Peer Review File

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Responses to the comments of Reviewer A

Comment 1: Line 125 Patients who needed multi lymph node punctures for lymph node staging were ineligible. Can you expand on why you only looked at adequacy/diagnosis for pathological samples and not also adequacy for negative lymph node samples in staging, which is a vital function of EBUS?

Reply 1:
We included patients with hilar/mediastinal lymphadenopathy, which were obviously suspected as representing lymph node metastasis from primary lung cancer, but not patients who needed mediastinal staging. EBUS-TBNA for mediastinal staging is conducted for determining the best curative treatment strategy such as surgical resection or chemoradiotherapy. So that the main purpose of EBUS-TBNA for mediastinal staging is not completely match that of EBUS-TBNA for patients who were highly suspected of having lymph node metastasis from lung cancer, of which purpose is making a definitive diagnosis of lung cancer and making a biomarker analysis.
We performed this study to determine which of a 22G needle or a 25G needle could be recommended based on a higher diagnostic rate and higher yield of larger tissue samples that may be appropriate for biomarker analysis. Thus, we only included patients with hilar/mediastinal lymphadenopathy which were highly suspected with malignant metastasis.
As reviewer mentioned, it is also important that comparing the efficiency for mediastinal staging in lung cancer between the 22G needle and 25G needle. We think further prospective evaluation will be needed in a population subset which included patients who need mediastinal staging of lung cancer. To explain these points, we have revised the following sentence in Discussion as described below.

Changes in the text:

In the Discussion, we added: “First of all, in our study, we included patients with hilar/mediastinal lymphadenopathy, which were obviously suspected as representing lymph node metastasis from primary lung cancer, but not patients who needed mediastinal staging. EBUS-TBNA for mediastinal staging is conducted for determining the best curative treatment strategy such as surgical resection or chemoradiotherapy. So that the main purpose is different from EBUS-TBNA for patients who were highly suspected of having lymph node metastasis from lung cancer, of which purpose is making a definitive diagnosis of lung cancer and making a biomarker analysis. This is why we only included patients with hilar/mediastinal lymphadenopathy which were highly suspected with malignant metastasis. Therefore, we did not compare the efficiency for mediastinal staging in lung cancer between the 22G needle and 25G needle and further investigation is needed.” (Page 31, line 1-12)

Comment 2: Line 159: Was the trainee/expert doing the needling or scoping? How many operators do you have when you practice EBUS, is it single or two operators (one to scope and the other to needle)?

Reply 2: Only single operator was doing the scoping and needling when performed EBUS-TBNA in our practice procedure. As with the practical method, doing the scoping and needling was also performed by the same operator in this study. To clarify this point, we have added the following sentence in Materials and methods as described below.

Changes in the text:
In Materials and methods, we added the text as following: “The same operator inserts and holds the bronchoscope, and also handles the needle passes. Another person assists the procedure.” (Page 12, line 2-4)

Comment 3: Lines 170-185 do we really need to describe this in so much detail as this is all fairly standard practice with which the readers should be familiar, this can be condensed in my opinion.

Reply 3: The description in Lines 170-185 is a standard procedure of EBUS-TBNA and we also considered the readers may be familiar to EBUS-TBNA. We agree with the comment of reviewer, we have removed the following text in line 170 through 184.

Changes in the text:

In Materials and methods, we removed the text as follows: "The patient's heart rate, blood pressure, and SpO2 were monitored via electrocardiography, sphygmomanometry, and pulse oximetry. Oxygen supplementation was provided, as needed, to keep the SpO2 at ≥90%. All procedures were performed via the oral route and no tracheal tube was inserted. The convex probe transducer was placed at the tip of the CP-EBUS bronchoscope and CP-EBUS scanning was conducted parallel to the direction of insertion of the bronchoscope. The ultrasound images were generated using a dedicated ultrasound scanner (Olympus, EU-MF2). When the target lymph node was visualized by CP-EBUS, the allocated TBNA needle with a central stylet was inserted through the working channel of bronchoscope and advanced through the tracheobronchial wall into the target lymph node under real-time EBUS visualization. Then, after removing the stylet, the needle was moved back and forth inside the target lymph node 20-30 times with aspiration via negative suction applied with a 20 mL syringe. After sampling, the suction was released and the needle was removed from bronchoscope"

Comment 4: Line 215: I am not sure this paper referenced, which is centred around cryobiopsy. is the best to quote relating to bleeding categories.

Reply 4: There was no validated scale about bleeding severity after flexible bronchoscopy that was widely accepted. In one study, the CTCAE was used to assess bleeding complications (J Thorac Oncol. 2019;14:445-458). In another study, bleeding was graded with mild defined as need for continuous suctioning, moderate as need for wedging the bronchoscope into the affected airway, and severe as needing any additional intervention (Chest. 2006;129:

734-737). Recently a new bleeding scale was published, which categorized bleeding as grade1-4 (Chest. 2020;158:393-400). Any scales were generally based on the level of intervention needed to control bleeding.
So that we think it was important that bleeding severity was defined according to the clinical interventions required. In this point, we thought that bleeding severity that defined in the paper (Eur Respir J. 2012;39:685-90) referenced was appropriate. To clarify this point, we have revised the following sentence in Materials and methods.

Changes in the text:
In Materials and methods, we revised the text as following: "Bleeding events were evaluated for each puncture; the bleeding events were categorized into three grades (mild, moderate, or severe) defined according to the clinical interventions required, which was previously reported definition.(12)" (Page 13, line 6-8)

Comment 5: Line 229: This is unclear as it appears from the description that the patients have four separate bronchoscopy procedures but I assume what is meant is that they had up to four EBUS Samples taken in one sitting/procedure. Please clarify.

Reply 5: The patients were undergone at least four punctures and four EBUS samples were taken in one setting. To clarify this point, we have revised and added the following sentence in Materials and methods.

Changes in the text:
In Materials and methods, we revised and added the text as following:
"Patients were scheduled to undergo EBUS-TBNA punctures four times according to the study protocol, and our primary interest was in the diagnostic properties of the first two punctures. EBUS-TBNA after the fourth puncture was also allowed as an out-of-protocol procedure(s). "EBUS-TBNA puncture" is a distinct entry and exit of needle through the air way wall, and each EBUS-TBNA puncture includes 20 to 30 agitations of the needle within the target

lymph node. All EBUS-TBNA punctures were done without withdrawal of the bronchoscopy." (Page 15, line 6-13)

Comment 6: Line 313: The rate of 75% yield of malignant cells seems low with two passes, is there any explanation for this? I am confused with the 94% quoted as adequate for histopathological diagnosis.

Reply 6: The yield rate of malignant cells was 75% after the one punctureusing the 22G needle, and 75% after the one puncture using the 25G needle, respectively. Furthermore, the yield rate of adequate sample was 94% after the one puncture using the 22G needle, and 89% after the one puncture using the 25G needle, respectively. These results were shown in table2. A previous report (Chest. 2008;134:368-374) addressed the association of number of needle punctures in EBUS-TBNA and diagnostic accuracy. In that study, the sensitivity for differentiating malignant from benign lymph node stations was 69.8%, 83.7%, 95.3%, and 95.3% for one, two, three, and four punctures, respectively. The rate of sample adequacy was 90.1% after the first puncture, 98.1% after two punctures, and reached 100% after three punctures.
Therefore, we though our results were comparable to previous study.
According to reviewer's comment, we have revised changed the sentence in Results.

Changes in the text:
In the Results, we revise the text as following: "The yield rate of histology specimens containing malignant cells obtained using the 22G needle was 75% (76/102 patients) in the 1st puncture of arm A and 2nd puncture of arm B, and that in the specimens obtained with the 25G needle was also 75% (77/102 patients) in the 2nd puncture of arm A and 1st puncture of arm B" (Page 20, line 7-11)

Comment 7: Line 387: Di Felice et al in Journal of Thoracic Disease have done a retrospective comparison of 22 vs 25G needles in EBUS, this study has to be mentioned in the discussion in my opinion. In addition, Sakaguchi et al also published a small retrospective comparison of 22 vs 25G needles which could be referenced in discussion.

Reply 7: The study reported by Di Felice et al (J Thorac Dis. 2019;11:3643-9) was already mentioned in the discussion (Page 25, line 2-11). But we thought this study was so important that we have also add the following sentence in introduction. Furthermore, the study reported by Sakaguchi et al (Respir Investig. 2021;59:235-239) were thought to be mentioned in the discussion, we have added the following sentence in Discussion.

Changes in the text:
In the Introduction, we added: "but there are only studies that evaluate the efficiency of 25 G needle retrospectively (7,8)" (Page 7, line 1-2)

In the Discussion, we added: "Furthermore, a small retrospective study which compared 22G and 25G needles among 10 patients who underwent EBUS-TBNA using both 22G and 25G needles.(8) In that study, the diagnostic yield of EBUS-TBNA using the 22G needle was 80% and 25G needle was 60%, respectively. The diagnostic yields of 22G and 25G needle were also thought to be comparable in that study." (Page 26, line 4-8)

Comment 8: Line 497-503 May need to say in the conclusions about the 22G potentially providing better material for NGS and molecular analysis

Reply 8: We agree with the reviewer's comment about in this point, we have revised the following sentence in Conclusion.

Changes in the text:
In the Conclusion, we added: "As compared to the 25G needle, the 22G needle was slightly more advantageous, in that it could obtained larger tumor

tissue samples and specimens containing higher numbers of malignant cells that potentially better samples for NGS and molecular analysis." (Page 32-33, line 15-1)

Responses to the comments of Reviewer B

This is a well-organized prospective randomized study to evaluate the diagnostic performance of EBUS-TBNA between 22G and 25G needle. Although the author reported a valuable and important result about selecting needle size during EBUS procedure, it would be good to improve a few points.

Comment 1: The authors reported that more tissue and cancer cells could be obtained when using the 22G needle than the 25G needle. In the current treatment of lung cancer, performing molecular testing including EGFR, ALK, PD-L1, and NGS panel is very important for treatment decisions. Therefore, the authors need to show whether there were differences in performing these molecular tests using tissues obtained through the 22G-and the 25G needle.

Reply 1: We regret that we are unable to provide the information requested by the Reviewer. We could not compare the success rate of molecular test including NGS in the same patients between 22G and 25G needle in our study, because the molecular tests were performed in clinical practice. The pathologist selected the specimens that were thought to be most suitable for NGS analysis, and NGS was performed in clinical practice. Among the patients who included in this study, there were 5 cases in which NGS was performed on specimens obtained within the protocol. Success rate of NGS was 100% (3/3) on the specimens obtained with 22G needle, and 50% (1/2) on the specimens obtained with 25G needle, but this was very small cohort and we could not interpret definitively. Some previous studies that evaluated that sample size, number of malignant cells in biopsy samples and the success rate of NGS, larger sample sizes and higher numbers of malignant cells in the samples were associated with higher success rates of NGS (Thorac Cancer. 2020;12:194-200, Cancer Cytopathol. 2014;122:822-32, Int J Clin Exp Pathol. 2018;11:3647-55) Thus, we had thought that the 22G needle

that yielded larger sizes of tissue samples and higher may be associated with an improved success rate of NGS.

On the other hands, as reviewer mentioned, the optimal needle size for the analysis of biomarker including NGS is a very important issue, we think further prospective study will be needed that evaluate which needle size is most suitable for biomarker analysis in the patients of lung cancer. According to reviewer's comment, we have added the following sentence in Discussion.

Change in the text:

In the Discussion, we added: "Fourth, it was considered that large sample size and high number of malignant cells are associated with the high success rate of NGS (25), in that point, we thought 22G needle was more suitable for NGS than 25G needle. But we did not directly compare the success rate of molecular test including NGS between the 22G and 25G needle. Therefore, to evaluate the optimal needle size for the analysis of biomarker including NGS, we think further prospective study will be needed." (Page 32, line 4-10)

Comment 2: (Page 14, line 441-4446) There was no difference of diagnostic accuracy between the experts and the trainee operator in the biopsy result using the 25G needle. However, it was questionable whether this result could be explained simply by the improved handleability and increased flexibility of 25G needle. Considering the results of table 4, the diagnostic yield of the 25G needle in the expert (76%) and trainee (75%) was very similar to that of the 22G needle in trainee (75%). Therefore, it seemed appropriate to interpret these results not as a different characteristics of needles, but because even experienced people do not have experience with 25G new needle. Please give the authors' opinions on this issue.

Reply 2: The diagnostic yield of 22G was significantly higher when performed by expert as compared trainee (82% vs. 75%, respectively), but that of 25G was not different between expert and trainee (76% vs. 75%). We thought the discrepancy was due to a different characteristics of needles. A 25G needle

improved handleability and increased flexibility, and the use of 25G needle may be easier for trainee operators.
On the other hands, as reviewer mentioned, 22G needles had been usually used in clinical practice use until 25G new needle became available. So that the experience in using 25G needle was less than that of 22G needle in expert operators. Therefore, another possible reason for the discrepancy, expert operators were not familiar with use of 25G needle as well as trainee operators, so that there was no difference of diagnostic yield with specimens obtained 25G needle between expert and trainee operators.
We have added the following sentence in Discussion.

Change in the text:
In the Discussion, we added: "On the other hands, 22G needles had been usually used in clinical practice until 25G needle became available. Therefore, expert operators had a little experience of using 25G needle, and it was possible that expert operators were not familiar with use of 25G needle as well as trainee operators. This was another possible cause that there was no difference of diagnostic accuracy between expert operators and trainee operators when using 25G needle." (Page 28, line 12-17)

Comment 3: The authors described only the criteria for inclusion and randomization. Please clarify the exclusion criteria for this randomized cross-over study.

Reply 3: To clarify the exclusion criteria for this randomized cross-over study, we have added the following sentence in Materials and methods.

Change in the text:
In the Materials and methods, we added: "Patients were ineligible if they had hypersensitivity to lidocaine, midazolam, flumazenil, and pethidine hydrochloride or serious hypersensitivity to other drugs, or had current treatment with antiplatelet agents or anticoagulants, serious complications or

comorbidities (heart disease, interstitial pneumonia, poorly controlled hypertension, among others).” (Page 9, line 13-17)

Comment 4: Was there a difference between LN diagnosed only at 22G (not 25G) and LN diagnosed only at 25G (not 22G)? Please provide the authors' opinions on the reasons for these discrepancies.

Reply 4: In the results of the 1st to 4th punctures, 4 (4%) lymph nodes were diagnosed as having malignant cells only at 22G but not 25G, 6 (6%) lymph nodes were diagnosed as having malignant cells only at 25G but not 22G. There was no statistically difference in the age, sex, smoking history, ECOG PS, distribution of the lesion location (#4L or not #4L), or lesion size between these lymph nodes. Because of small number of lymph nodes which were diagnosed only 22G or 25G needle, we could not identify the reasons for those discrepancies. To clarify this point, we have revised the following sentence in Results.

Change in the text:
In the Results, we added: “There were 4 patients whose specimens containing malignant cells only obtained by 22G needle, and 6 patients only obtained by 22G needle. There was no statistically difference in the age, sex, smoking history, ECOG PS, distribution of the lesion location (#4L or not#4L), or lesion size between those patients.” (Page 21, line 2-6)

Responses to the comments of Reviewer C
Comment 1: Overall, the paper is very well designed. Considering the limited data available which comparing yields of 22 vs 25-gauge needles for EBUS, this paper would definitely contribute to the current literature. Prior studies are largely retrospective which also adds to the strength of this paper.
As the authors stated, a major limitation of the paper is that the patient population was already highly suspected of having malignant metastasis. Of interest would be to have a similar method of study to evaluate differences in

diagnostic yield in a population subset which included disease such as sarcoidosis etc. making this study more robust.

Reply 1: As the reviewer mentioned, we included only patients who were highly suspected of having malignant metastasis in our study. Therefore, we did not compare the diagnostic accuracy for benign disease such as sarcoidosis.

This study was designed as a prospective trial and the pre-defined accumulation of cases had already been completed. Therefore, it is difficult to add new patient who is suspected to have benign lymphadenopathy such as sarcoidosis. We think similar designed study among patients with benign disease need to be considered. According to reviewer's comment, we have added the following sentence in Discussion.

Change in the text:

In the Discussion, we added: "Furthermore, as the same reason of our patient inclusion criteria, the diagnostic accuracy for benign diseases, such as sarcoidosis and tuberculous lymphadenitis, between the 22G needle and 25G needle are unclear. Therefore, we think further prospective evaluation in a population subset which included benign diseases such as sarcoidosis will be needed." (Page 31, line 13-17)

Responses to the comments of the Editorial Comments

Comment 1. Please follow the attached "Submission Checklist for Authors" and revise your paper if needed. Here are some additional points:

Comment 1a. The article already followed a Checklist for reporting standards. Please place "Y" in the "Submission Checklist".

Response: This has been done as requested.

Comment 1b. “Data Sharing Statement” is a statement made by authors to confirm their willingness of sharing raw data/patient information related to the article with others. We attached a template for your reference.

Response: The Data Sharing Statement is included in our manuscript file, and the form was provided with our original submission.

Comment 1c. Conflict of Interest (COI) Form must be provided, as suggested by ICMJE: (http://www.icmje.org/conflicts-of-interest/). Each author should submit a separate form and is responsible for the accuracy and completeness of the submitted information. COI form download link: https://cdn.amegroups.cn/static/public/coi_disclosure.docx. We also attached two templates for your reference.

Response: COI forms have been completed by each author and we are uploading these with our revised submission.

Comment 1d. Please indicate if any of the authors serves as a current Editorial Team member (such as Editors-in-Chief, Editorial Board Member, Section Editor) for this journal.

Response: We confirm that none of the authors of this manuscript serve as a current Editorial Team member for Translational Lung Cancer Research.

Comment 1e. Please confirm that all figures/tables/videos in this manuscript are original; if not, permission is needed from the copyright holder for the reproduction.

Response: We confirm that all of the display items included in our submission are original and that no permissions are needed to reproduce any of them.

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Response: The Submission Checklist for Authors has been completed following the guidelines above, and has been uploaded with our manuscript submission.