Endobronchial ultrasound guided transbronchial needle aspiration combining with immunohistochemistry and genotype in lung cancer: A single-center, 55 cases retrospective study

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HIGHLIGHTS

- EBUS-TBNA enables minimally invasive mediastinal and hilar lesions sampled successfully.
- EBUS-TBNA is a safe and efficient method with high sensitivity and specificity in the diagnosis of lung cancer.
- Uniquely combining with Immunohistochemistry and molecular testing has significant clinical value in lung cancer.

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ABSTRACT

Objective: The purpose of this study was to evaluate the utility of EBUS-TNA for mediastinal mass or suspected lung cancer patients with mediastinal or hilar lymph node enlarged. Further to investigate the clinical value of EBUS-TBNA combining with immunohistochemistry and genotype in lung cancer.
Methods: A total of 55 patients with mediastinal, and/or hilar lymphadenopathy, and/or mediastinal mass previously detected by CT or PET/CT scan and who underwent EBUS-TBNA. An additional immunohistological analysis was performed for establishing a reliable diagnosis and sub classification when necessary. Some samples were tested for the EGFR and/or ALK mutations to provide suitable mutational genotyping for adenocarcinoma by using the PCR assays.
Results: Of the 55 patients, the sensitivity and diagnostic accuracy of EBUS-TBNA in the diagnosis of lung cancer were 92.5% (37/40) and 94.5% (52/55), respectively. 37 samples were further confirmed and obtained particular type by Immunohistochemistry. 6 cases of EBUS-TBNA samples from patients with lung adenocarcinoma referred for EGFR testing were analyzed, 4 patients were found to have EGFR gene mutations. The procedure was uneventful without any complications.
Conclusion: EBUS-TBNA is a safe and efficient method with high sensitivity and specificity in the diagnosis of lung cancer. Uniquely combining with Immunohistochemistry and molecular testing has significant clinical value in subtype diagnosis and guiding the treatment strategy in lung cancer.

1. Introduction

Lung cancer is one of the most common malignancies, and the early clinical symptoms almost are not typical. The majority of

patients with treatment had occurred mediastinal or hilar lymph node metastasis when seeing the doctor. Accurate and early diagnosis of such diseases is crucial for the appropriate treatment and prognosis [1,2]. To identify the lesions, we routinely use the bronchoscopic biopsy and CT-guided lung biopsy, which are suitable for the performance of central lung lesions and some peripheral lung cancer, but no intraluminal mucosal clear violations of the lung tumor lesions or only with simple mediastinal or hilar lymph nodes suspected lung cancer, more than two means are very limited at this time need to mediastinal or hilar lymph node biopsy.

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Endobronchial ultrasound guided transbronchial needle aspiration (EBUS-TBNA) is widely used minimally invasive sampling technique that has been shown to have a high sensitivity and diagnostic and safe for diagnosis and staging of lung cancer [3,4].Multiple studies have shown that EBUS-TBNA can improve the diagnostic yield for sampling mediastinal and hilar lymph nodes when compared with other available alternatives, such as mediastinoscopy, video-assisted thoracoscopy and conventional TBNA [5,6]. However, the specimens obtained by EBUS-TBNA are less than bronchoscopic, or mediastinoscopy biopsy is owing to negative pressure with fine-needle aspiration [7]. Sometimes, it is always mixed with more blood so that we can get the tissue information less than conventional pathological examination. To a certain extent, it is more difficult to diagnosis and histological type with only morphology. In recent years, the popularity of IHC and genetic testing provide some practical methods for lung cancer diagnosis [8–10].

Fortunately, EBUS-TBNA sampling can be a reliable tool to obtain a sufficient amount of tumor cells for IHC for cancer typing and molecular analysis. When used appropriately, IHC staining can allow tumor subtype to be inferred when histologic morphology is nonspecific, to further distinguish between adenocarcinoma and squamous cell carcinoma when some small biopsy specimens for morphological typing difficult [11–13]. Meanwhile, molecular analysis can be routinely performed on the majority of cytological samples obtained by EBUS-TBNA [10,14]. The presence of somatic mutations in epidermal growth factor receptor (EGFR) predicts the effectiveness of EGFR tyrosine kinase inhibitors (TKIs). It would be ideal if an EGFR mutation could be detected in biopsy samples since the majority of non-small cell lung cancer patients are inoperable at the time of presentation. EBUS-TBNA enables the sampling of histologic cores, which can be used for genetic analysis (i.e. EGFR, KRAS, and ALK).

Increasing evidence indicates that IHC and gene typing are the relevant references in some small biopsy specimens. However, we are unaware of studies the clinical value of EBUS-TBNA combining immunohistochemistry and molecular testing in lung cancer. In the present study, we investigated tissue samples obtained by EBUS-TBNA with immunohistochemistry and gene detection technology to provide a more precise tissue typing or genotyping to guide the protocols and assess of prognosis for lung cancer to provide an essential reference in the clinical.

2. Methods

2.1. Study design and patients

This was a retrospective review of consecutive patients who underwent EBUS-TBNA in Affiliated tumor Hospital of Guangxi Medical University between May 2013 and October 2015. Before each procedure, 5 mm slice chest CT scan or PET/CT was done (Fig. 1-A) on all patients for central parenchymal, mediastinal or hilar metastasis of a previously known and treated or concurrent extrathoracic malignancy, then that were recommended for EBUS-TBNA because of the clinical suspicion and could not be confirmed the diagnosis with the bronchoscopic biopsy. The baseline characteristics of those 55 patients in this study were summarized in Table 1. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee [15] and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards(researchregistry2747). Eventually, the diagnosis of all patients was confirmed with the biopsy of punctures such as EBUS-TBNA, supraclavicular lymph node biopsy, CT-guided tumor puncture and surgical pathology or cytology, and who failed to

obtain pathology and cytology verified by more than six months following-up with imaging.

2.2. Procedures

All examinations were performed by the same bronchoscopist (Aiqun Liu). After local anesthesia to the pharynx with 4% lidocaine spray (10 mL), each patient was placed under moderate sedation with intravenous midazolam or Propofol with monitoring of heart rate and oxygen saturation. The convex probe EBUS (CP-EBUS; BF-UC260FW, Olympus, Tokyo, Japan) was inserted through the oral route, with intermittent instillation of 2 mL aliquot doses of 2% lidocaine according to the response of patients. Scanning was done on a 10 MHz frequency ultrasound bronchoscope, and images were generated using the ultrasound unit (EU-ME1 processor, Olympus). The location, number, and size of the lymph nodes or mass were recorded. Vascular structures were confirmed using the color Doppler function. A dedicated aspiration needle (Olympus NA-201XS-4022, 22-gauge) was then placed in the working channel and advanced into the lymph node or mass, the stylet was withdrawn, suction was applied to the needle and the needle was then moved to and fro within the lymph node or mass. From two to five passes per lymph node or mass were obtained (Fig. 1-B, Fig. 1-C).

The aspirates obtained by EBUS-TBNA were placed into a bottle with liquid-based preparation, and an internal stylet was advanced to facilitate the removal of tissue from within. The tissues obtained by EBUS-TBNA were fixed in formalin and stained with hematoxylin and eosin for further histological examination (Fig. 1-D). The remainder of the aspirates was smeared onto glass slides, immediately fixed in 95% ethanol, and stained with Papanicolaou stain (Fig. 1-E). If the pathology of the EBUS-TBNA samples resulted in a definite diagnosis, this was judged as a true-positive finding. No further tissue confirmation was requested in these cases. In some cases, immunohistochemistry was performed for additional information. Immunohistochemical staining with an antibody to the cytokeratins (e.g. CK7 and CK20), thyroid transcription factor-1(TTF-1), p63, Synaptophysin(Syn), CD56 (also known as neural cell adhesion molecule or N-CAM), Ki-67, Chromogranin A(CgA), or Epidermal Growth Factor Receptor(EGFR) and so on were used whenever these were needed for tumor origin identification. A part of lung adenocarcinoma cases have detected the presence of mutations in the Epidermal Growth Factor Receptor (EGFR) with Real-time quantitative PCR technology (their screen EGFR RGQ PCR Kit, QIAGEN Manchester Ltd).

3. Statistical analysis

The diagnosis was confirmed by histological examination. Statistical analysis was conducted using SPSS for Windows (version 17.0; SPSS Inc., Chicago, IL, USA). Sensitivity, specificity, and positive and negative predictive values were calculated using standard formulas.

4. Results

The baseline characteristics of all the LNs or mass evaluated in this study were summarized in Table 2. A total of 80 hilar and mediastinal LNs or mass were identified for sampling based on the presence of the following features on EBUS. In 55 patients, 178 biopsies were performed in the following stations, and the average puncture number of each group lymph node was 2.09 times.

We evaluated 55 cases of EBUS-TBNA during the study period. Diagnoses made using EBUS-TBNA included lung cancer (n = 37): Small cell carcinoma (n = 6), adenocarcinoma (n = 19, Fig. 1-F), Squamous cell carcinoma (n = 6), Lung cancer-NOS (not otherwise
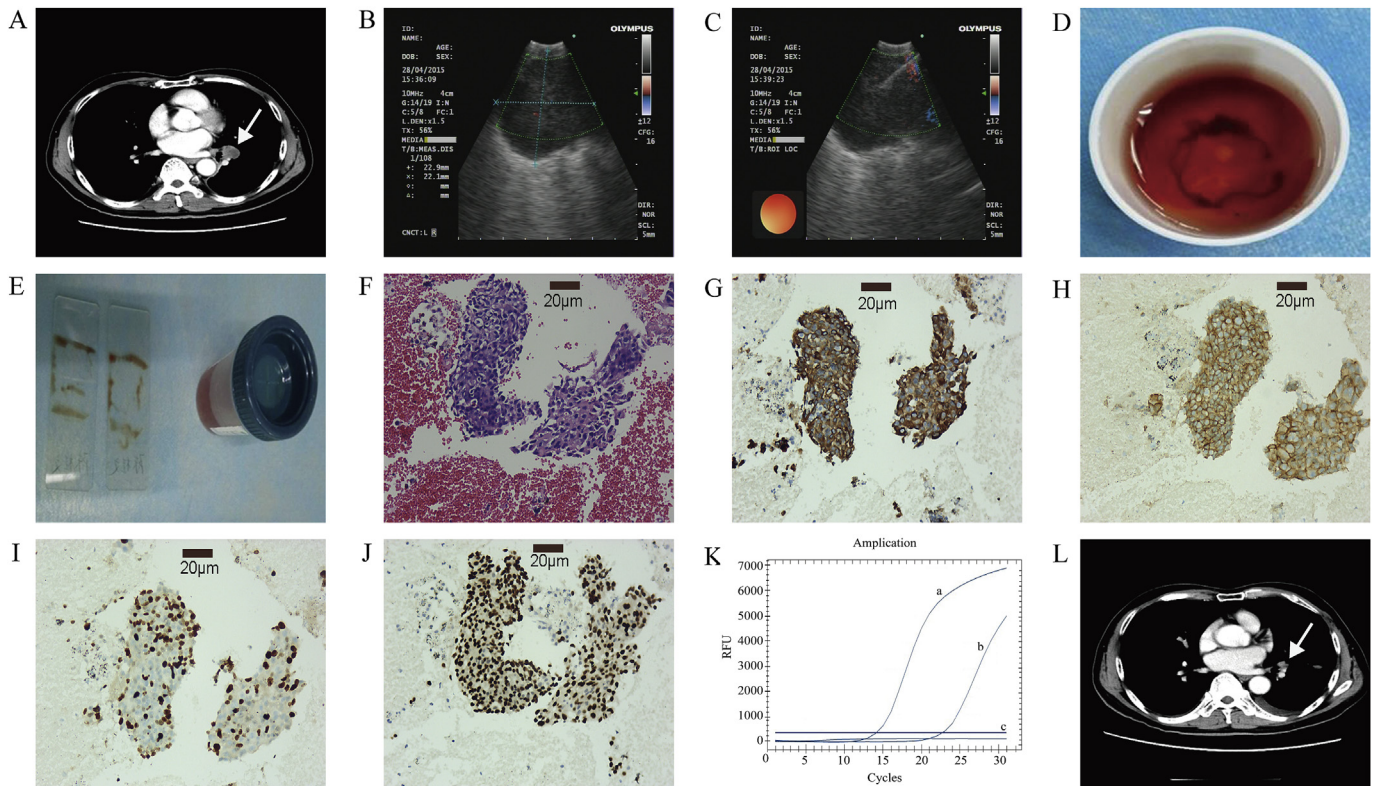
Fig. 1. EBUS-TBNA combined with immunohistochemistry and genotype on a 69-year-old man in the left lower lobe lung lesion (station 12L). A: CT scanning showed the left lower lobe lung lesion (station 12L, showed by white arrow). B: EBUS image showed a hypoechoic mass on station 12L. C: Puncture the lesion by EBUS-TBNA. D: The strip tissue by EBUS-TBNA. E: The air-dry cell smears on the glass slides and liquid-based preparation in the tube. F: Hematoxylin and eosin stained for histopathological diagnosis (lung adenocarcinoma, original magnification ×400). G: Immunohistochemical staining showed CK-7 positive both membranous and nucleus (SP, original magnification ×400). H: Immunohistochemical staining showed EGFR positive on membranous (SP, original magnification×400). I: Immunohistochemical staining showed Ki-67 positive on nucleus (SP, original magnification×400). J: Immunohistochemical staining showed TTF-1 positive on nucleus (SP, original magnification×400). K: Real-time RT-PCR detect EGFR gene mutation (exon 21, L858R, a: positive control, b: EBUS-TBNA sample, c: negative control). L: Followed up by CT scanning after targeted EGFR gene therapy one month later, the lesion significantly reduced. (Showed by white arrow).

Table 1
Baseline characteristics of patients.

Characteristic	N or Average Number	Percentage, %
Age (years), Mean ± SD (range)	55.2 ± 11.5(28−82)	
Sex		
Women	42	76%
Men	13	24%
Smoking status>20 pack/d, >10y	22	41%
Symptom		
Pure cough>3w	15	27%
Chest pain>3w	16	29%
Dyspnea>3w	7	13%
hemoptysis	7	13%
hoarseness>3w	6	11%
Hinder of sawllow	2	4%
Facial edema	1	2%
Other sites of metastases symptoms (headache, abdominal pain, etc.)	6	11%

specified), poorly differentiated neoplasms or carcinomas (n = 4), NSCLC-NOS (n = 2), Metastasis carcinoma (n = 3), Malignant lymphoma (n = 1), Lymphoma (n = 1), Chronic inflammation (n = 1), tuberculosis (n = 5). Regardless of procedure purpose, all diagnostic accuracy of EBUS-TBNA was 87.3%. The diagnostic accordance rate of EBUS-TBNA for each disease was as follows (Table 3). 7 undiagnosed patients underwent more invasive procedures including mediastinoscopy (n = 1), subclavian lymph node biopsy (n = 3), CT-guided tumor puncture (n = 2) and followed-up with anti-tuberculosis treatment (n = 1). They were eventually

diagnosed with benign cyst (n = 1), lung cancer (n = 3), malignant mesothelioma (n = 1), ganglioneuroma (n = 1), and tuberculosis after anti-tuberculosis treatment with CT imaging showing the mediastinal lymph nodes shrinked (n = 1). The diagnostic sensitivity, accuracy for EBUS-TBNA in lung cancer are 92.5% (37/40) and 94.5% (52/55), respectively.

Among 55 EBUS-TBNA specimens, immunostaining was performed in 37 cases that were needed for origin identification, and 29 lung cancer cases of them got the clear typing with different antibodies. 17 cases were adenocarcinoma, 6 cases were the

Table 2
Location of the lymph nodes or mass in the mediastinum as per the IASLC classification and biopsy results.

Location	Samples	Positive biopsies	Diagnostic accuracy
1R	1	0	0
2R	9	7	77.8%
4R	31	24	77.4%
4L	6	4	66.7%
7	16	12	75.5%
10R	5	5	100.0%
10L	7	7	100.0%
11R	3	2	66.7%
11L	1	1	100.0%
12R	1	1	100.0%
Mediastinal mass	3	3	100.0%
Hilar mass	2	2	100.0%
Total	85	68	80.0%

Table 3
Diagnostic Accuracy of EBUS-TBNA According to different lesions.

Final diagnosis	Total No.	Diagnostic yield [n (%)]
malignant		
Small cell carcinoma	6 (10.9)	6 (100.0%)
Adenocarcinoma	21(38.2)	19(90.5%)
Squamous cell carcinoma	7 (12.7)	6 (85.7%)
Lung cancer-NOS	4 (7.3)	4 (100.0%)
NSCLC-NOS	2 (3.6)	2 (100.0%)
Metastasis carcinoma	3 (5.5)	3 (100.0%)
Malignant lymphoma	1 (1.8)	1 (100.0%)
Sarcoidosis	1 (1.8)	1 (100.0%)
Malignant mesothelioma	1 (1.8)	0
Ganglioneuroma	1 (1.8)	0
Lymphoma	1 (1.8)	1(100.0%)
Benign		
Chronic inflammation	1 (1.8)	1 (100.0%)
tuberculosis	5 (9.1)	4 (80.0%)
cyst	1 (1.8)	0
Total	55(100)	48(87.3%)

squamous carcinoma, 6 cases were small cell carcinoma. 3 cases were metastasis carcinoma, and 1 case was soft tissue sarcoma. Immunohistochemical analyses were carried out at the pathology department of Affiliated Tumor Hospital of Guangxi Medical University (Xingqing Ye). The immunostaining techniques varied with the antibodies used as follow (Table 4). Lung adenocarcinoma showed CK7 positive (Fig. 1-G), EGFR positive (Fig. 1-H), Ki-67 with strong positive (>50%, Fig. 1-I), and TTF-1 positive (Fig. 1-J); Squamous cell carcinoma showed CK5/6 and P63 positive; Small cell carcinoma showed both Syn and CD56 positive, and with high expression of Ki-67. Among 3 cases metastasis carcinoma, 1 case diagnosed echinoderms endometrial carcinoma with lung metastasis showed both CK7 and CK5/6 positive combined with the history of endometrial adenocarcinoma adenoid and hysterectomy five years ago, 1 case diagnosed with hepatocellular carcinoma of lung metastases showed both CKpan, CK18, CK19 and Hepatocytepositive with CT imaging found liver mass lesions with multiple mediastinal and supraclavicular lymph nodes enlarged, 1 case diagnosed with invasive ductal carcinoma lung metastasis

showed estrogen receptor positive and HER-2 strong positive combined with the history of breast cancer. One case diagnosed of soft tissue sarcoma showed EGFR high positive and Vimentin positive.

6 cases of EBUS-TBNA samples were analyzed from 19 cases lung adenocarcinoma referred for EGFR gene testing and 1 case with ALK fusion gene testing together. 4 cases were found to have EGFR gene Exon 21 L858R mutant (Fig. 1-K), including 2 cases were found EGFR wild type and 1 case showed ALK fusion gene negative.

All patients received the conventional chemotherapy or radiotherapy according to the pathological examination combined with sub-type diagnosis by IHC. The targeted therapy was carried out on patients with mutated EGFR. Major of patients symptoms improved or alleviated, especially patients targeted therapy to EGFR gene mutations (Fig. 1-L).

5. Discussion

Patients with mediastinal lymphadenopathy or suspected lung cancer require the accurate diagnosis to determine their optimal treatment. EBUS-TBNA is a new technique which can be used to obtain cytological and histological samples of lesions adjacent to the tracheobronchial tree, especially in Lung cancer diagnosis and staging [16]. It has been reported as having a cumulative sensitivity of 88–93% and a cumulative specificity of 100% [1,17]. In a prospective direct comparison of EBUS-TBNA and mediastinoscopy to date in patients with suspected lung cancer, EBUS-TBNA demonstrated significantly superior sensitivity (91 vs. 78%, P = 0.007) [18]. Lee et al. retrospectively evaluated the diagnostic accuracy of EBUS-TBNA for lung cancer. They reported excellent accuracy and sensitivity of 98% and 97%, respectively [19]. In our study, 55 cases suspected lung cancer with occupied mediastinal lesions or hilar or mediastinal lymph node metastasis diagnosed with EBUS-TBNA. Combined with cytology smear sheet and histopathological examination, we found the sensitivity in the diagnosis of lung cancer by EBUS-TBNA was 92.5% (37/40), and the accuracy was 94.5% (52/55), which is accorded with previous reports.

Meanwhile, EBUS-TBNA has been applied as the first-line procedure for the staging of lung cancer and in establishing a definitive diagnosis of mediastinal and hilar lymphadenopathy. It can identify patients who are right candidates for surgery [20]. Although mediastinoscopy or surgery is considered to be the gold standard for diagnosis [21], some patients are unable to tolerate the procedural injury and intravenous anesthesia. Therefore, efforts are focused on establishing a method that is easy to use, minimally invasive and efficient in providing the critical information required. In this study, the procedure of all 55 patients by EBUS-TBNA was uneventful without any complications. It demonstrates that EBUS-TBNA is a highly useful and safe diagnostic modality. However, mediastinoscopy, CT-guided tumor puncture, and follow-up are recommended after negative EBUS-TBNA, which is an important consideration when obtaining consent from patients for the procedure. In our study, still have 7 cases negative EBUS-TBNA; other methods were used to diagnose.

Lung cancer divided by traditional pathological type is non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).

Table 4
Expression of different antigens in different lesion tissues with immunohistochemical staining method in samples by EBUS-TBNA cases [n (%)].

Group	CK5/6	P63	TIF-1	CK7	Syn	CD56	EGFR	Ki67(>50%)
Lung adenocarcinoma (n = 17)	1(5.9)	0(0.0)	17(100.0)	16(94.1)	1(5.9)	0(0.0)	8(47.1)	4(23.5)
Lung squamous cell carcinoma (n = 6)	4(66.7)	4(66.7)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(33.3)
Lung small cell carcinoma (n = 6)	0(0.0)	0(0.0)	2(33.3)	0(0.0)	6(100.0)	4(66.7)	0(0.0)	4(66.7)

Recently, with the individualized treatment of lung cancer, especially lung cancer targeted therapy, conventional tissue typing is facing a challenge. The association of international society classified the lung cancer with a new standard. NSCL should be classified as adenocarcinomas and squamous cell carcinoma [22]. To gross specimens with surgical resection, the immunohistochemical techniques are not critical, but for a small biopsy sample, it's hard to identify the morphologic classification and need some immuno-histochemical indicators such as TTF-1, P63, CK5/6 and so on to distinguish further between adenocarcinoma and squamous cell carcinoma [12,13]. Navani et al. [6] reported that 774 cases of suspected non-small cell lung cancer patients who underwent EBUS-TBNA examination combined with related indicators such as CK5/6, p63, and TTF-1 with immunohistochemistry, also showed that can significantly increase the typing, thus reducing the proportion of cases of non-small cell lung cancer could not be typed. Our study revealed that adenocarcinoma cells have CK7, TTF-1 positive expression, and lung squamous cell carcinoma showed a high CK5/6, p63 positive expression. However, small cell lung cancer cells showed Syn, CD56 high positive expression rate. Which is consistent with previous reports [12,13].

As we know, lung cancer is a high malignancy; nearly 40 percent of diagnosed with lung cancer is IV stage unresectable [23]. However, the incidence of NSCLC is approximately 85 percent in all lung cancer, and adenocarcinoma has high proportion about 50 percent in NSCLC [24]. In recent years, targeted therapy for advanced lung cancer especially in patients who had mutated EGFR with tyrosine kinase inhibitors (TKI) has changed the treatment modalities of lung cancer. The status of EGFR gene mutation can predict the patient's therapy and prognosis [14,25]. Compared with conventional chemotherapy or radiotherapy, the targeted therapy significantly improved the efficacy with fewer adverse reactions. Some researchers had reported the efficacy of patients with EGFR mutations by targeted treatment was 73.7%, so anti-EGFR targeted therapy had been recommended as first-line treatment program with EGFR mutations of advanced lung cancer [25,26]. The efficacy of targeted drug is better, but more expensive, so molecular testing significantly affected the treatment and prognosis of advanced lung cancer. A recent European consensus [27] showed the EGFR gene mutations tissue should use surgery or biopsy samples hardly use the cell samples, but advanced cancer cases had not chance to operate or get the sample by routine examination, EBUS-TBNA can be available to get the tissue sample for gene testing. Molecular analysis can be routinely performed on the majority of cytological samples obtained by EBUS-TBNA and conventional TBNA but largely depends on the absolute number of tumor cells (preferably > 100), the percentage of tumor cells present in the material, the degree of preservation of tumor cells, and the type and sensitivity of the molecular test that is being utilized [28–30]. In general, the material obtained by EBUS-TBNA is suitable for molecular analysis, which can be performed in 88–96% of the samples [7,31,32]. In this investigation, only six cases tissue specimens by EBUS-TBNA tested the EGFR gene mutation; however, the feasibility was 100%. The sample for gene testing is fewer. There may be several reasons for this. First, the tissue type of EGFR or ALK gene mutation with high mutation rate majorly existed on lung adeno-carcinoma, hardly on other kinds of carcinoma, only patients diagnosed with lung adenocarcinoma were recommended to have the gene testing. Secondly, the expensive cost for gene testing and target drug, some patients cannot afford to refuse. Thirdly, the part of tissue by EBUS-TBNA is insufficient or poor quality and cannot be tested.

To our knowledge, this is the first and more comprehensive report that evaluates the value of EBUS-TBNA combined with immunohistochemistry and molecular testing for the diagnosis of lesions with central parenchymal, mediastinal or hilar metastasis. The immunohistochemistry used in this study played a significant role in types of lesions and origination. Although some research had reported for the diagnosis and staging of mediastinal lymph nodes in patients with lung cancer, most are large specimens with surgical resection [27]. Righi et al. [33] retrospectively analyzed 103 cases of NSCLC-NOS diagnosed morphologically on FNA cytology and then performed immunohistochemistry. The results were compared them with surgical specimens analyzed on morphology. Use of immunohistochemistry reduced the NSCLC-NOS diagnostic category from 36% to 14%. Accuracy was improved by the addition of cell blocks and immunohistochemistry [9,34–36]. In this study, 37 cases samples of all with EBUS-TBNA got the clear diagnosis, including 17 cases of lung adenocarcinoma, 6 cases of lung squamous cell carcinoma, 6 cases of small cell lung cancer, 3 cases of metastasis carcinoma, 1 case of soft tissue sarcoma with different antibodies. Meanwhile, 4 cases of samples by EBUS-TBNA could not be diagnosis due to some reasons. First, the approach to some hilar lesions is not always easy, and anatomical factors such as the interposition of large vessels of aerated tissue can interfere with the puncture. Especially the upper lobe masses, located too peripherally can be out of reach of the scope making the different selection of the patient a crucial issue. Secondly, the histopathological analysis of various extrathoracic malignancies might be more difficult as compared with lung cancer samples. These are all kinds of antibodies in different tumors, so we must combine with the history of the patients to choose. There was not a particular tumor type prone for this false-negative result of EBUS-TBNA and need a confirmatory intervention or follow-up. Finally, to further good therapy and prognosis, gene testing can be carried out in lung adenocarcinoma with EGFR or ALK gene mutation.

It should be noted that several limitations apply to this study. First, the single center, retrospective of the study implies a selection bias of the investigators allowing the patient for EBUS-TBNA. Prospective, randomized multi-center trials are needed in the future to overcome this. Secondly, the cases of this study are fewer, which must more cases to confirm. Finally, high-quality tissue samples must be acquired by EBUS-TBNA for more gene testing.

5.1. Conclusions

EBUS-TBNA is a safe and efficient method with high sensitivity and specificity in the diagnosis of mediastinal and hilar lesions. Uniquely combining with IHC and genotype has important clinical value in subtype diagnosis and guiding the treatment strategy. Further, more and high-quality tissue samples by EBUS-TBNA need to have a generalization and a lower cost in gene testing for individualized treatment.

Ethical approval

The protocol of current study was approved by the Ethics Committees of the Affiliated Tumor Hospital of Guangxi Medical University.

Author contribution

A-Q L,X-L L and Y-X Z designed the study. A-Q L and L-W Q processed clinical samples. Z Y and N Y performed immunohistological and genotype detection analysis. A-Q L wrote the manuscript.

Conflicts of interest

The authors no conflict of interest.

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