Evaluation of Tissue Adequacy in Patients with Malignant Intrathoracic Lymphadenopathy undergoing Combined Endobronchial Ultrasound-Guided Miniforceps Biopsy (EBUS-MFB) and Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration (EBUS-TBNA) compared to EBUS-TBNA alone

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จุฬาลงกรณ์มหาวิทยาลัย
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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Medicine
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การศึกษาปริมาณและสัดส่วนของจำนวนเซลล์มะเร็งในชิ้นเนื้อเยื่อจากผู้ป่วยที่มีต่อมน้ำเหลืองในทรวงอกโตที่มีสาเหตุจากมะเร็งที่ได้จากการส่องกล้องอัลตราซาวด์ทางหลอดลมด้วยวิธีใช้เข็มเก็บเนื้อเยื่อควบคู่กับวิธีใช้เข็มหัวปากคีม เปรียบเทียบกับวิธีใช้เข็มเก็บเนื้อเยื่อเพียงวิธีเดียว

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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พิภู ถาวรชีวิน : การศึกษาปริมาณและสัดส่วนของจำนวนเซลล์มะเร็งในชิ้นเนื้อเยื่อจากผู้ป่วยที่มีต่อมน้ำเหลืองในทรวงอกโตที่มีสาเหตุจากมะเร็งที่ได้จากการส่องกล้องอัลตราซาวด์ทางหลอดลมด้วยวิธีใช้เข็มเก็บเนื้อเยื่อควบคู่กับวิธีใช้เข็มหัวปากคีม เปรียบเทียบกับวิธีใช้เข็มเก็บเนื้อเยื่อเพียงวิธีเดียว. (Evaluation of Tissue Adequacy in Patients with Malignant Intrathoracic Lymphadenopathy undergoing Combined Endobronchial Ultrasound-Guided Miniforceps Biopsy (EBUS-MFB) and Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration (EBUS-TBNA) compared to EBUS-TBNA alone) อ.ที่ปรึกษาหลัก : อ. นพ.นพพล ลีลายุวัฒนกุล, อ.ที่ปรึกษาร่วม : รศ. นพ.ปุญช วิษฐ์ จันทรานุวัฒน์

ความเป็นมา: การส่องกล้องอัลตราซาวด์ทางหลอดลมด้วยวิธีใช้เข็มเก็บเนื้อเยื่อเป็นวิธีวินิจฉัยโรคที่เป็นมาตรฐานในการตรวจเนื้อเยื่อต่อมน้ำเหลืองในทรวงอก การรักษาโรคมะเร็งมีความหลากหลายและจำเพาะขึ้นกับลักษณะความผิดปกติทางอณูพันธุศาสตร์ จึงมีความจำเป็นที่จะต้องใช้เนื้อเยื่อที่มีปริมาณมากและคุณภาพดีเพื่อนำไปสู่การรักษาที่เหมาะสม วัตถุประสงค์: เพื่อศึกษาการส่องกล้องอัลตราซาวด์ทางหลอดลมด้วยวิธีใช้เข็มเก็บเนื้อเยื่อควบคู่กับวิธีใช้เข็มหัวปากคีมว่าสามารถเพิ่มคุณภาพของเนื้อเยื่อสำหรับการตรวจทางอณูพันธุศาสตร์ เปรียบเทียบกับวิธีเข็มเก็บเนื้อเยื่อเพียงอย่างเดียว วิธีการศึกษา: วิจัยเก็บรวบรวมข้อมูลผู้ป่วยที่มีต่อมน้ำเหลืองในทรวงอกโต เข้ารับการส่องกล้องอัลตราซาวด์ทางหลอดลมด้วยวิธีใช้เข็มเก็บเนื้อเยื่อควบคู่กับวิธีใช้เข็มหัวปากคีมในโรงพยาบาลจุฬาลงกรณ์ สภากาชาดไทย ตั้งแต่เดือนกุมภาพันธ์ ปี 2565 จนถึง เดือนธันวาคม ปี 2565 และนำผลพยาธิวิทยามาเปรียบเทียบปริมาณและสัดส่วนของจำนวนเซลล์มะเร็งในชิ้นเนื้อเยื่อ โดยกำหนดนิยามคุณภาพชิ้นเนื้อที่เหมาะสมต้องประกอบด้วยปริมาณเซลล์มะเร็งมากกว่า 100 เซลล์ และสัดส่วนของจำนวนเซลล์มะเร็งในเนื้อเยื่อเทียบกับปริมาณเซลล์ทั้งหมดมากกว่าร้อยละ 25 ผลการศึกษา: พบว่าผู้ป่วย 57 รายที่มีต่อมน้ำเหลืองในทรวงอกโตผิดปกติได้รับการทำหัตถการส่งตรวจชิ้นเนื้อทั้งสองวิธี พบว่ามีผู้ป่วย 21 รายมีต่อมน้ำเหลืองในทรวงอกโตมีสาเหตุจากมะเร็ง โดยร้อยละ 90.5 ของเนื้อเยื่อที่ได้จากการตรวจด้วยเข็มปกติมีลักษณะคุณภาพที่เหมาะสม เปรียบเทียบกับวิธีเข็มเก็บเนื้อเยื่อควบคู่กับวิธีใช้เข็มหัวปากคีมพบคุณภาพของเนื้อเยื่อมีลักษณะคุณภาพที่เหมาะสมร้อยละ 95.2 โดยไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ (p=0.317) แต่ปริมาณเซลล์มะเร็งที่ได้จากเข็มเก็บเนื้อเยื่อปกติพบว่ามีสัดส่วนที่มีปริมาณเซลล์มะเร็งที่มากกว่า 1,000 เซลล์ ซึ่งมากกว่าวิธีใช้เข็มหัวปากคีมอย่างมีนัยสำคัญทางสถิติ (ร้อยละ 80.9 และร้อยละ 47.6 ตามลำดับ, p=0.039) ในแง่การวินิจฉัยโรคพบว่าวิธีการใช้เข็มเก็บเนื้อเยื่อปกติควบคู่กับวิธีใช้เข็มหัวปากคีมทำให้สามารถวินิจฉัยโรคได้มากขึ้นอย่างมีนัยสำคัญ (98.2% และ 87.7%; p=0.031) โดยพบว่าร้อยละ 20.7 สามารถได้รับการวินิจฉัยเพิ่มเติมจากวิธีใช้เข็มหัวปากคีม ภาวะแทรกซ้อนไม่แตกต่างจากวิธีใช้เข็มปกติแบบปกติ สรุปผลการวิจัย: การส่องกล้องอัลตราซาวด์ทางหลอดลมด้วยวิธีใช้เข็มเก็บเนื้อเยื่อปกติควบคู่กับวิธีใช้เข็มหัวปากคีมพบว่าไม่ได้เพิ่มคุณภาพของเนื้อเยื่อในการตรวจทางอณูพันธุศาสตร์ แต่วิธีใช้เข็มเก็บเนื่อเยื่อปกติมีปริมาณเซลล์มะเร็งที่มากกว่าอย่างมีนัยสำคัญทางสถิติ การใช้เข็มเก็บเนื้อเยื่อเพิ่มเติมด้วยวิธีใช้เข็มหัวปากคีมสามารถให้การวินิจฉัยที่แม่นยำได้มากยิ่งขึ้น เป็นหัตถการอีกทางเลือกหนึ่งที่สามารถใช้เสริมในทางปฏิบัติได้อย่างปลอดภัย

สาขาวิชา	อายุรศาสตร์	ลายมือชื่อนิสิต .....
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KEYWORD: Tissue adequacy, Molecular analysis, Endobronchial Ultrasound-Guided Miniforceps Biopsy, Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration, Malignant Intrathoracic lymphadenopathy

Pipu Tavornshevin : Evaluation of Tissue Adequacy in Patients with Malignant Intrathoracic Lymphadenopathy undergoing Combined Endobronchial Ultrasound-Guided Miniforceps Biopsy (EBUS-MFB) and Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration (EBUS-TBNA) compared to EBUS-TBNA alone. Advisor: Nophol Leelayuwatanakul, M.D. Co-advisor: Assoc. Prof. POONCHAVIST CHANTRANUWATANA, M.D.

RATIONALE: EBUS-TBNA has become an effective way of tissue assessment for evaluating mediastinal lymph nodes. Identifying molecular mutations is a key to personalized management in malignant disease. The success of molecular analysis depends on adequate tissue specimens consisting of an absolute number of tumor cell counts and the neoplastic cell percentage (NCP) estimation. This study aimed to evaluate the efficacy of EBUS-MFB added on EBUS-TBNA to improve the tissue adequacy and the overall diagnostic yield. In this prospective study, patients with enlarged intrathoracic lymph nodes underwent EBUS-TBNA followed by EBUS-MFB. The tissue adequacy for molecular analysis required that the tissue samples met both a tumor cell count of more than 100 cells and an NCP estimation of more than 25%. RESULTS: Fifty-two patients (57 nodes) with enlarged intrathoracic lymphadenopathy were enrolled. Twenty-one of fifty-seven nodes were diagnosed with malignant disease by both EBUS-TBNA and EBUS-MFB. The tissue adequacy of EBUS-TBNA was 19/21 (90.5%) comparable to EBUS-MFB added on EBUS-TBNA, which was 20/21 (95.2%) with no statistical significance (p=0.317). EBUS-TBNA resulted in higher tumor cell counts; more than 1,000 cells were shown in 17/21 (80.9%) compared to EBUS-MFB 10/21 (47.6%) (p=0.039). The EBUS-MFB added on EBUS-TBNA significantly improved the overall diagnostic yield compared to EBUS-TBNA alone (98.2% vs 87.7%; p=0.031). The discordant cases between EBUS-TBNA and EBUS-MFB were 19 of the 29 nodes (65.5%). Within these, 6/29 (20.7%) nodes were misdiagnosed with EBUS-TBNA, but EBUS-MFB demonstrated a valid diagnosis including three anthracotic lymph node, two granulomatous nodes, and one silicotic node. No serious adverse events were observed, only 2 patients out of 52 (3.84%) had minor bleeding. CONCLUSION: The tissue adequacy for molecular analysis by EBUS-TBNA and EBUS-MFB added on EBUS-TBNA were not different. However, EBUS-TBNA showed better tumor cell counts of specimens. Also, the EBUS-MFB added on EBUS-TBNA is a feasible and safe procedure which may provide more diagnostic yield, particularly in nonmalignant disease.

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Academic Year: 2022 Advisor's Signature .....

Co-advisor's Signature .....

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Pipu Tavornshevin

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CHAPTER 1
INTRODUCTION

1.1 Historical background

Endobronchial ultrasound transbronchial needle aspiration (EBUS-TBNA) is a minimally invasive diagnostic procedure which permits real-time visualization of intrathoracic lymph node tissue sampling (1, 2). The diagnostic yield of EBUS-TBNA ranges widely from 50-81%, depending on the study population, diseases, procedural techniques, and cytological techniques (3-7). Endobronchial ultrasound-guided miniforceps biopsy (EBUS-MFB) is a novel technique of using miniforceps biopsy to obtain tissue from intrathoracic lymph nodes (3). EBUS-MFB provides more adequate histological specimens of intrathoracic lymph nodes which could increase the diagnostic yields and may add value for molecular analysis of lung cancer cases (5).

Lung cancer is the most common cancer and is also the most common cause of cancer mortality worldwide (8). Personalized treatments in advanced stage lung cancer are nowadays mainly based on histologic features and oncogenic alterations (9). The development of targeted therapies in personalized treatment has led to an increasing need for molecular testing for which obtaining adequate samples is an important key (10). However, the absolute number of tumor cells is one of the factors determining tissue adequacy for molecular testing (11). For example, the EGFR-mutation requires a minimum of 1,000 cells (approximately at least 5 ng DNA) and the ALK/ROS1 translocations may need tissue containing at least 100 cancer cells for adequate testing (12). Moreover, tumor cell proportion in sampling tissue is another important factor. Recent studies showed that the increasing proportion of non-tumor cells in sampling may potentially dilute the proportion of tumor DNA and cause false-negative results (13, 14). Up to now, the minimum neoplastic cell percentage (NCP) required for genetic mutation testing has yet to be elucidated. Depending on molecular testing, some techniques may require at least 40-50% of tumor cells, but newer

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methods of genetic sequencing such as direct sequencing, pyrosequencing, or amplification refractory mutation system (ARMS) may only need 1-25% of tumor cells (15). However, the NCP estimation can be under- or overestimated with a significant variation of about 20% between pathologists (14, 16).

Previous studies have shown that EBUS-TBNA provided sufficient samples for molecular analysis of EGFR and ALK mutations (17). However, there are limited studies on tissue adequacy for molecular testing, especially newer methods that need a large amount of tumor cell count and NCP such as next-generation sequencing (NGS). This study was conducted to preliminary assess the efficacy of EBUS-MFB added on EBUS-TBNA to improve tissue adequacy in patients with malignant intrathoracic lymphadenopathy compared to EBUS-TBNA alone.

1.2 Research questions

Primary questions: Can EBUS-MFB added on EBUS-TBNA increase the adequacy of tissue samples in malignant intrathoracic lymphadenopathy?

Secondary questions

1. Can EBUS-MFB added on EBUS-TBNA increase the diagnostic yield in patients with intrathoracic lymphadenopathy?
2. What are the factors that improving the tissue adequacy or the diagnostic yield from EBUS-MFB added on EBUS-TBNA?
3. Are the complications from EBUS-MFB added on EBUS-TBNA equal to EBUS-TBNA alone?

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1.3 Objectives

1. To evaluate the adequacy of samples of EBUS-MFB added on EBUS-TBNA compared with EBUS-TBNA alone in malignant intrathoracic lymphadenopathy
2. To evaluate the diagnostic yield of EBUS-MFB added on EBUS-TBNA in patients with intrathoracic lymphadenopathy

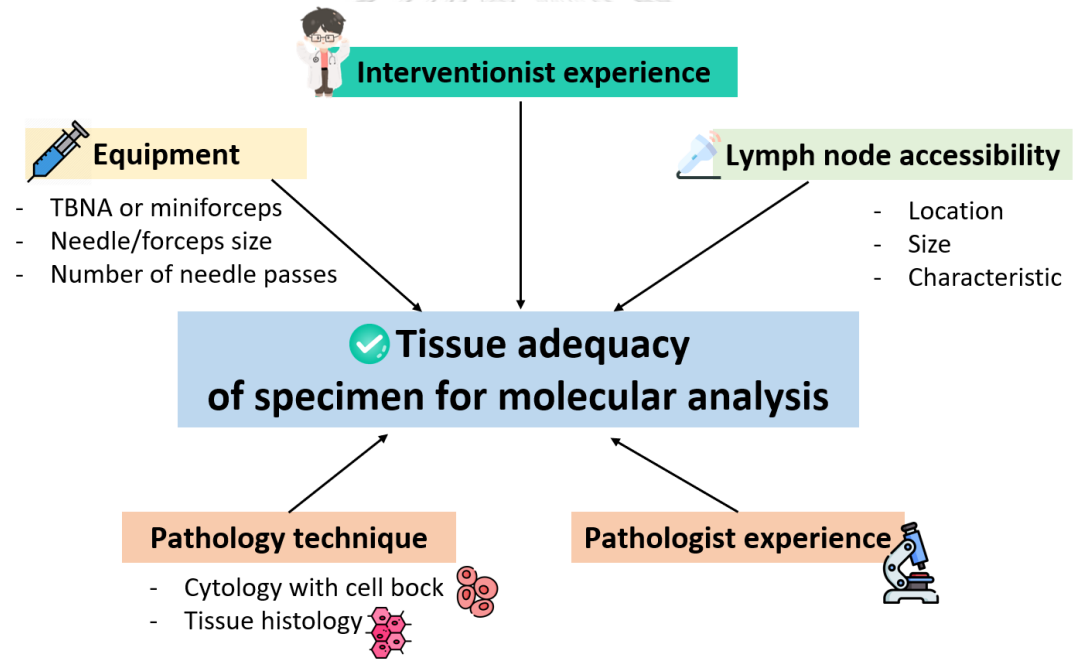
3. To identify factors that improving the tissue adequacy or the diagnostic yield from EBUS-MFB added on EBUS-TBNA.
4. To evaluate the safety outcome of EBUS-MFB added on EBUS-TBNA

1.4 Hypothesis

Primary hypothesis: The EBUS-MFB added on EBUS-TBNA increase the adequacy of tissue samples for molecular analysis in malignant intrathoracic lymphadenopathy

Secondary hypothesis: The EBUS-MFB added on EBUS-TBNA has higher diagnostic yield and better tissue adequacy than EBUS-TBNA alone. The complications from EBUS-MFB added on EBUS-TBNA may not higher than EBUS-TBNA alone.

1.5 Conceptual framework



1.6 Definitions

The procedure time started when the bronchoscopy was passed through the trachea and ended when the bronchoscopy was withdrawn from the patient. Intrathoracic lymph nodes are defined as several groups of nodes associated with lymphatic drainage of the lungs and conducting airway. They include: the pretracheal and paratracheal nodes located anterior to, and along the sides of the

trachea, respectively; the hilar nodes, located in the hilum of the lungs where the main-stem bronchi enter the lungs; the intrapulmonary nodes, deep in the hilum and surrounding the bronchi; and the subcarinal nodes inferior to the carina of the trachea. The lymph node stations were defined according to the 8th edition of the International Association for the Study of Lung Cancer (IASLC). Enlarged lymph nodes are defined by a lymph node size greater than 10 mm in the short axis on chest CT or lung positron emission tomography (PET) scan. The index lymph nodes were defined as enlarged lymph nodes suitable for miniforceps biopsy during the procedure. Each lymph node sample was evaluated for adequacy and a specific diagnosis based on cytological and histological results. Diagnostic lymph node specimens were defined as follows:

1) Malignant if tumor cells present

2) Nonmalignant in those demonstrating granulomatous inflammation, or evidence of specific diseases

3) Benign reactive lymphadenopathy in those demonstrating a sufficient number of benign lymphocytes, or anthracotic pigment-laden macrophages, and no other diagnosis

A positive result of malignancy was accepted as evidence of cancer. Immunohistochemistry was performed if needed. Non-diagnostic lymph node specimens were defined if there was no lymphoid stroma and no specific diagnosis. In those patients in whom bronchoscopy was not diagnostic or in whom tissue specimens were inadequate for molecular testing, subsequent secondary procedures further clarified the diagnosis (surgical procedure, mediastinoscopy, biopsy of another location, repeat bronchoscopic procedure, etc.). Otherwise, patients underwent follow-up with chest CT imaging within 3 months.

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Tissue adequacy for molecular analysis

To demonstrate a good tissue sample in all molecular analysis methods, we defined tissue adequacy for molecular analysis as 'tissue adequate' if the tissue samples meet both of the following criteria:

1. Tumor cell count more than 100 cells
2. The neoplastic cell percentage (NCP) estimation more than 25% versus all cells in the dissection zone

To determine the amount of tumor cell count, only the viable tumor or degenerating tumor cell with pyknotic nuclei were counted manually; the result was scored in categories of 1-100, 101-500, 501-1,000, 1,001-3,000, and >3,000 tumor cells. The reported diagnostic threshold for different techniques requires approximately 10-25% tumor cells to detect mutations(15). Testing samples with insufficient NCPs may lead to false negative results. Therefore, we defined the NCP estimations scored in categories of 0-10%, 11-25%, 26-50%, 51-100%. The study's board-certified pathologist had more than 10 years of experience in pulmonary pathology, and molecular pathology and performed routine evaluations at least five days per week. To standardize the NCP estimation in this trial, the pathologist followed the recommendations from a modified Delphi study to improve the accuracy of NCP estimates to ensure a correct interpretation of the test results(18). To validate the reliability of the results, the pathologist was blinded to re-evaluate the total tissue samples for a second time. The results were considered for interpretation, and the average results were used in each parameter. 'Discrepancy' was recorded if the results of those were in different categories. A third interpretation was performed, and the results were documented in the categories confirmed by two identical results of the three.

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The overall diagnostic yield for the EBUS-TBNA and the EBUS-MFB were calculated as follows:

EBUS-TBNA overall diagnostic yield (%)

$$= \frac{number\ of\ TBNA\ diagnosed\ (malignant + benign + reactive\ lymph\ node)}{number\ of\ TBNA\ diagnosed + nondiagnosed} \text{ x100\%}$$

EBUS-MFB overall diagnostic yield (%)

$$= \frac{number\ of\ MFB\ diagnosed\ (malignant + benign + reactive\ lymph\ node)}{number\ of\ MFB\ diagnosed + nondiagnosed} \text{ x100\%}$$

1.7 Ethical consideration

The study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (COA No. 0138/2022, IRB No.923/64) and written informed consent was obtained before bronchoscopy from all patients. The investigators comply with the following conditions:

1. Respect for person: the patients were informed all information without bias and discussed the benefit and risk before consent in this trial

2. Beneficence/Non-maleficence: the patients were informed and consent if this procedure would have the benefits more than the risk. After consent, the data was collected according to the basic principles of patient confidentiality.

3. Justice: patients were included and excluded according to the criteria and no bias to inform to include in this trial

1.8 Limitation

- The EBUS-MFB added on EBUS-TBNA, the complicated technique required advanced bronchoscopic skills and leads to an extended procedural time and may needed more sedation

- During COVID-19 pandemic, some patients were not able to assess and performed the bronchocoscopic procedure and some patient delayed for the investigation

1.9 Expected or Anticipated Benefit Gain

The EBUS-MFB added on EBUS-TBNA will increase the adequacy of tissue samples for molecular analysis in malignant intrathoracic lymphadenopathy and improve the diagnostic yield. This procedure would decrease the patient's risk to perform other procedure for obtaining more tissue samples.

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CHAPTER 2
LITERATURE REVIEW

TBNA is used to sample intrathoracic lymph node, but the value may be limited by the small specimen size obtained. Herth et al (19) enrolled 75 patients with subcarinal masses that larger than 2.5 centimeters without known or suspected non-small cell lung cancer. A specific diagnosis was made in 36% of patients with EBUS-TBNA and in 88% with EBUS-MFB. The diagnostic yield with EBUS-MFB was significantly increased in patients with both benign and malignant disease without complications. Chrissian et al (3) enrolled 50 patients with mediastinal or hilar lymphadenopathy who underwent EBUS-TBNA and EBUS-MFB of 74 lymph node stations. When both techniques were combined, the overall diagnostic yield was 97% ($p<0.001$) compared to EBUS-TBNA and EBUS-MFB which were 81% and 91%, respectively. Granulomatous and lymphoproliferative disorders may require examination of tissue architecture for their diagnosis. Therefore, suggestions have been made that cytologic analysis from TBNA may not be sufficient. Based on these studies, in patients presenting with mediastinal or hilar lymphadenopathy and a low likelihood of non-small cell lung carcinoma, EBUS-MFB may be used to obtain tissue specimens which the diagnostic yield may be superior to EBUS-TBNA alone.

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EBUS-TBNA is a useful technique for cytological assessment of enlarged mediastinal lymph nodes with a high diagnostic yield for lung cancer. However, the small sample volume can be problematic in diagnosing benign diseases and for molecular analysis of malignant tumors. In 2013, Darwiche et al (5) conducted a study in which they evaluated the addition of EBUS-MFB to EBUS-TBNA in 55 patients with intrathoracic lymph node larger than 10 millimeters. The study demonstrated that the overall diagnostic yield was higher when EBUS-MFB was used in conjunction with EBUS-TBNA, as compared to using EBUS-TBNA alone. Furthermore, the study revealed that in patients with lung cancer, the sensitivity was not improved with EBUS-MFB compared

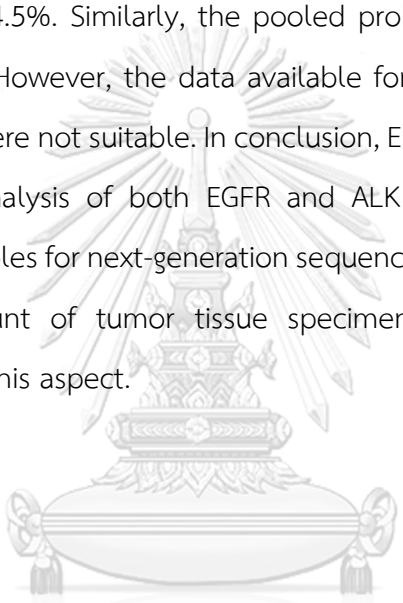
to EBUS-TBNA. Additionally, EGFR mutation testing could only be performed on three of the samples and all them showed a wild type result. Therefore, the EBUS-MFB procedure may increase the diagnostic yield in benign conditions and add value in molecular analysis of non-small cell lung cancer.

In a retrospective cohort study by Wang et al (20), 227 patients underwent concurrent EBUS-TBNA and EBUS-MFB procedures. The study found that the diagnostic yield of EBUS-TBNA cytology was comparable to that of EBUS-MFB (95% and 94%, respectively). EBUS-TBNA cytology was deemed a more effective diagnostic modality compared to EBUS-MFB. Despite the comparable diagnostic yields, there may be instances during an EBUS-TBNA procedure performing a concurrent EBUS-MFB on the specific lesion of interest becomes necessary for various reasons, such as molecular analysis.

Agrawal et al (21) conducted a systematic review and meta-analysis from 6 observational studies involving 443 patients who underwent a total of 467 biopsies. The pooled overall diagnostic yield was 67% (312/467) for EBUS-TBNA and 92% (428/467) for EBUS-TBNA combined with EBUS-MFB. The rates of complications including pneumothorax (1%), bleeding (0.8%), pneumomediastinum (1%), were higher compared to EBUS-TBNA alone, which reported rates of pneumothorax (0.03%) and bleeding (0.68%). However, the morbidity associated with EBUS-guided lymph node sampling was lower than that of mediastinoscopy(22). The trial had certain limitations. First, given the nature of the data, the author could not provide information on the diagnostic yield for each malignancy. Second, the meta-analysis did not clarify whether EBUS-MFB offered any advantage over EBUS-TBNA in terms of the amount of tissue for molecular analysis in malignancies. In summary, based on the analysis of the available studies, the addition of EBUS-MFB to EBUS-TBNA improved overall diagnostic yield, particularly for diagnosing sarcoidosis and lymphoma. Comprehensive molecular testing in cancer patients requires a larger amount of tissue acquisition during EBUS-guided tissue sampling. The decision to use EBUS-MFB should consider the balance

between its benefit and risk in increasing tissue specimens for molecular analysis. Further prospective multicenter randomized controlled trials are necessary to assess the generalizability of these findings.

The adequacy of samples obtained by EBUS-TBNA for molecular analysis in patients with non-small cell lung cancer was assessed in a systematic review and meta-analysis conducted by Gonzalo et al. (17) A total of 33 studies involving 2,698 patients were analyzed. The pooled probability of obtaining a sufficient sample for identifying EGFR mutations was 94.5%. Similarly, the pooled probability for the identifying ALK mutations was 94.9%. However, the data available for meta-analysis regarding ROS1 and PD-L1 mutations were not suitable. In conclusion, EBUS-TBNA demonstrated a high yield for molecular analysis of both EGFR and ALK mutations. Nevertheless, the suitability of TBNA samples for next-generation sequencing remains uncertain, as it may require a larger amount of tumor tissue specimen. Further studies should be conducted to explore this aspect.



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CHAPTER 3
MATERIAL AND METHODOLOGY

3.1 Study Design and Population

We conducted a single-center, prospective, cross-sectional, diagnostic study at King Chulalongkorn Memorial Hospital, The Thai Red Cross Society, Bangkok. The patients were recruited from February 1, 2022, to December 31, 2022. The study protocol was approved by the Institutional Review Board of Chulalongkorn University (IRB number 923/64).

We included all patients who were 18 years or older with an evidence of enlarged mediastinal or hilar lymph nodes greater than 10 mm in the short axis from chest computed tomography (CT). Informed consent was obtained from all patients before enrollment. Patients were excluded if they had contraindications or were considered high risk for the procedure (hemodynamic instability, impending respiratory failure, or respiratory compromise), thrombocytopenia (platelets less than 75,000 cells/mm^3), or coagulopathy (an international normalized ratio (INR) more than 1.5). They were also excluded from the study if they declined to participate, could not tolerate the procedure, had inaccessible lymph nodes by miniforceps biopsy, suspected severe bleeding during EBUA-TBNA, lymph node size less than 10 millimeters from EBUS findings, or needed further tissue assessment but were previously included in this trial.

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3.2 Bronchoscopic Techniques: EBUS-TBNA and EBUS-MFB

Endobronchial ultrasound was advanced, and an intrathoracic lymph nodes evaluation was performed. EBUS-TBNA and EBUS-MFB were performed using a real-time EBUS-TBNA bronchoscope by well-trained, board-certified interventional bronchoscopists. The patients underwent EBUS-TBNA with 30 agitations per pass through the airway wall and into the lymph node using the 21-guage needle, with an

outer diameter of 0.82 mm (Model No. NA-201SX-4021; Olympus, Japan). First pass of TBNA was collected for traditional smear cytology. Subsequently, three passes of TBNA were obtained, the guide sheath was advanced against bronchial wall at the same TBNA puncture site to increase its diameter. Then, miniforceps (standard fenestrated) with an external diameter of 1.5 mm (Model No. FB-233D; Olympus, Japan) were inserted through the working channel of the endoscope. Then, the miniforceps was advanced through the puncture site into the lymph node which can be visualized on the ultrasound image. After penetration, the miniforceps was opened and slightly advanced to biopsy the lymph node tissue, then closed and retracted the miniforceps (Figure 1 and 2.). Three samples were obtained by miniforceps biopsy. When the patient had multiple lymph nodes, EBUS-TBNA and EBUS-MFB were performed if possible.

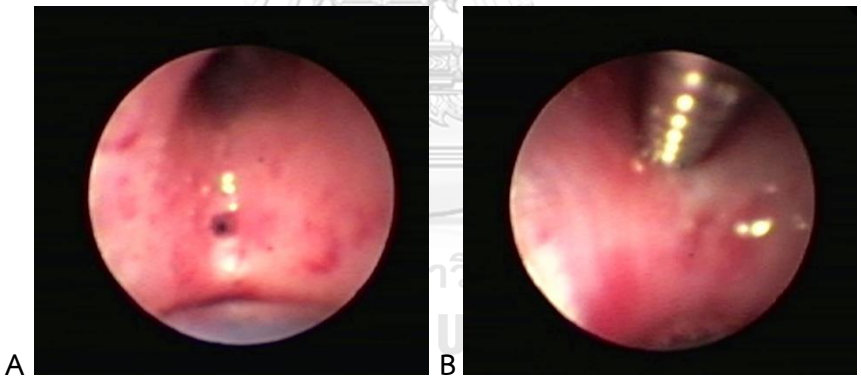

Figure 1 Endoscopic view of a lymph node biopsy

(A) The puncture site created by the guide sheath of TBNA. (B) Miniforceps extended out of the working channel and were inserted into the bronchial wall and entered the right interlobar space.

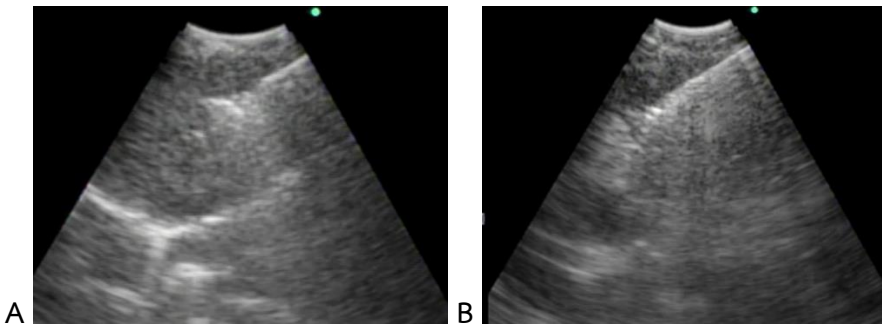

Figure 2 EBUS-MFB procedure

(A) Endobronchial ultrasound image showed right interlobar lymph node and the opening of miniforceps clearly visible within the lymph node. (B) The miniforceps was opened and slightly advanced to biopsy the lymph node tissue.

Before bronchoscopic procedures, patients were evaluated by laboratory tests to keep platelets more than 75,000 cells/mm^3 and an INR of less than 1.5. The severity of bleeding was graded as minor bleeding and major bleeding. Minor bleeding is defined as bleeding that is managed by routine bronchoscopic maneuvers; suction, cold saline, or adrenaline instillation. Major bleeding is defined as bleeding which requires blood transfusion. Also, pneumothorax or pneumomediastinum, respiratory failure, hemodynamic instability, or unscheduled admission were recorded. The procedure was terminated immediately if complications occurred. After the procedure, all patients were observed for 1 hour in the recovery room and a chest radiograph was performed to monitor complications of pneumothorax or other procedure-related adverse events. The principal investigator will contact the patients 48-96 hours after the procedure to identify chest pain, hemoptysis, blood-stained sputum, or the patient's need to re-visit the hospital.

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3.3 Specimen Handling

All TBNA samples, three passes of TBNA not included the traditional smear cytology, were placed into a 10% buffered formaldehyde solution. The cell-block technique was performed on all TBNA samples. The tissue specimens obtained by the miniforceps were placed immediately into a 10% buffered

formaldehyde solution, sent to the pathology department, and embedded in paraffin for histological sectioning. An experienced pathologist evaluated hematoxylin and eosin (H&E) staining of TBNA cell blocks and MFB tissue fragments.

3.4 Data Collection

We collected the demographic data of patients, including sex, age, patients' comorbidities (known malignancy, diabetes mellitus, hypertension, dyslipidemia, chronic kidney disease, chronic obstructive pulmonary disease, coronary artery disease), and smoking histories of at least 10 pack-years. We reviewed and collected lymph node characteristics from chest CT (stations, size, radiographic features). The pathological results diagnoses were recorded as malignant, nonmalignant with specific disease (granulomatous disease) , benign reactive lymphadenopathy, or non-diagnostic/questionable. In malignant disease, we also collected the tumor cell count and neoplastic cell percentage to assess the tissue adequacy. We identified the complications of bronchoscopic procedures: pneumothorax, pneumomediastinum, respiratory failure, bleeding (major and minor bleeding), complications delayed within 24-48 hours (chest pain, hemoptysis or blood-stained sputum, re-visit to the hospital) or patients requiring further tissue assessment within 3 months (EBUS-TBNA, EBUS-MFB, miniprobe biopsy, transbronchial needle biopsy, transthoracic needle biopsy, surgical procedure or mediastinoscopy, tissue diagnosis at other sites).

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3.5 Outcome Measures

The primary outcome of the study was the tissue adequacy of EBUS-MFB added on EBUS-TBNA compared to EBUS-TBNA alone in malignant intrathoracic lymphadenopathy. The secondary outcomes were the tumor cell characteristics between EBUS-TBNA and EBUS-MFB, the diagnostic yield of EBUS-MFB added on EBUS-TBNA in patients with intrathoracic lymphadenopathy, the factors that improve the tissue adequacy or the diagnostic yield from EBUS-MFB added on EBUS-TBNA, and the safety outcomes of EBUS-MFB added on EBUS-TBNA.

3.6 Statistical Analysis

Demographic data were described using descriptive statistical analysis. Continuous variables were described using mean with corresponding standard deviation. The categorical variable was presented in number and percent. The tissue adequacy for molecular analysis and the diagnostic yield of EBUS-MFB added on EBUS-TBNA were compared using the McNemar test for dependent samples. The tumor cells count and neoplastic percentage were compared using the marginal homogeneity tests. The kappa-statistic measure of agreement was used to assess the inter-rater reliability between EBUS-TBNA and EBUS-MFB. For intermediate values, we suggested the following interpretations: below 0.0 was poor agreement, 0.0-0.20 was slight agreement, 0.21-0.40 was fair agreement, 0.41-0.60 was moderate agreement, 0.61-0.80 was substantial agreement, and 0.81-1.00 was almost perfect agreement(23).

No studies have determined a clinically significant difference in tissue adequacy between EBUS-MFB with EBUS-TBNA versus EBUS-TBNA alone. A p-value <0.05 was considered statistically significant, and an alpha level of 0.05 and a power of 0.80 were used. The tissue adequacy yield for EBUS-TBNA is around 70%, and we predicted the clinically significant difference was considered to be 20% more tissue adequacy with EBUS-MFB. The sample size needed for this trial is estimated to be 66 index lymph nodes.

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The factors of successful biopsy of EBUS-MFB were analyzed using a univariable logistic regression. Subsequently, the factors that resulted in a p-value of less than 0.1 were analyzed in a multivariate logistic regression. The association between variables and successful biopsy of EBUS-MFB was reported as an odds ratio with a corresponding 95% CI. The safety outcome of EBUS-TBNA added on EBUS-MFB using descriptive statistical analysis. A p-value <0.05 was considered statistically significant. All analyses were performed using STATA software version 16.0 (StataCorp LLC, Texas, USA).

CHAPTER 4
RESULT

Initially, a total of 52 patients with enlarged intrathoracic lymph node were enrolled in the study. However, 12 patients were subsequently excluded from the protocol for various reasons. Out of these exclusions, six cases experienced a failure of the miniforceps to penetrate the index node during the procedure. Two cases encountered significant bleeding after EBUS-TBNA, three cases had lymph node sizes smaller than 10 mm as determined by EBUS, and one case was unable to tolerate bronchoscopy due to severe coughing during EBUS-TBNA (Figure 3). The mean age of the remaining patients was 63.7±12.5 years, with 76.9% of them being males. Among the 52 patients, 26 (50%) had underlying malignancies, including lung cancer (19.2%), breast cancer (7.7%), colorectal cancer (7.7%), head and neck cancer (7.7%), and lymphoma (5.8%). Additional information regarding other medical illnesses are shown in Table 1.

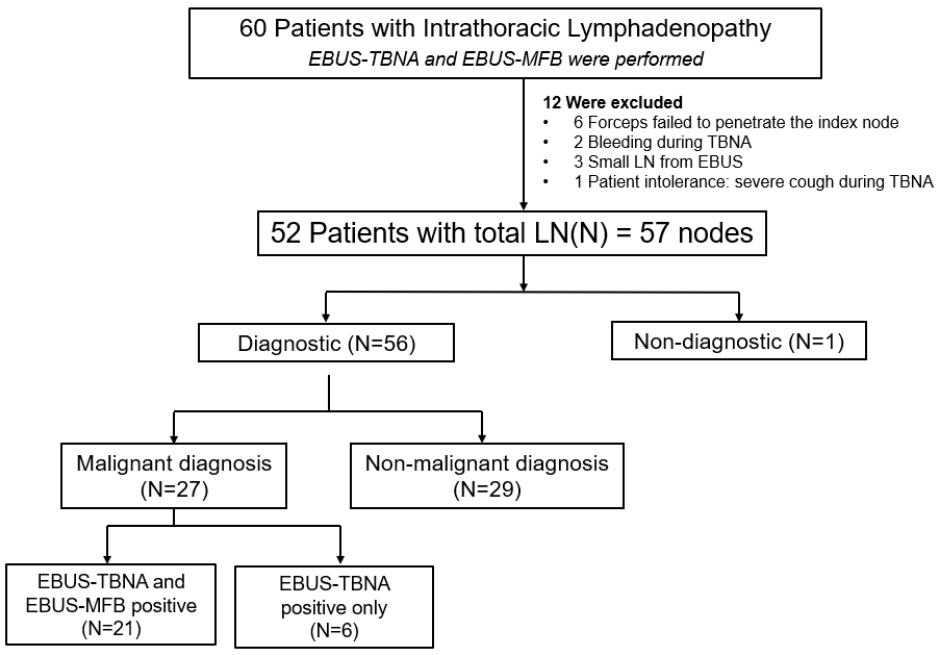

Figure 3 Diagnostic flow diagram

Table 1 Characteristics of patients with intrathoracic lymphadenopathy

	Total (N=52)
Age (years), mean ±SD	63.7 ± 12.5
<50 years, n(%)	9 (17.3%)
51-70 years, n(%)	26 (50.0%)
>70 years, n(%)	17 (32.7%)
Male sex, n(%)	40 (76.9%)
Comorbidities, n(%)	
Malignancy	26 (50.0%)
Hypertension	18 (34.6%)
Diabetes mellitus	16 (30.8%)
Dyslipidemia	13 (25.0%)
Chronic obstructive pulmonary disease	6 (11.5%)
Chronic kidney disease	2 (3.8%)
Coronary artery disease	1 (1.9%)
Malignant disease, n(%)	
Lung cancer	
- Adenocarcinoma	10 (19.2%)
- Small cell lung carcinoma	1 (1.9%)
Breast cancer	4 (7.7%)
Colorectal cancer	4 (7.7%)
Head and neck cancer	4 (7.7%)
Lymphoma	3 (5.8%)

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The index lymph nodes selected for both EBUS-TBNA and EBUS-MFB consisted of 57 nodes, with the majority located in the subcarinal region (56.2%). The distribution of index lymph nodes was as follows: right lower paratracheal (22.8%), right interlobar (14%), and left interlobar (7%) nodes. The mean size of the index lymph node as determined by CT imaging was 15.4 mm (range: 10-29 mm), while the mean size based on EBUS findings was 19.9 mm (range: 10.1-41 mm), as shown in Table 2. Radiographic findings of the index lymph nodes indicated that 22.8% showed necrosis, 5.3% demonstated calcification, and 3.5% exhibited a fatty hilum. The EBUS findings of the index lymph nodes revealed that 52.6% had a round shape, 73.7% displayed

heterogeneous echogenicity, 52.6% had a distinct margin, 66.7% lacked a central hilar structure, and 43.9% showed the presence of vascular structure. When EBUS-TBNA was used, 27 nodes (47.4%) was diagnosed as malignant, whereas only 21 nodes were diagnosed using EBUS-MFB. Among the 27 lymph nodes diagnosed as malignant, 18 (66.7%) were diagnosed as adenocarcinoma, 5 (18.5%) as poorly differentiated carcinoma, 2 (7.4%) as small cell lung carcinoma, and 2 (7.4%) as lymphoma (Table 7).

Table 2 Index Lymph node characteristics

	Total (N=57)
Lymph node station, n(%)	
Subcarinal node	32 (56.2%)
Right lower paratracheal node	13 (22.8%)
Right interlobar node	8 (14.0%)
Left interlobar node	4 (7.0%)
Lymph node size (mm), mean ±SD	
From Computed tomography	15.4 ± 5.3
From Endobronchial ultrasound	19.9 ± 8.3
Radiographic characteristics, n(%)	
Necrosis	13 (22.8%)
Calcification	3 (5.3%)
Fatty hilum	2 (3.5%)
Sonographic findings, n(%)	
Round shape	30 (52.6%)
Heterogenous echogenicity	42 (73.7%)
Distinct margin	30 (52.6%)
Absence of central hilar structure	38 (66.7%)
Presence of vascular structure	25 (43.9%)

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In cases of malignant intrathoracic lymphadenopathy, there were concordant results for tissue adequacy for molecular analysis between EBUS-TBNA and EBUS-MFB in 19 out of the 21 nodes. Table 3 provides details of the lymph nodes diagnosed with malignant disease by these procedures (N=21). The analysis showed that the tissue

adequacy of EBUS-TBNA was 19 out of 21 nodes (90.5%), which was comparable to tissue adequacy achieved with EBUS-MFB added on EBUS-TBNA, where 20 out of 21 nodes (95.2%) were considered adequate (p-value=0.317). Furthermore, one node was initially classified as non-diagnosed based on EBUS-TBNA samples. However, with the use of EBUS-MFB, an adequate tissue sample was obtained, leading to the diagnosis of diffuse large B cell lymphoma (as shown in Figure 4A and 4B).

Table 3 Tissue adequacy of EBUS-TBNA and EBUS-MFB added on EBUS-TBNA

	EBUS-TBNA, n (%)	EBUS-MFB added on EBUS-TBNA, n (%)	p-value*
Tissue adequate#	19	20	0.317
Tissue inadequate	2	1	
Total (N=21)	19 (90.5%)	20 (95.2%)	

*p-value was calculated from McNemar test

#Tissue adequate required that the tissue samples meet both tumor cell count>100 cells and NCP>25%

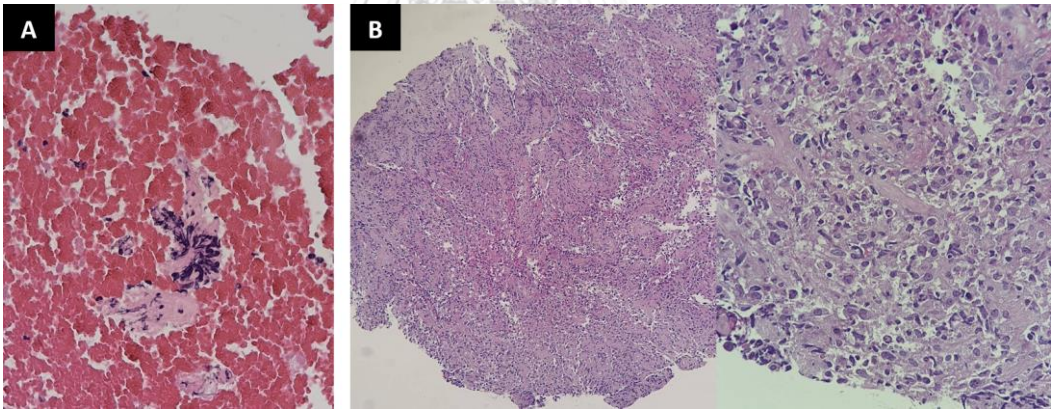

Figure 4 Multiple intrathoracic lymphadenopathies diagnosed as diffuse large B cell lymphoma.

(A) EBUS-TBNA showed tiny fragments of crushed probable lymphoid stroma (original mag. 400X). (B) EBUS-MFB revealed diffuse, highly cellular infiltration of malignant small round cells. The tumor cell count was >3,000 cells and the NCP estimation was 51-100% demonstrating an adequate tissue adequacy for molecular analysis. (left figure, original magnification 100X; right figure, original magnification 400X)

Table 4 Tumor cell results in malignant intrathoracic lymphadenopathy (N=21)

Tumor cell count of EBUS-MFB (cells)	Tumor cell count of EBUS-TBNA (cells)					p-value*
	1-100	101-500	501-1,000	1,001-3,000	>3,000	
1-100	0	0	1	0	0	0.073
101-500	1	0	1	2	1	
501-1,000	0	0	0	3	2	
1,001-3,000	0	0	0	3	3	
>,3000	1	0	0	0	3	
Neoplastic cell percentage of EBUS-MFB (%)	**Neoplastic cell percentage of EBUS-TBNA (%)**					
	1-10%	11-25%	26-50%	51-100%		
1-10%	0	1	1	0		0.834
11-25%	0	0	1	0		
26-50%	0	0	1	4		
51-100%	1	0	1	11		

*p-value was calculated from the marginal homogeneity test

The tumor cell count and the NCP from EBUS-TBNA and EBUS-MFB are shown in Table 4. The marginal homogeneity test showed the tumor cell count, and the NCP from EBUS-TBNA and EBUS-MFB were not significantly different (p=0.073 and p=0.834, respectively). The observed agreement between EBUS-TBNA and EBUS-MFB were 80.95% of the results and the kappa statistic was calculated as 0.236 (0.034-0.438). This indicated that there was fair agreement between the two techniques. When analyzing cases where the tumor cell count was greater than 100 cells or NCP of more than 25%, no statistical differences were found between EBUS-TBNA and EBUS-MFB (p=0.564) However, EBUS-TBNA resulted in higher tumor cell count; more than 1,000 cells were shown in 17/21 (80.9%) compared to EBUS-MFB in 10/21 (47.6%) (p=0.039) in table 5.

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Table 5 Tumor cell characteristics between EBUS-TBNA and EBUS-MFB (N=21)

	EBUS-TBNA	EBUS-MFB	p-value*
Tumor cell count, N(%)			0.564
1-100 cells	2	1	
>100 cells	18	20	
Tumor cell count, N(%)			0.039
1-1,000 cells	4	11	
>1,000 cells	17	10	
Neoplastic cell percentage, N(%)			0.564
<25%	2	3	
26-100%	19	18	

*p-value was calculated from McNemar test

Among the 57 lymph nodes that were obtained, adequate histology were obtained, and a diagnosis was determined from histological analyses in all cases except one case. In this particular case, the EBUS-TBNA procedure yielded an inadequate tissue biopsy , showing crushed lymphoid tissue, while the EBUS-MFB showed bronchial tissue. Follow-up CT scan were conducted on this case for one year, and no significant change in the mediastinal lymphadenopathy were observed, indicating a benign condition. The overall diagnostic yield of EBUS-TBNA was 50 out of 57 nodes (87.7%). Within the EBUS-TBNA group, initially, six nodes remained undiagnosed, but the diagnoses were subsequently determined using EBUS-MFB. The pathological results of these undiagnosed cases revealed anthracotic lymph nodes in three nodes, granulomatous disease in two nodes, and one silicotic node. On the other hand, the overall diagnostic yield of EBUS-MFB was 37 out of 57 nodes (65.9%). Within the EBUS-MFB group, 19 nodes were undiagnosed due to inadequate specimens. The reasons for inadequate specimens included 13 cases of no lymphoid stroma, three cases of bronchial tissue, two cases of fibroadipose tissue, and one case of uncertain cell composition.

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The addition of EBUS-MFB to EBUS-TBNA significantly improved the overall diagnostic yield compared to EBUS-TBNA alone (98.2% vs 87.7%; p=0.031). When considering non-malignant diseases, this combined approach had a diagnostic yield of 96.6%, while EBUS-TBNA alone had a yield of 76.6% (Table 7). Among the nonmalignant disease, 29 nodes (50.8%) were diagnosed using both procedures. The pathological results revealed eight anthracotic lymph nodes, three nodes with granulomatous disease, three silicotic lymph nodes, and ten nodes with reactive lymphadenopathy (Table 7). In three cases, necrotic material obtained from EBUS-TBNA was confirmed as tuberculosis. Two of these cases had a positive polymerase chain reaction for the *M. tuberculosis* complex. Another case was diagnosed with granulomatous disease by EBUS-MFB. One case of the EBUS-TBNA specimen displayed the presence of lymphoid stroma and fungal hyphae in the EBUS-TBNA specimen. However, EBUS-MFB did not show lymphoid stroma. Subsequently, the patient underwent a radial probe-EBUS biopsy, which revealed pulmonary aspergilloma. The interpretation of the presence of a fungal organism in a lymph node requires caution and careful consideration of the patient's clinical context. In this particular case, the patient was immunocompetent, with no evidence of invasive aspergillosis or necrotic nodes observed in imaging. Based on these findings, the index lymph node was classified as reactive lymphadenopathy and the presence of fungal hyphae in the lymph node was likely a result of colonization from the airways rather than invasive aspergillosis.

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The discordant diagnoses between EBUS-TBNA and EBUS-MFB for non-malignant diseases were observed in 19 of the 29 nodes (65.5%) (Table 8). Out of these, 13 nodes (44.8%) were diagnosed with EBUS-TBNA but remained undiagnosed by EBUS-MFB. The reasons for these discrepancies included histological findings of no lymphoid stroma in nine nodes, bronchial tissue in two nodes, fibroadipose tissue in one node, and uncertain cell composition in one node. Among these cases, six nodes

out of the total 29 nodes (20.7%) were misdiagnosed with EBUS-TBNA, but EBUS-MFB provided a valid diagnosis (Figure 5).

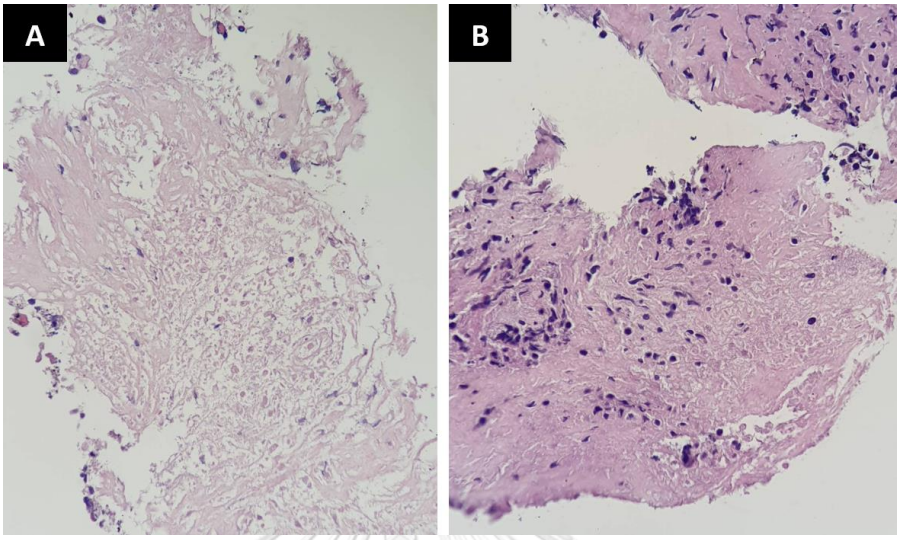

Figure 5 A sample node with discordance between the diagnosis of EBUS-TBNA and EBUS-MFB in mediastinal tuberculous lymphadenitis.
(A) EBUS-TBNA of a mediastinal lymph node. Histology showed entirely granular eosinophilic necrotic material. No granuloma is seen (original magnification 400X). (B) EBUS-MFB demonstrated an area of necrosis with vague aggregate of epithelioid histiocyte, suspected granulomatous inflammation is shown (original magnification 400X).

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The univariable analysis revealed that the patients with underlying malignancy and radiographic findings of a necrotic node had a higher likelihood of successful biopsy using of EBUS-MFB. The results of the multivariable logistic regression model adjusted for other factors, are presented in Table 6. The presence of underlying malignancy was found to be significantly associated with a greater success rate of EBUS-MFB with an odd ratio (OR) of 10.09, a 95% confidence interval (CI) ranging from 2.52 to 40.43 (p=0.001). Similarly, the presence of a necrotic node observed on CT imaging was associated with an increased likelihood of successful biopsy using EBUS-MFB with an OR of 14.57, and a 95% CI ranging form 1.55 to 137.19 (p=0.019).

Table 6 Diagnostic yield of EBUS-TBNA, EBUS-MFB, and Combined approach

	EBUS-TBNA, n (%)	EBUS-MFB, n (%)	Combined, n (%)	p-value*
Overall diagnostic yield (N=57)	50[a] (87.7)	37 (64.9)	56[b] (98.2)	0.031
Malignant disease (N=27)	27 (100)	21 (77.7)	27 (100)	-
Lung cancer				
- Adenocarcinoma	16 (59.3)	12 (44.4)	16 (59.3)	
- Poorly differentiated carcinoma	5 (18.5)	4 (14.8)	5 (18.5)	
- Small cell lung carcinoma	2 (7.4)	1 (3.7)	2 (7.4)	
Breast adenocarcinoma	2 (7.4)	2 (7.4)	2 (7.4)	
Lymphoma	2 (7.4)	2 (7.4)	2 (7.4)	
Nonmalignant disease (N=30)	23 (76.6)	16 (53.3)	29 (96.6)	0.031
Anthracotic lymph node	6 (20)	6 (20)	8 (26.7)	
Necrotic material[#]	3 (10)	0 (0)	3 (10)	
Granulomatous disease	2 (6.7)	3 (10)	4 (13.3)	
Silicotic lymph node	1 (3.3)	3 (10)	3 (10)	
Reactive lymphadenopathy	11 (36.6)	4 (13.3)	11 (36.6)	

SD: standard deviation; EBUS: endobronchial ultrasound; TBNA: transbronchial needle aspiration; MFB: miniforceps biopsy.

*EBUS-TBNA compared to EBUS-MFB added on EBUS-TBNA

[a]6 cases were excluded due to misdiagnosis in TBNA group: 3 anthracotic node, 2 granuloma, 1 silicotic node

[b]1 node was undiagnosed due to inadequate tissue biopsy in EBUS-TBNA showed crushed lymphoid tissue and EBUS-MFB showed bronchial tissue

[#]Necrotic material: 2 cases PCR for tuberculosis were positive, another case was diagnosed with granulomatous disease by EBUS-MFB

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The mean procedural time for the EBUS-TBNA and EBUS-MFB procedures was 37.7± 14.3 minutes. During the procedures, the following medications were administered: fentanyl with a mean dose of 86.1± 37 mcg, midazolam with a mean dose of 3.9±1.7 mg, and endobronchial lidocaine with a mean dos of 121.9±40.2 mg (Table 9). The overall complication rate was 3.5%. The only acute complication reported was minor bleeding during EBUS-MFB procedure, which was successfully controlled by endobronchial instillation of epinephrine. There were no severe complications observed during the procedure, such as pneumothorax, pneumomediastinum, respiratory failure, or hemoptysis.

Table 7 The discordant cases by TBNA and MFB diagnosis for nonmalignant disease

	Discordant cases	The diagnosis
TBNA diagnosed + MFB undiagnosed	13/29 (44.8%)	6 Lymphoid stroma 5 Anthracotic node 1 Granuloma 1 Necrotic tissue
TBNA undiagnosed + **MFB diagnosed**	6/29 (20.7%)	3 Anthracotic node 2 Granuloma 1 Silicotic node

Table 8 Logistic regression for the factors associated with successful biopsy of EBUS-MFB (N=37)

	Unadjusted OR (95% C.I.)	p-value	Adjusted OR (95% C.I.)	p-value
Male sex	0.90 (0.20-3.59)	0.868	-	-
Age >65 years	1.29 (0.38-4.44)	0.647	-	-
Underlying malignancy	7.39 (1.80-35.50)	0.001	10.09 (2.52-40.43)	0.001
LN size >20 mm from CT	2.46 (0.69-9.49)	0.121	-	-
Radiographic characteristics				
Necrosis	9.12 (1.12-409.91)	0.019	14.57 (1.55-137.19)	0.019
Calcification	1.09 (0.05-67.38)	0.948	-	-
Fatty hilum	1.09 (0.05-67.38)	0.948	-	-
Sonographic findings				
Round shape	2.20 (0.64-7.78)	0.160	-	-
Heterogenous echogenicity	1.33 (0.32-5.22)	0.642	-	-
Distinct margin	1.60 (0.47-5.54)	0.396	-	-
Absence of central hilar structure	0.45 (0.13-1.66)	0.170	-	-
Present of vascular structure	1.76 (0.51-6.43)	0.322	-	-
Lymph node station				
Subcarinal node	1.07 (0.31-3.67)	0.899	-	-
Right lower paratracheal node	1.29 (0.30-6.63)	0.710	-	-
Right interlobar node	1.74 (0.27-19.26)	0.519	-	-
Left interlobar node	0.16 (0.01-2.20)	0.083	-	-

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Table 9 Procedure details and complications

	Number (%)
Mean operation time(minutes) ± SD	37.7 ± 14.3
Dose of sedative drugs, mean (SD)	
Fentanyl (mcg)	86.1 ± 37.0
Midazolam (mg)	3.9 ± 1.7
Endobronchial lidocaine (mg)	121.9 ± 40.2
Acute complications	
Pneumothorax or pneumomediastinum	0
Respiratory failure	0
Major bleeding	0
Minor bleeding	2 (3.5)[#]
Delayed symptoms within 48-96 hours	
Blood-stained sputum	4 (7)*
Chest pain	2 (3.5)
Emergency visit	0

[#]Minor bleeding was controlled by endobronchial instillation of epinephrine

*Delayed symptoms resolved spontaneously within 96 hours with conservative treatment

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CHAPTER 5
DISCUSSION AND CONCLUSION

5.1 Discussion

EBUS-TBNA is a valuable technique for evaluating enlarged intrathoracic lymph nodes. However, its limited sample volume poses a challenge for molecular analysis in cases of malignant diseases. Molecular mutation profiling plays a crucial role in guiding treatment decisions for patients with advanced lung cancer. Currently the assessment of tissue adequacy for molecular testing using EBUS-MFB is insufficient. Ensuring tissue adequacy is essential for reliable molecular analysis, as it requires an adequate quality of tumor cells and NCP. This is the first study to examine tissue adequacy for molecular analysis using tissue pathology. We present the findings on tissue adequacy of EBUS-MFB added on EBUS-TBNA, according to defined criteria, to assess the suitability of the tissue sample before conducting molecular analysis. Our results supported the notion that EBUS-TBNA can provide an adequate specimen that is more suitable for further molecular analysis.

In current clinical practice, routine mutation analysis performed for specific mutations such as EGFR, ALK, ROS1, KRAS, and RET (24-26). A previous meta-analysis study has shown that EBUS-TBNA provides high diagnostic adequacy for EGFR and ALK mutations analysis, with pooled probabilities of obtaining sufficient samples being 94.5% and 94.9%, respectively (17). However, these studies did not specifically address tissue adequacy in terms of tumor cell count and NCP. Different molecular analysis tests have varying minimum tissue adequacy criteria. For example, pyrosequencing requires at least 10% of NCP, direct sequencing requires 25% of NCP, while some reverse transcription Real-Time polymerase chain reaction (PCR) tests are applicable only to tumor specimens with an NCP of at least 30% (15). In our study, we defined tissue adequacy as having a tumor cell count greater than 100 cells and more than 25% of NCP to evaluate the suitability of the tissue sample before conducting molecular analysis. To ensure accurate interpretation of the test results and minimize

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the risk of tumor cell under- and overestimation, the pathologist followed recommendations from a modified Delphi study to improve NCP estimates (18). The pathologist performed a re-evaluation of the total tissue samples in a blinded manner, and discrepancies were found in only 3 out of 21 cases (14.3%). When comparing EBUS-MFB added on EBUS-TBNA to EBUS-TBNA alone, our results showed no significant difference in tissue adequacy (90.5% and 95.2%, p=0.317). This indicates that tissue samples obtained from both techniques are acceptable for various molecular techniques. Tumor cell count is also crucial for molecular mutation analysis, but the specific requirements of EBUS-TBNA samples for NGS or whole-genome sequencing (WGS) are uncertain. It is estimated that approximately 167 cells of normal human (diploid)cells are needed to obtain 1 nanogram(ng) of DNA (27). Therefore, the use of NGS requires a larger amount of tumor tissue, exceeding the 50 ng of DNA obtained from FFPE samples. It is estimated that approximately 8,000 cells are needed to test multiple mutations (28). We performed a subgroup analysis based on tumor cell count, which revealed that EBUS-TBNA collects a significantly larger number of tumor cells (>1,000 cells) compared to EBUS-MFB (p=0.039). We believe that the use of repetitive agitations per pass of EBUS-TBNA can enhance the volume of tumor cell collection. As a result, EBUS-TBNA samples may be more suitable for molecular analysis, particularly for molecular techniques that require a high amount of tumor DNA.

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EBUS-TBNA is commonly used to sample intrathoracic lymph nodes, but its value can be limited due to the small size of the obtained specimen. For certain disorders, such as granulomatous and lymphoproliferative disorders, examining tissue architecture is crucial for accurate diagnosis. Previous studies have evaluated the use of EBUS-MFB added on EBUS-TBNA and have shown a higher diagnostic yield compared to EBUS-TBNA alone (3, 5, 7, 19, 21). For example, Chrissian et al. (3) conducted a study with 50 patients with mediastinal or hilar lymphadenopathy who underwent EBUS-TBNA and EBUS-MFB of 74 lymph node stations. The overall diagnostic yield of EBUS-MFB added on EBUS-TBNA was 97% (p<0.001) compared to EBUS-TBNA and EBUS-MFB

which were 81% and 91%, respectively. In another retrospective cohort study by Wang et al. (20), 227 patients who underwent concurrent EBUS-TBNA and EBUS-MFB were studied. The diagnostic yields of EBUS-TBNA cytology and EBUS-MFB were not significantly different (95% and 94%), but there were discordant diagnoses between EBUS-TBNA and EBUS-MFB in 19 of the 227 cases (8.37%). In summary, a pooled overall diagnostic yield of 67% for EBUS-TBNA and 92% for EBUS-TBNA combined with EBUS-MFB has been reported (21). EBUS-MFB added on EBUS-TBNA is superior to EBUS-TBNA alone and our results are consistent with previous studies. The addition of EBUS-MFB to EBUS-TBNA significantly increased the overall diagnostic yield from 87.7% to 98.2% (p=0.031). Utilizing EBUS-MFB added on EBUS-TBNA provides a more reliable and valid diagnosis. Furthermore, the diagnostic yield of EBUS-MFB is influenced by various factors, including the type and size of forceps, lymph node size, specimen handling technique, and cytopathology practices. In our study, we specifically examined factors that contributing to a higher success rate in biopsy procedures, aiming to improve the overall diagnostic yield. We observed that the patients with necrotic lymph nodes identified on imaging or those with underlying malignancy had a higher likelihood of a successful miniforceps biopsy. These findings suggest that these factors can serve as potential predictors for selecting patients who would benefit from EBUS-MFB in addition to EBUS-TBNA.

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According to the findings from a systematic review and meta-analysis (21), the overall incidence of complications in patients undergoing both EBUS-TBNA and EBUS-MFB was approximately 3-4%. These complications included pneumothorax or pneumomediastinum (2%), bleeding (0.8%), and respiratory failure (0.6%). It is important to note that the complication rates were higher compared to EBUS-TBNA alone. However, when compared to other interventions, EBUS-guided lymph node sampling was considered a less invasive procedure with lower morbidity than mediastinoscopy (22). In our study, we observed a relatively low incidence of complications, with only minor bleeding reported. No cases of pneumothorax or

pneumomediastinum were identified on the follow-up chest x-ray conducted at 1 hour after the procedure. Additionally, delayed symptoms such as chest pain and blood-stained sputum occurred in only 10% and resolved spontaneously within 96 hours with conservative treatment. Based on these findings, it can be concluded that EBUS-MFB is safe procedure and a low complication rate.

Our study has several strengths that contribute to its validity. Firstly, it was conducted in a single tertiary care center, ensuring consistency in the procedures performed by interventional bronchoscopists and minimizing the influence of operator variability. Secondly, we used the same size of aspiration needle and type of miniforceps, reducing device-related variability. Thirdly, we performed the same number of needle passes for both EBUS-TBNA and EBUS-MFB. Lastly, the pathologic results were carefully validated in all cases, and we observed a low rate of discrepancies, indicating a high level of reliability. These strengths enhance the relevance and robustness of our results, which are consistent with previous studies demonstrating that EBUS-MFB added on EBUS-TBNA can significantly improve the overall diagnostic yield.

5.2 Conclusion

To the best of our knowledge, this is the first report that evaluates tissue adequacy for molecular analysis based on the histological tumor cell characteristics of EBUS-TBNA and EBUS-MFB added on EBUS-TBNA. Our results provide support for the hypothesis that EBUS-TBNA can provide an adequate specimen and is more suitable for further molecular analysis.

5.3 Limitation

1) The small number of patient in our study limited the statistical power. Factors such as the COVID-19 pandemic, technical challenges of the EBUS-MFB procedure, and unsuccessful pathological results in the miniforceps biopsy constributed to the limited sample size. Therefore, the advantage of EBUS-MFB added EBUS-TBNA in terms of tissue adequacy for molecular analysis remains unclear.

2) The EBUS-MFB procedure requires precise technical skills, and we encountered multiple cases where the procedure was unsuccessful. The type and size of the miniforceps used in our clinical practice differed from those used in other studies. Challenges such as accurate localization of the target lymph node and poor visualization of the miniforceps within the lesion posed obstacles during the procedure. Additionally, performing EBUS-MFB after EBUS-TBNA may lead to aspiration artifacts and a decrease in the amount of tissue sample obtained by EBUS-MFB.

3) The diagnostic yield observed in our study may differ from that reported in other studies due to variations in patient population and disease prevalence. We did not perform surgical evaluation such as thoracotomy or video-assisted thoracic surgery (VATS) to confirm the diagnosis, which could have influenced the diagnostic outcomes of our study. Additionally, the small sample size of malignant diseases prevented us from drawing definite conclusions regarding the diagnostic yield of each specific malignancy.

5.4 Recommendation

In malignant diseases, EBUS-TBNA alone provided a higher tumor cell count in specimens, making them more suitable for molecular analysis. On the other hand, EBUS-MFB added on EBUS-TBNA demonstrated to be a valuable and safe procedure for achieving a more accurate diagnosis, especially in nonmalignant diseases. These findings have practical implications and can be implemented in clinical practice. Ti illustrate this, we have included a proposed algorithm (Figure 6) that outlines the suggested approach based on the diagnostic yield and suitability of the techniques.

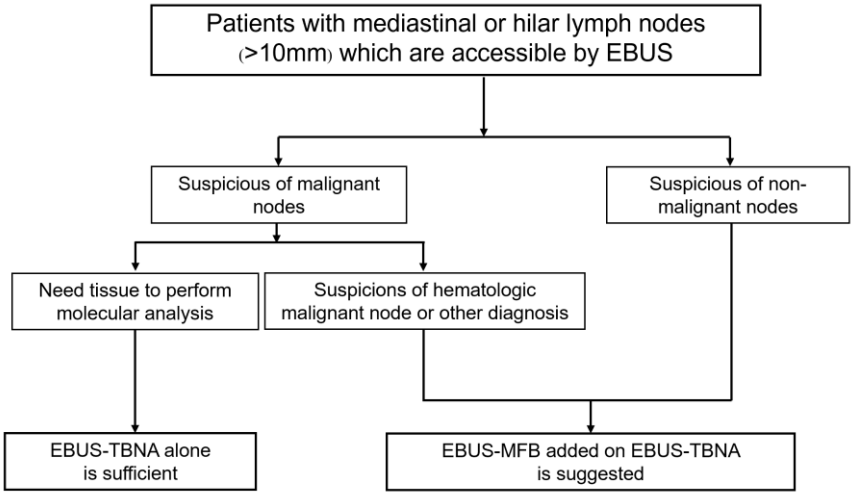

Figure 6 Proposed schematic diagram for clinical application in patients with intrathoracic lymphadenopathy

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