TBNA specimens. Further studies are needed to confirm this evidence.

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AUTHOR CONTRIBUTIONS

JG and LS: study conception and design; and materials or referral of patients. LS: study conception and design. FDM and SMD: materials or referral of patients. EGR, CDT, and MB: data collection/assembly. LB and MC: data analysis and interpretation. GS: administrative support. All authors: drafting and revision of the manuscript; and approval of the final version.

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