Peer Review File

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Reviewer A

The paper "Cathepsin Detection To Identify Malignant Cells During Robotic Pulmonary Resection" is a well-designed and well-written original manuscript.
I have some comments:

Comment 1:
in the introduction, the possible applications of VGT-309, based on the activation of cathepsins, in lung surgery should be discussed, describing also the possible false negative situations (benign tumor? undifferentiated cancer?)

Reply 1:
Thank you for this valuable comment. We made sure to include the phase 2 trail that has been done in Australia as well as future work using VGT-309. We also helped explain in general possible reasons for false negatives in IMI in general and using VGT-309 specifically.

Changes in the text:
(page 4, line 97-101) These enzymes are overly expressed in almost all solid tumors and have been the target of cancer therapies.(1, 2) This helps to minimize the resection of normal parenchyma surrounding the tumor. However, it is important to note that other non-cancerous inflammatory processes might also cause an increased cathepsin expression and could potentially be a cause of a false positive.

(page 4, line 108-115) Initially, a Phase 2 safety randomized, double-blind, placebo-controlled, single ascending dose study was performed in healthy subjects in Australia (anzctr.org.au; registration number ACTRN12621000301864).(3) This was performed to evaluate the safety, tolerability, and pharmacokinetics of VGT-309. Recently, our group completed a single center phase 2 study for patients with suspected cancer in the lungs (ClinicalTrials.gov Identifier: NCT05400226) which showed promising results in using VGT-309 with near-infrared light (NIR) to localize lesions not seen under white light, identification of synchronous lesions, and identifying positive or close margins.

Comment 2:
several methods for the intraoperative identification of pulmonary nodules have been developed in the last decades, such as the radio-guided technique or methylene blue staining. According to the literature, these methods are able to detect small and deep nodules. Could you discuss this argument, explaining the pros and cons of your technique?

Reply 2:
Indeed, several molecules have been developed and used for identification of pulmonary nodules. We had a recent bi-annual congress that summarized these molecules and have cited it in the

manuscript. The pros of this molecular agent is its ability to be activated within tissue that have increased cathepsin activity and hence increasing its specificity. Another limitation is the long operative times typically needed for nodule localization without IMI without necessarily identifying the nodule in question. More traditional techniques that use transthoracic localization are also associated with a high degree of morbidity such as pneumothoraxes or require an additional procedure. Furthermore, having ICG as its fluorophore makes it compatible with NIR cameras that many hospital systems already carry. It also has a very safe toxicity profile.

Changes in text:
(page 8, line 291-306)
Over the past decade, a few fluorescent probes have been introduced to the market such as indocyanine green (ICG) and a folate-specific fluorescent probe while probes that get activated within a specific pH or quenched-activity based probes(qABP) such as VGT-309 are in clinical development. A cathepsin-targeted qABP such as VGT-309 can have a better selectivity to tumors, since tumors have a heightened cathepsin expression, thereby decreasing background fluorescence in non-malignant tissue.(4) An additional advantage to the VGT-309 molecule is that the fluorophore is ICG, which can be detected by widely-available near-infrared (NIR) cameras used in most hospital systems. Fluorescent probes also allow for less operative time by identifying tumors more rapidly. It also avoids the complications associated with traditional techniques that use transthoracic localization such as pneumothoraxes and avoids the needs for an additional procedure.(5) Finally, as a qABP, VGT-309 only gets activated when it encounters the increased enzymatic activity of cathepsin. In our study, we present a novel concept in IMI that focuses on identifying increased enzymatic activity in tumor cells to localize them. While enzymatic activity had been a staple of therapeutic options in cancer treatment, it has only recently been applied in the realm of imaging. The fluorescent probe VGT-309 is a quenched activity-based probe that is activated in the presence of cathepsins.(3)

Comment 3:
• In your opinion, what does VGT-309 add to the already established technique using ICG in the localization of malignant pulmonary nodules?

Reply 3:
Thank you for this important comment. Certainly, ICG was a cornerstone in terms of developing IMI. However, the important advancements that VGT-309 as a fluorophore compared to ICG have been addressed while answering the previous reviews. VGT-309 has a higher specificity to cathepsin-expressing cancer cells, it has better tissue penetration, and allows for less background fluorescence than traditional ICG. It is still advantageous that ICG-compatible devices can be used to detect VGT-309 since it is conjugated to ICG as the fluorophore.

Changes in text:
(page 8, line 291-306)
Over the past decade, a few fluorescent probes have been introduced to the market such as indocyanine green (ICG) and a folate-specific fluorescent probe while probes that get activated within a specific pH or quenched-activity based probes(qABP) such as VGT-309 are in clinical

development. A cathepsin-targeted qABP such as VGT-309 can have a better selectivity to tumors, since tumors have a heightened cathepsin expression, thereby decreasing background fluorescence in non-malignant tissue.(4) An additional advantage to the VGT-309 molecule is that the fluorophore is ICG, which can be detected by widely-available near-infrared (NIR) cameras used in most hospital systems. Fluorescent probes also allow for less operative time by identifying tumors more rapidly. It also avoids the complications associated with traditional techniques that use transthoracic localization such as pneumothoraxes and avoids the needs for an additional procedure.(5) Finally, as a qABP, VGT-309 only gets activated when it encounters the increased enzymatic activity of cathepsin. In our study, we present a novel concept in IMI that focuses on identifying increased enzymatic activity in tumor cells to localize them. While enzymatic activity had been a staple of therapeutic options in cancer treatment, it has only recently been applied in the realm of imaging. The fluorescent probe VGT-309 is a quenched activity-based probe that is activated in the presence of cathepsins.(3)

Comment 4:

• In discussion, the possible future applications of RIMI in thoracic surgery should be inserted.

Reply 4:

This is a very important point. We do believe it is important to integrate RATS into the future of the field more especially that it has similar outcomes to VATS while allowing the surgeon a more ergonomic procedure, more degrees of freedom, and a higher resolution image. However, it is important to bypass the added cost and the steep learning curve. As such, we believe that integrating robotic training into formative years of surgical training can account for the increased cost accrued with the learning curve and is where the field is directed. With increasing robotic adaptation, we can then encourage the use of RIMI as an adjunct the same way VIMI is an adjunct to VATS.

Changes in text:

The points mentioned above have already been addressed on p.9-line 339-345.

Comment 5:

• Moreover, please describe the current limits of this study (small sample, non-homogeneous histology of lung lesions...)

Reply 5:

Thank you for this point. We did add a paragraph at the conclusion that addresses the previously mentioned limitations and explains how that limits our ability to also do a subgroup analysis.

Changes in text:

(p.9-10, 350-357) The study does have some limitations. On one hand, we have a small sample size of n=10. As such, we recommend replicating this study on a larger cohort to improve the power of this study. Also, the tumor histologies that were studied were heterogenous (4

adenocarcinoma, 3 neuroendocrine, benign/fibrosis 2, and 1 invasive mucinous). It was hence more challenging to do a subgroup analysis with a smaller number.

Reviewer B

The authors used a cathepsin-quenched activity-based optical probe to report the intraoperative tumor target NIR probe.

Comment 1:
- Black pigmented pleural is usually expected in patients with a smoking history, which could mask the NIR-positive lesion. What is your solution for this patient?

Reply 1:
That is certainly a concern and a very important point to make. Indeed, smoking and environmental pollutants can cause changes in the color of the nodes and lesions often making them more anthracitic. In fact, we created an ex-vivo lymph node model that specifically addresses that in a manuscript we recently submitted and has been accepted with revisions.
We emulated anthracosis with black food dye. Frequently, with these fluorescent probes, there is some level of autofluorescence of normal tissue that might dampen the contrast between the actual lesion and surrounding normal tissue. Adding black food coloring dampened this auto-fluorescence and renders minute differences in fluorescence more detectable.
As such, we are expect a more pronounced difference between normal tissue and lesions in question. Many of the patients included in the phase 2 clinical trial were smokers and we have not noticed a significant impact on the intraoperative fluorescence.

Changes in text:
(p9, lines 333-337) However, while we expect to see these anthracitic changes in smokers, sometimes that may elicit a more pronounced signal in the lesion compared to background autofluorescence. In other words, while the VGT-309 signal might be slightly dampened, the signal from the background will be more significantly dampened, thereby increasing the tumor-to background ratio.

Comment 2:
Table 1 seems not to match with the manuscript; Subject 3, adenoid cystic...

Reply 2:
Thank you for pointing that out to our direction. We did make the corresponding changes in table 1 as well as the corresponding portions of the text.

Changes in the text:

(p.7,8 - line 263-267 and p.12-13 table 1) The resected specimen was then histologically analyzed. (n=4, 40%) were invasive adenocarcinoma, two were neuroendocrine, two were mucinous carcinoma, one was fibrotic tissue, and one was adenocarcinoma in situ. The slides were then sent for cathepsin staining. Tumors had the highest intensity of fluorescence under fluorescent

microscopy. These areas directly correlated with areas that had a higher cathepsin expression as seen on IHC. (Figure 3)

Comment 3:
- Adenoid cyst was visible under NIR?

Reply 3:
We apologize for the confusion. There were no adenoid cystic lesions included in our study. Subject 3 was in fact fibrotic tissues. Those were not visible under NIR.

Changes in the text:

(p.7,8 - line 257-265 and p.12 table 1) The resected specimen was then histologically analyzed. (n=4, 40%) were invasive adenocarcinoma, two were neuroendocrine, two were mucinous carcinoma, one was fibrotic tissue, and one was adenocarcinoma in situ. The slides were then sent for cathepsin staining. Tumors had the highest intensity of fluorescence under fluorescent microscopy. These areas directly correlated with areas that had a higher cathepsin expression as seen on IHC. (Figure 3)

Reviewer C

Since tactile sensation is not available in RATS, tumor identification is a critical issue. The fact that research has been conducted from the basics and applied clinically is highly commendable.

Thank you for this feedback. We appreciate your taking the time to read and review our manuscript.

Reviewer D:

I would like to congratulate the authors of the interesting manuscript entitled “Cathepsin detection to identify malignant cells during robotic pulmonary resection”.
In their study, the authors assess the possibility of using the VGT-309 detected by near-infrared imaging for identification of lung nodules during robotic-assisted thoracic surgery.
The study was well planned, the manuscript is well written and the method has great potential for clinical application.
I have only a few comments on the article.

Comment 1:
Currently, there are many methods of localization of lung nodules used during minimally invasive procedures. In order to better show the background of the conducted scientific research, I would suggest to briefly discuss their advantages and limitations in the introduction.

Reply 1:
One of the most classical methods to localize nodules and that is commonly used is trying to estimate location based on the CT scan usually reviewed and displaced intraoperatively.

Furthermore, there are many types of fluorescent probes that have been implemented to localize lesions with different mechanisms of action. We have added these different mechanisms of action to the conclusion when we talked about the advantages and disadvantages.

Changes in text:
(p.8, lines 291-306)
Over the past decade, a few fluorescent probes have been introduced to the market such as indocyanine green (ICG) and a folate-specific fluorescent probe while probes that get activated within a specific pH or quenched-activity based probes(qABP) such as VGT-309 are in clinical development. A cathepsin-targeted qABP such as VGT-309 can have a better selectivity to tumors, since tumors have a heightened cathepsin expression, thereby decreasing background fluorescence in non-malignant tissue.(4) An additional advantage to the VGT-309 molecule is that the fluorophore is ICG, which can be detected by widely-available near-infrared (NIR) cameras used in most hospital systems. Fluorescent probes also allow for less operative time by identifying tumors more rapidly. It also avoids the complications associated with traditional techniques that use transthoracic localization such as pneumothoraxes and avoids the needs for an additional procedure.(5) Finally, as a qABP, VGT-309 only gets activated when it encounters the increased enzymatic activity of cathepsin. In our study, we present a novel concept in IMI that focuses on identifying increased enzymatic activity in tumor cells to localize them. While enzymatic activity had been a staple of therapeutic options in cancer treatment, it has only recently been applied in the realm of imaging. The fluorescent probe VGT-309 is a quenched activity-based probe that is activated in the presence of cathepsins.

Comment 2:
Methods of visual nodule localization are most often limited by the depth of the location of the lesions in the lungs. I propose to briefly discuss this limitation.

Reply 2:
Indeed, the longer the distance fluorescence has to cross, the less likely it will be detected by the NIR cameras. This new fluorescent probe appears to have improved penetrance but certainly shares that limitation. Although this can be better appreciated with the larger phase 2 trial, we added this to our manuscript to highlight this important point.

Changes in text:
(p.10, lines 352-354) *Lastly, deeper tissue penetrance remains a concern with fluorescent probes. While in this study, the sample size is not large enough to address this question, it is something that needs to be further studies in larger scale studies and compared to other imaging modalities.*

Comment 3:
The most accurate method of intraoperative evaluation of the completeness of the resection is histopathological examination. In order to emphasize the value of the method of assessing the completeness of resection proposed by the authors, I would suggest briefly discussing the limitations of the intraoperative frozen section.

Reply 3:

Thank you for your comment. Intraoperative frozen section has been a staple in lung surgery and often a reliable adjunct to decide on the extent of the resection and the corresponding lymp node harvest. One of the problems, is the delay between sending the specimen to frozen section and receiving a call from the pathologist. This delay can add unnecessary. Furthermore, frozen section might miss foci of disease depending on the location where the specimen is resected (false negative) and would only be truly identified during permanent formal evaluation of the specimen. That patient might have benefited from a more extensive resection had the surgeon known that information intraoperatively.

Changes in text:
(p.8, lines 282-289)
As such, tumor visualization is increasingly becoming one of the most critical steps enabling an R0 resection. Currently, intraoperative frozen section is used to help surgeons decide the extent of the needed dissection. However, it adds to the overall operative time as it requires sending the specimen to pathology, processing it, and then calling the surgeon back. Also, there is a risk for a false negative if the foci of tumors are undetected on frozen and only confirmed on permanent, which might have altered the surgeons' decision making. To address the previous concerns, IMI uses a fluorescent probe that helps localize the tumor in question in real time. It has had promising results in VIMI and RIMI in localizing occult disease.

Comment 4:
I would also suggest briefly discussing the complications that may result from the use of the VGT-309.

Reply 4:
In our complete safety analysis in the phase 2 clinical trial, we had no adverse effects with the use of VGT-309 and that has also been the case in the phase 2 trial in Australia. It has proven to be a safe drug.

Changes in text:
(p.4, lines 115) It has also proven to be a safe drug with no adverse effects noted.

Comment 5:
In order to emphasize the importance of research, the authors could discuss future directions of development and application of the method. I can think of such applications as identification of lesions during resection of lung metastases, identification of pleural lesions during the diagnosis of pleural tumors for targeted biopsy, evaluation of lymph nodes for intraoperative localization of metastases. The method can also be used in the diagnosis and treatment of extrapulmonary cancers, for example, the assessment of R0/R1 during esophageal or tracheal cancer surgery.

Reply 5:
Indeed, this imaging technology has potential applications outside of primary lung cancers. We have worked on a preclinical model to highlight the utility of using this technology in identifying positive lymph nodes. We have also evaluated its used in assessing neuroendocrine tumors. Finally, tumors with increased cathepsin expression, even if they are not primary lung tumors, might also be a target for this fluorescent probe.

Changes in text:
(p.10, lines 363-367) There is potential for using this technology not only for primary pulmonary malignancies, but also to identify tumors that have metastasized to the lung especially if the tumors have an increased cathepsin expression. It may also help in localizing lesions for targeted biopsies, localizing positive lymph nodes, and localizing other primary malignancies in the chest such as esophageal or tracheal cancer.

Once again, congratulations on a very interesting scientific study with great potential for clinical application.

Reviewer E

Dr. Samra and his colleagues reported that identification of pulmonary nodules during robotic surgery using a new fluorescence imaging technique conventionally applied in VATS. They planned a phase II clinical trial and tried this new technology on 10 patients via RATS. As a result, it was possible to identify four lesions that could not be identified with normal white light. On the other hand, two lesions could not be identified using the new technology. They concluded that this technology is a valuable option for visualization of occult disease in RATS as well.
Intraoperative identification of small pulmonary nodules in robotic surgery without tactile sensation is an important challenge and unmet need. The authors conducted this study as a solution to this issue. The authors are to be commended, and I read the article with great interest.

I have some comments.

Major comments:

Comment 1:
Has the surgical time been reduced before and after the introduction of this new technology?

Reply 1:
This is worthwhile endeavor but something that is beyond the scope of our paper. We believe that with implementing IMI, surgeons can perform a more targeted resection and often if they get the required margins on wedge resection, should not need to do a formal resection that would take more time. We will certainly try to address this in our clinical trial manuscript. Our main question here was comparing the use of RIMI and VIMI in identifying lesions.
It is important to note that the other element of comparison is RATS vs VATS surgery as RATS is technically also a newer technology. Robotic surgery is more recent and hence has an associated learning curve. Since most surgeons are more comfortable with VATS, it may take them less operative time.

Changes in text:
none

Comment 2:
How did the margin of resection change before and after the introduction of the new technology?

Reply 2:
This is another valuable and clinically very significant question. The role of IMI in detecting margin proximity has been evaluated previously and aided surgeons in their decision of either pursuing a more extensive resection or being satisfied with the margin taken. Previous studies have shown very promising correlation between the margins identified on IMI and those from pathology. We also have shown that in our phase 2 clinical trial that we are drafting. This particular point is beyond the scope of this study.
However, in this study, we explain how IMI aided in minimizing the extent of the procedure needed by confirming negative margins upon a wedge or sublobar resection and allowing the surgeon to be comfortable not to pursue a more extensive anatomic resection. This was explained in the text.

Changes in text:
(p.9, lines 315-318) IMI helps to address the R1 margins by fluorescently labelling lesions, providing additional information to the surgeon during margin assessment. This was the case in 4 lesions in our study. These patients all underwent R0 resections and benefited from a more parenchymal-sparing wedge resections as opposed to more extensive surgery.

Comment 3:
Is there any concern that the use of fluorescence to identify tumors will result in larger than necessary resection margins?

Reply 3:
The reviewer brings up a great concern of whether we could have false positives and end up resecting more parenchyma than needed. While this has been an issue with previous fluorescent probes, this has not been an issue that we observed with VGT-409. In fact, it helped in avoiding unnecessary resection of normal parenchyma by being selective to tumors that overexpress cathepsins. However, the false positives that we may come across are other conditions that might have an increased cathepsin expression such as inflammatory processes.

Changes in text:
(p.4, line 96-101)
Essentially, this unique contrast agent is an indirect measure of cathepsin activity. In other words, areas that fluoresce are areas that have increased cathepsin activity. These enzymes are overly expressed in almost all solid tumors and have been the target of cancer therapies.(1, 2) This helps to minimize the resection of normal parenchyma surrounding the tumor. However, it is important to note that other non-cancerous inflammatory processes might also cause an increased cathepsin expression and could potentially be a cause of a false positive.

Minor comments:

Comment 4:
Abstract

Abbreviations are used extensively and are very difficult to read. Some of them have no explanation. The use of abbreviations should be avoided in abstracts as much as possible. Please revise.

Reply 4:
We apologize for that. We made sure all abbreviations mentioned for the first time have a clear explanation.

Changes in text:
(p.2, lines 57-61) *The MFI of tumors visualized by RIMI was 115.81 A.U. (SD 58.57) compared to 95.6 AU. (SD 14.81) by VIMI. (p=0.41). The mean TBR of tumors visualized by RIMI was 9.20 (SD 9.12) compared to 2.29 A.U. (SD 1.11) using VIMI.(p=0.1) The mean TBR of tumors visualized by RIMI was 9.20 (SD 9.12) compared to 2.29 A.U. (SD 1.11) using VIMI.(p=0.1)*

Comment 5:
Methods
Line107: "ongoing. ." There are two periods.

Reply 5:
This has been addressed. The trial was concluded, and this was reworded and rectified to reflect that.

Changes in text: (p.4 line 129-130)

Our study is part of a completed phase 2 clinical trial (**NCT05400226)** that began in June 2022 (patients included up to June 2023) at The Hospital of the University of Pennsylvania.

Comment 6:
Line 112-113: Target lesions were limited to peripheral lesions close to the visceral pleura. Please specify this point.

Reply 6:
The tumors were mostly superficial with most located on the pleural surface (6/10) and the deepest being 1.4cm away. This point has been removed.

Changes in text
none