PROTOCOL

TITLE:	Transbronchial needle aspiration with cryobiopsy in the diagnosis of mediastinal disease: a randomised trial
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Confidentiality Statement

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1. Background

Mediastinal and hilar lymphadenopathy can be manifested in both malignant and benign disorders. Accurate timely diagnosis of mediastinal disease is essential for choosing appropriate treatment and for predicting prognosis, which usually requires sufficient samples qualified for pathological and molecular assessment. Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is a minimally invasive and safe technique that is widely employed for the sampling of mediastinal lesions[1]. Clinical guidelines recommended that EBUS-TBNA is a preferred and initial modality for the invasive staging of non-small cell lung cancer (NSCLC)[2, 3]. However, the relatively limited amount of intact tissue obtained might restrict its diagnostic yield in mediastinal diseases of other etiologies[4, 5].

We recently demonstrated that transbronchial mediastinal cryobiopsy provides larger amounts of intact tissue and improves the overall diagnostic yield as compared to EBUS-TBNA[6, 7]. The excellent performance of this approach, particularly in non-lung cancer diseases, suggests a potentially additive value of mediastinal cryobiopsy to the standard sampling strategy[7].

2. Objective

We aim to prospectively evaluate the diagnostic accuracy and the safety of adding transbronchial mediastinal cryobiopsy to standard sampling in mediastinal diseases.

3. Trial design and procedure

This is a prospective, mutlicentre, randomised, comparative, diagnostic accuracy study, which is designed to evaluate diagnostic value and safety of combined application of EBUS-TBNA and mediastinal cryobiopsy. The study is designed and reported following the Consolidated Standards for Reporting Trials (CONSORT).

3.1 Trial Participants

Consecutive individuals who need bronchoscopic diagnosis based on suspicion of either benign or malignant disease in mediastinal or hilar lymph nodes or masses, and who have completed preoperative examinations, are prospectively enrolled by the team member from each of the participating centres according to the inclusion criteria. Those individuals not suitable for bronchoscopy or mediastinal biopsy or who meet the exclusive criteria are excluded from study. The details of the inclusive and exclusive criteria are presented below.

3.1.1 Inclusive criteria

Aged ≥ 15 years old;

Patients with at least one mediastinal lesion with a short-axis ≥ 1 cm that is detected by thoracic image;

Patients with recently discovered mediastinal lesions, clinical respiratory symptoms of cough, expectoration, thoracalgia, apnea, or complicated lung lesions implicated by thoracic image, which indicates a need for biopsy to identify the underlying etiology;

Patients should have undergone necessary preoperative laboratory examinations and other examinations such as cardiac ultrasound or CTA when necessary, in order to exclude potential contradictions;

Patient is willing and able to give informed consent for participation in the study.

3.1.2 Exclusive criteria

Patients with contradictions to endoscopic examination, such as severe cardiopulmonary diseases, coagulation disorders, intolerance to anesthesia or endoscopic operation, psychiatric disorders, or severe neurosis, and so on;

The EBUS procedure fails to detect the mediastinal lesion;

The mediastinal lesions are actually cysts or abscess;

Patients need for additional procedures other than EBUS examination (such as endobronchial biopsy);

Patients could not provide full informed consent;

Patients have been previously randomised to an arm of the present trial or involved in other clinical trials in the recent 3 months;

Patients have any other conditions that are considered to be inappropriate to be involved in this study.

3.2 Sample size

Sample size of this study is calculated by Power Analysis version 11.0 and Sample Size version 11.0 software using two independent proportions analysis.

Two Independent Proportions (Null Case) Power Analysis

Numeric Results of Tests Based on the Ratio: P1 / P2
H0: P1/P2=1. H1: P1/P2=R1<>1. Test Statistic: Z test with pooled variance

Power	Sample Size Grp 1 N1	Sample Size Grp 2 N2	Prop|H1 Grp 1 or Trtmnt P1	Prop Grp 2 or Control P2	Ratio if H0 R0	Ratio if H1 R1	Target Alpha	Actual Alpha	Beta
0.9015	130	130	0.9348	0.7990	1.000	1.170	0.0500		0.0985

Note: exact results based on the binomial were only calculated when both N1 and N2 were less than 100.

Sample size estimation is conducted according to the findings of our prior transbronchial mediastinal biopsy report indicating the similarly low operational risks of EBUS-TBNA and cryobiopsy (no serious adverse events have been observed in both groups), and thus the number of the required cases is calculated mainly on the basis of potential diagnosis benefit[7]. Assuming that the increase in the overall diagnostic yield by the combined approach is 13.5% (a threshold of 79.9%), α=0.05, β=0.1, power is 0.9, N is calculated to be 130 for each group. The result report is presented above.

3.3 Randomisation and blinding

Patients will be randomised using a computer-generated blocked randomisation scheme (block size four based on a random table from an independent statistician who has no further

involvement in the actual clinical study). The allocation is stratified at the centre level. Once study participants are deemed eligible, a member of the trial team will randomise the participant. The random allocation sequence will not be disclosed to patients and consenting investigators until the interventions are assigned. Because of the nature of the intervention, neither patients nor study personnel could be masked to group assignment. The radiologists and the pathologists are unaware of the patient recruitment for a clinical trial. The paper-based case forms are applied to record study data, and important information such as patient-related information and the diagnostic results, are also recorded in an electronic system. The data are further collected by an independent research assistant. Data monitoring is conducted at each of the centres on the basis of the the local ethic committee recommendation.

3.4 Patient withdrawal

All patients reserve the right to withdraw from the study at any time. For those who lack appropriate decision-making ability, the expression of dissent in any form will be taken as an indication that they do not wish to be included and these individuals are then withdrawn.

4. Trial interventions

All the procedures are performed by the same experienced bronchoscopist from each centre.

4.1 Sedation

Patients are in supine position. The upper airway topical anesthesia is achieved by 2% lidocaine, and conscious sedation is achieved by intravenous injection of midazolam and fentanyl. Oxygen is initially administrated at 1-2 L/min and increased when oxygen saturation is lower than 90%. Patients' vital signs and pulse blood oxygen saturation are continuously monitored.

4.2 EBUS procedure

In our procedure, rigid bronchoscopy is not used. An EBUS bronchoscope (BF-UC260F-OL8 or BF-UC260F, Olympus, Tokyo, Japan) is inserted into the trachea through the nose (if the nasal airway is too narrow for the EBUS bronchoscope to pass through or there are other contradictions, the oral way can be the alternative option). As the EBUS bronchoscope has been inserted in, the glottis, the trachea, the bronchuses are probed in turn. After the examination of the airway, the EBUS bronchoscope is then contacted with the airway wall, and a systematic examination of all mediastinal and hilar lymph node stations is sequentially performed according to the Mountain-Dressler lymph node map. For each lesion, the blood supply is identified by the Doppler Ultrasound and the size is measured by its long axis and short axis.The most suspicious target (lesion with disease characteristics implicated by imaging data [such as significantly enlarged size, contrast enchantment on CT imaging, high FDG uptake on PET/CT imaging, et al.]) with relatively less biopsy risk is chosen for biopsy sampling. The ultrasonic detection duration is defined as the time interval from the insertion of the bronchoscope into the nose or mouth to the moment when the mediastinal lesion is detected.

After the target is localized, EBUS-TBNA (step 4.3) with or without transbronchial mediastinal cryobiopsy (step 4.4) is performed according to the randomised group. To avoid the risk of severe bleeding, the vascularization status of the lesion is assessed by Doppler-mode blood flow imaging as previously described by Nakakima and coworkers[8, 9]. Lesions are classified as follows: grade 0: no blood flow or small amounts of flow; grade I: a few main vessels running toward the center of the lesion; grade II: a few punctiform or rod-shaped flow signals, a few small vessels found as a long strip of a curve; and grade III: rich flow, more than four vessels found with different diameters and a twist or helical-flow signal. If the lesion is too abundant in blood supply (Grade III), too close to large vessels, or other conditions detected by EBUS that indicates high risk of biopsy, another appropriate lesion would be considered for sampling.

4.3 EBUS-TBNA

4.3.1 As the target lesion has been visualized by ultrasound, a dedicated TBNA needle (21-gauge or 22-gauge, NA-201SX-4021 or NA-201SX-4022, Olympus, Tokyo, Japan, which are the standard biopsy instruments for EBUS-TBNA as recommended by the current guideline) is passed through the working channel of the EBUS bronchoscope, and advanced through the tracheobronchial wall into the lesion under real-time ultrasound visualization, avoiding areas with abundant blood supply or massive necrosis[10]. After the central stylet has been removed, the suction is applied using a syringe while the needle is manipulated back and forth for 25 times within the lesion. The specimen collected in the lumen of the needle is pushed out using the central stylet and then blown by air with a syringe onto a glass slide. The specimen on the glass slide is smeared and fixed in 95% alcohol for cytologic examination. The visible tissue fragment on the glass slide and the residual specimen in the lumen of the needle are collected and transferred into the containers filled with formalin for cell block analysis. Both cell block analyses and glass slide-based cytology analyses are performed.

4.3.2 Repeat 4.3.1 for 4 times. The operation duration for TBNA is recorded from the insertion of the needle into the bronchoscope to its final exit from the working channel. Patients assigned to the combined group will receive one-time mediastinal cryobiopsy after the completion of EBUS-TBNA, which is specified as below.

4.4 Transbronchial mediastinal cryobiopsy after EBUS-TBNA

4.4.1 Opening of a window on the airway wall

The target lesion is supervised by ultrasound, and a high-frequency electric needle knife (Olympus KD-31C-1, Olympus, Tokyo, Japan) is inserted through the working channel of the EBUS bronchoscope, and an incision is then made (about 2 - 3 mm) on the airway wall adjacent to the target lesions. The knife is then advanced into the lesion under real-time ultrasound visualization, avoiding areas with abundant blood flow or massive necrosis. After

being confirmed within the lesion and the measurement of its insertion depth, the needle knife is then withdrawn. The time from the insertion of the needle knife into the bronchoscope to its removal from the work channel is recorded as the window-opening duration.

4.4.2 Cryobiopsy

An Erbe frozen probe (Erbe 20402-401, 1.1 mm, Erbe, Tübingen, Germany) is then passed through the working channel of the EBUS bronchoscope, and advanced through the window on the airway wall into the lesion under real-time ultrasound visualization, avoiding areas with abundant blood flow or massive necrosis. After measuring the insertion depth of the cryoprobe, the probe is activated to cool down with nitrous oxide for around 7 seconds. Then the bronchoscopist gently extracts the cryoprobe tip with the rapidly frozen biopsy specimen attached to it, and then withdraws the bronchoscope and the cryoprobe. The frozen specimen is released from the cryoprobe by thawing in saline and it is then fixed in formalin.

4.4.3 One-time cryobiopsy is performed. Cryobiopsy time is recorded from the insertion of the cryoprobe into the bronchoscope to its final exit from the working channel. The size of each specimen is measured and recorded by its long axis and area.

4.5 Postbiopsy

Hemorrhage within the airway is assessed through the bronchoscope, especially in the biopsy site. The procoagulants are locally used when necessary. Record all the procedure-associated adverse events during the procedure, including bleeding, severe hypoxemia, cardiac arrhythmia, hypotension, etc. The overall procedure duration is measured from the insertion of bronchoscope to its removal from the patient’s nose or mouth.

4.6 Procedure interruption

The EBUS procedure will be interrupted accordingly in the following situations:

1) Patients are intolerant to the bronchoscopic procedure;

2) The ultrasound fails to detect the lesion within 20 minutes;

3) The blood supply is so abundant in the lesion that is considered inappropriate to continue biopsy due to the high risk;

4) The ultrasound identifies that the lesion is a cyst or abscess;

5) Severe adverse events occur during operation, such as severe bleeding. Bleeding is classified as follows: grade 0, traces of blood not requiring suctioning; grade 1, bleeding only requiring suctioning and hemostatic wedging for up to 2 minutes (two 1-minute cycles); grade 2, bleeding requiring hemostatic wedging for 3 minutes or more; grade 3, bleeding requiring topical instillation of epinephrine or ice cold saline; and grade 4, bleeding requiring hemodynamic support, transfusion of blood products, selective mainstem intubation, bronchial blocker, hospital admission, or surgical intervention[11, 12].

4.7 Histological assessment

All the specimens obtained from patients are sent to the Department of Pathology and appropriately processed. The pathologic evaluation is made by the pathologists at each of the centres, who are unaware of the patient recruitment for a clinical trial. In our study, the pathological diagnosis is performed following the local workflow. All specimens are examined by two pathologists.

4.8 Patient follow-up

Immediately after the operation, the patients are inquired about whether there are any discomforts, and it is recorded if there are any. Chest X-ray or other imaging examinations are performed within 24 hours to detect whether there is pneumothorax, pneumomediastinum, or mediastinitis. Twenty-four hours after operation, a follow-up is conducted and the symptoms are recorded, including fever, coughing, hemoptysis, chest pain, dyspnea, and so on. Perioperative severe adverse events include moderate-to-severe bleeding (grade 3 or 4), oversedation requiring ventilatory support or sedation reversal, pneumothorax with persistent air leakage (> 5 days), unplanned hospital readmission, and death. ICU transfer within 48

hours after the procedure is also considered a severe adverse event. One month later, a second follow-up is conducted, and examinations such as X-ray or CT scan would be arranged according to patient symptom. The complications relative to the operation are recorded. Patients with an indefinite diagnosis (especially for benign disorders) are followed up continuously or receive surgical procedures (such as mediastinoscopy or surgical lymph node dissection), and the final diagnosis should be revised at any time if more reliable evidence supports new diagnosis. Six months later, imaging reexaminations are required for patients with an indefinite diagnosis (especially for benign disorders).

5. Study endpoints

The co-primary outcomes are the diagnostic yield and safety of the EBUS procedure. The diagnostic yield of the EBUS procedure is defined as the percentage of patients for whom the EBUS procedures provided a definite diagnosis. Suspicious findings from the biopsy procedures are regarded as negative cases. Safety is evaluated with regard to the prevalence of the procedure-related adverse events.

Secondary end-points include the diagnostic yield of transbronchial mediastinal cryobiopsy, specimen adequacy and size, suitability of samples for molecular genetic assay, and duration of the bronchoscopic procedure.

6. Data analysis

The full analysis set, including all the patients who do not violate the inclusion criteria and have undergone the biopsy, is used for demographic summaries and safety analyses. Inter-individual diagnostic analyses are performed based on the full analysis set, and the intra-individual diagnostic analyses include subjects receiving both EBUS-TBNA and mediastinal cryobiopsy, which are performed in concern of the potential intergroup differences[13]. Subgroup analyses are performed on disease etiology. Analyses of the

secondary outcomes are done in the combined biopsy population, except the analysis of procedural duration that is based on the full analysis set.

Statistical analyses are performed using IBM SPSS 21.0 software (IBM Corp., Armonk, NY, USA). Categorical variables are reported as counts and percentages, and continuous variables as means and standard deviation. Pearson's Chi-squared or Fisher's exact test is used to compare proportions, as appropriate. For continuous data, between-group comparisons are performed by Student´s t test or the Mann-Whitney U-test for parametric or non-parametric data, respectively. A $P<0.05$ is considered to denote statistical significance. Because of discussions within the research and statistical team about the need for more details on how analyses are to be carried out, amendments to the data analysis section of the protocol are made on Feb 8th 2022 before our analyses are started. The changes made are that more specified information on the populations for the primary and subgroup analyses are provided in this part.

7. Ethical considerations

The research group will ensure that this study is conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. All participants will be asked to provide informed consent.

8. Risks and benefits

Similar to the application of standard needle aspiration biopsy, bleeding may occur during the combined mediastinal sampling. In most situations, it requires no additional interventions. Other adverse events include pneumothorax, infection, and hematoma or emphysema in mediastinum; however, these occurrences are much less frequent[7]. The bronchoscopist will try the best to operate carefully to avoid the damage to blood vessels and other important tissues and organs. Patients will be reexamined by Chest X-ray or other imaging

examinations within 24 hours to detect whether there is pneumothorax, or emphysema, hematoma, or infection in mediastinum.

Compared with the routine application of EBUS-TBNA alone, the operative duration of the combined sampling may be prolonged by approximately 5 minutes (the duration required for cryobiopsy), which may increase the discomfort of the patients. During the procedure, conscious sedation, local anesthesia, and continuous vital signs and oxygen saturation monitoring will be used to minimize the discomfort and ensure the safety of the patients.

Compared with the traditional EBUS-TBNA, the operation scheme of the combined approach helps to obtain more intact tissues and improve diagnostic utilities. This lessened the need for more invasive examinations and surgical procedures.

9. Privacy and personal information protection

The research group hereby promises to protect the privacy and personal information of the subjects that participate in the study. The security measures for protecting the privacy and confidentiality of personal information include hiding information that can identify the subjects during data reports, restricting access to such information, data anonymity, and so on.

Legally, there are exceptions for researchers to protect the privacy and personal information of subjects when inspections are required by administrative authorities, or ethics committee, etc.

10. Funding

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