Peer review file

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Reviewer A

- Comment 1: Would recommend authors discussing in discussion section if such technique could also be used in esophageal adenocarcinoma.
- Reply 1: Thank you for your helpful comment. Our previous data of ICG imaging from the lung cancer showed that the fluorescence intensity did not differ according to the pathology of the pulmonary tumor (Eur J Cardiothorac Surg. 2016 May;49(5):1497-502.). And another research team revealed the fluorescence intensity was independent of pathologic variables (Cancer. 2019 Mar 1;125(5):807-817). Therefore, we assumed that ICG also can used to detect esophageal adenocarcinoma. If it is possible to conduct a multi-center trial in our country, we would like to do to confirm the effectiveness of ICG for detection of the esophageal cancer regardless of the type in a large number of patients. We added contents in discussion section (please see Page 12, line 248-252).
- Changes in the text: And, although our results are helpful for clinical use, the power of the study is limited because of the small number of patients who have squamous cell carcinoma only involved and ex vivo specimens. Therefore, there is a need to conduct further research with larger patient populations regardless of the pathology and staging to optimize clinical use and analyze the correlation between pathology or stage and the intensity of fluorescence in the esophageal tumor.

- Comment 2: Can authors comment if the stage of tumor makes a difference in the degree of ICG uptake or if the uptake is uniform irrespective of the stage.
- Reply 2: Thank you for your important question. We couldn't analyze a difference in the degree of ICG uptake as the stage of tumor because the small number of patients participated in this study. However, some researchers including our group concluded that there was no correlation between size and the intensity of fluorescence from another thoracic cancers (Ann Thorac Surg. 2014 Oct;98(4):1223-30., Eur J Cardiothorac Surg. 2016 May;49(5):1497-502., Cancer. 2019 Mar 1;125(5):807-817.). It would be good to analyze this issue in the esophageal tumor in a future study, although there was no correlation in another thoracic cancer. We added our opinion

about this issue in discussion section (please see please see Page 12, line 248-252).
- Changes in the text: And, although our results are helpful for clinical use, the power of the study is limited because of the small number of patients who have squamous cell carcinoma only involved and ex vivo specimens. Therefore, there is a need to conduct further research with larger patient populations regardless of the pathology and staging to optimize clinical use and analyze the correlation between pathology or stage and the intensity of fluorescence in the esophageal tumor.

- Comment 3: Moving forward in future, it would be interesting to see the possible use of ICG for detection of esophageal cancer in patients with Barrett's Esophagus using endoscopy, but more studies like these are needed.
- Reply 3: Thank you for your thoughtful comment. We agree with your opinion. We also think ICG can be helpful to detect the Barrett's Esophagus during endoscopy. Therefore, we would like to develop fluorescent imaging system for endoscopy and evaluate the possibility of system for using ICG detection in the future.
- Changes in the text: No changes in the text.

- Comment 4: Introduction: The first paragraph is not adding much to the topic of the paper. I recommend authors to focus on "Image-guided surgery using near-infrared (NIR) fluorescent imaging using ICG". Can consider starting from second paragraph or can shorten the first paragraph and merge with second paragraph (Line 61, Page 2)
- Reply 4: Thank you for your suggestion. However, the minimal invasive surgery (MIS) is important because image-guided surgery using near-infrared (NIR) fluorescent imaging with ICG has increased to overcome the limitations of MIS. Therefore, we think information about MIS is necessary in the first paragraph of introduction section.
- Changes in the text: No changes in the text.

- Comment 5: Method: Recommend authors to mention if the pathologists were blinded from the study and also if it was a single pathologist who evaluated all the surgical specimens.
- Reply 5: Thank you for your kind recommendation. Pathologists were blinded from the study because they performed the procedure as usual regardless of this study. Two pathologists evaluated all the surgical specimens. we have added relevant information

in results section (please see Page 9, line 177-178).
- Changes in the text: Two pathologists analyzed all the surgical specimens with the procedure as usual regardless of this study.

- Comment 6: Discussion: Recommend explaining what are the allergies associated with ICG and which ones increase with increasing the dose.
- Reply 6: Indocyanine green contains sodium iodide, so this may cause anaphylaxis including a rash, itching, swelling of the face, tongue, and throat, trouble breathing or swallowing, or chest pain after intravenous injection to the patient especially who have the allergy to iodides. We added this information in discussions section (please see Page 9-10, line 199-202).
- Changes in the text: The allergic reaction, such as anaphylaxis including a rash, itching, swelling of the face, tongue, and throat, trouble breathing or swallowing, or chest pain, after intravenous injection to the patient, frequency from ICG is reported to be 0.003% when the ICG concentration in the vein is less than 0.5 mg/kg.

Reviewer B

- Comment 1: They did two studies, I suggest that they use subsections for their animal and human studies specially in the materials and methods and the results sections for the sake of the clarity.
- Reply 1: Thank you for your suggestion. To clarify, I added subtitle for the animal and human study each in methods and results section (please see Page 4, line 78 and 85 / Page 6, line 110 and 125 / Page 7, line 134, 138, 148, and 153 / Page 8, line 160 and 173 / Page 9, line 182).
- Changes in the text: Making subtitles in methods and results section.

- Comment 2: The final version is rather put together carelessly, for example, they do not refer to figure 3 in their text which seems important and for the figure 4, they only have figure 4B while A is not mentioned in the text but shown, and C in mentioned and not shown.
- Reply 2: Thank you for pointing this out and I’m very sorry that it makes you confuse.

We have changed figure 4 to figure 3 in results section (please see Page 9, line 185 and 187).

- Changes in the text: Figure 4A and 4B changed with Figure 3A and Figure 3B.

- Comment 3: In the materials and methods, the TNR and region of interests should be defined clearly since just by looking at the figure 2C, I was unable to judge whether the fluorescent was successful in the classification of the tumour, especially for 48h:1mg/kg, 2mg/kg, and 5mg/kg; 24h: 1mg/kg, 3h: 2 and 5 mg/kg. You should either support your finding quantitatively but providing clear method that you used or qualitatively by showing better figures. Right now you are lacking both.

- Reply 3: Thank you for pointing out. We measured the fluorescence intensity of a region of interest on the normal tissue and tumor tissue each and calculated the signal of tumor to normal ratio. If the signal is greater than 1, which means the intensity of tumor is higher than the normal, we thought that the tumor has signal. As you can see in figure 2C, all specimens have value greater than 1, so they all have fluorescent signal of the tumor. However, the more signal is close to 1, the less difference is in between cancer and normal tissue. And we also considered that it can be easily distinguished the cancer and normal tissue by the fluorescent when the signal of TNR is higher than 2 (JAMA Surg. 2020 Aug 1;155(8):732-740.). Therefore, it is difficult to identify a tumor with only fluorescent signal, if the signal is less than 2. I'm sorry that we didn't write this concept and methods we used in detail. I added these methods in methods sections (please see Page 6, line 116-122).

- Changes in the text: The ICFIS images of the esophageal tumor were used to measure a fluorescent intensity. Each intensity of a region of interest on the tumor and normal was measured using Image J software (64-bit Java 1.8.0_172, National Institute of Healthcare, Maryland, U.S.A.), and the signal of tumor-to-normal tissue ratio (TNR) was calculated. The signal greater than 1 means the intensity of tumor is higher than the normal, and the tumor has signal. Also, it can be easily distinguished the cancer and normal tissue by the fluorescent when the signal of TNR is higher than 2 (14).

- Comment 4: In the materials and methods, you introduced your patient population, while crucial details are missing regarding their gender, age and etc. However, I found those information later on in the result section. lines 159-163 belong to the M&M in

line 119. Same goes for table 1 that has to move to the M&M section.
- Reply 4: Thank you for your suggestion. However, the detail information of patients was analyzed after completing study. Therefore, we thought it is better that the details are in the results section.
- Changes in the text: No changes.

- Comment 5: Regarding Table1, I think each patient should have a unique name, which also called by that throughout the text. In this way the reader can correspond which patient you are referring to and will give extra information. Redesigning the whole table is important, also mentioning the patient numbers in the text.
- Reply 5: Thank you for your helpful comment. I redesigned the table as you suggest. I have totally changed the table 1. (please see Page 16).
- Changes in the text: Table 1 was changed with a new form.

- Comment 6: I think your statement in lines 177 and 178 are bit strong based on the results that you showed in this paper. This could be if you clearly show your point as discussed in point 2. Same goes for Line 191-192, again I can see that you can draw such a conclusion with the presented results. Line 226: your conclusion is a bit optimistic regarding the presented results.
- Reply 6: Thank you for your comments. As I explained in the Reply 3, we measured the fluorescence intensity of a region of interest on the normal tissue and tumor tissue each and calculated the signal of tumor to normal ratio. If the signal is greater than 1, which means the intensity of tumor is higher than the normal, we thought that the tumor has signal. As you can see in figure 2C, all specimens have value greater than 1, so they all have fluorescent signal of the tumor. However, the more signal is close to 1, the less difference is in between cancer and normal tissue. And we also considered that it can be easily distinguished the cancer and normal tissue by the fluorescent when the signal of TNR is higher than 2 (JAMA Surg. 2020 Aug 1;155(8):732-740.). Therefore, it is difficult to identify a tumor with only fluorescent signal, if the signal is less than 2. We hope this concept could make you understand our results easier.
For the conclusion, we supposed that if intraoperative tumor imaging with ICG could be applicable in clinical fields, it can be helpful to find the tumor for surgeons during the operation. We hope to proceed the further study with large number of patients in a

near future and to evaluate the various attempts that have not been done in this study. I added these methods in methods sections (please see Page 6, line 116-122).

- Changes in the text The ICFIS images of the esophageal tumor were used to measure a fluorescent intensity. Each intensity of a region of interest on the tumor and normal was measured using Image J software (64-bit Java 1.8.0_172, National Institute of Healthcare, Maryland, U.S.A.), and the signal of tumor-to-normal tissue ratio (TNR) was calculated. The signal greater than 1 means the intensity of tumor is higher than the normal, and the tumor has signal. Also, it can be easily distinguished the cancer and normal tissue by the fluorescent when the signal of TNR is higher than 2 (14).

- Comment 7: Try to change the abstract accordingly.

- Reply 7: Thank you for the kind comment. I edited the abstract (please see Page 2, line 33 and 35).

- Changes in the text: The rabbits received intravenous injection of ICG at doses of 1, 2, or 5 mg/kg at 3, 6, 12, 24, or 48 h before surgical removal of esophagus. Twelve patients scheduled to undergo esophagectomy were also enrolled, and all received 2 mg/kg of ICG intravenously at 3, 6, 12, or 24 h before surgical removal of esophagus

- Comment 8:

Line 52: Do not use the acronym (MIS) for minimally invasive surgery. You do not mention it much in the manuscript and it adds to the confusion.

Line 79: mention where the animals are stored.

Line 83: Provide a number of applicable.

Line 91: for which procures you used anaesthesia, e.g. hair removal, make it clear.

Line92: you mentioned the incision size in the figure, add it here as well.

Line 101: Vague, try to make a table for the rabbits (if possible) and name them and mention the names here.

Line 106: Mention figure 1B here

Line 125: provide a sentence why you decided to exclude neoadjuvant therapy from your study for clarification.

Line 128: replace the word “surgery” with “surgical removal of the esophagus”

Line 131: Mention for which patients and how

Line 151: I am not sure if I agree with this point, of course signals are visible but no

tumour classification can be easily comprehended.

Line 155: you mentioned TNR value of 1mg/kg at 12h was too low, only this one

Line 173: change the word “specimen” with “specimens”

Line 190: mention what is the depth of penetration of ICG.

- Reply 8: Thank you for your detailed comments. I have changed or add contents as followed by your comments in each sections (please see Page 3, line 52 / Page 4, line 80-81 / Page 4, line 84 / Page 5, line 92 / Page 5, line 94 / Page 5, line 103 / Page 5, line 108 / Page 7, line 132-133 / Page 7, line 137 / Page 7, line 142 and 144 / Page 9, line 194 / Page 10, line 217-218). And for line 151 and 155, we explained on Reply 3 and 6. Please see the replying.

- Changes in the text:

Line 52: The use of minimally invasive surgery for the treatment of various types of solid cancers has increased in recent years because it offers several advantages over traditional open surgery.

Line 79: The animals were purchased from Doo Yeol Biotech in South Korea and they were housed in a metallic rabbit cage each.

Line 83: All animal experiments and protocols were approved by the institutional animal care and received committee approval from Korea University (KOREA-2016-0224).

Line 91: All rabbits were anesthetized with xylazine (5 mg/kg intramuscularly; RompunTM, Bayer Korea Inc., Seoul, Korea) and alfaxalone (5 mg/kg intravenously; Alfaxan®, Jurox Pty Ltd., NSW, Australia) to minimize suffering during all procedures.

Line 92: The abdomen was opened with 5 cm incision site, and the lower thoracic esophagus was pulled into the abdominal cavity and exposed.

Line 101: Two weeks after VX2 cells injection, tumor formation was evaluated using five randomly selected rabbits (number 1, 3, 12, 26, and 35)

Line 106: PET images were displayed and analyzed (Figure 1B) on a dedicated workstation.

Line 125: Patients who had received neoadjuvant therapy were excluded from this study because of the possibility that the tumor would not remain after neoadjuvant therapy.

Line 128: an ICG dose of 2 mg/kg was administered intravenously to esophageal cancer patients at 3, 6, 12, or 24 h before surgical removal of the esophagus.

Line 131: Standard video-assisted thoracoscopic surgery is our routine procedure. For

patients with middle and lower thoracic esophageal cancer (patient no. 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12), intrathoracic esophagogastrostomy was performed; in patients with upper thoracic esophageal cancer, esophagogastrostomy was performed through a left cervical incision (patient no. 2 and 3).

Line 151: No changes.

Line 155: No changes.

Line 173: The optimal ICG injection doses and times of injection for the detection of esophageal tumors were additionally evaluated with a rabbit and human ex vivo specimens.

Line 190: The fluorescent signal in the esophagus before resection was weak due to the depth limitation of ICG, which means that signals are not easily visible at depth of more than 2 cm of the tissue.

Reviewer C

- Comment 1: The authors have not shown the concordance between the pathologic tumor border and the tumor-normal border by NIR with ICG.
- Reply 1: Thank you for pointing this out. We would like to proceed the further studies and to compare the pathologic tumor border with NIR fluorescent border. If we can show the NIR fluorescent with ICG can accurately distinguish the boundary between cancer and normal tissue, we expect NIR imaging with ICG to be used to more easily obtain the enough negative margin in real time during the surgery. We added this plan in discussion section (please see Page 11, line 231-232).
- Changes in the text: For these results, there will be needed to compare the pathologic tumor border and the border by NIR fluorescent with ICG.

- Comment 2: Frequent intraepithelial spread is one of the pathologic features in squamous cell carcinoma of the esophagus. Therefore, to obtain the safety resection margin, an intraoperative diagnosis of the extent of intraepithelial spread is essential. It remains a problem if NIR with ICG can precisely identify the extent of intraepithelial spread.
- Reply 2: Thank you for your important comments. We agree with your opinion. As

with Reply 1, if we can show the extent of intraepithelial spread through fluorescence microscopy in a future study, we expect this will also really help remove cancer tissue from patients during surgery without any remaining cancer tissue. We added this in discussion section (please see Page 11, line 232-234).
- Changes in the text: Also, it is necessary to obtain results on whether NIR fluorescent signal with ICG could accurately identify the extent of intraepithelial spread.

Reviewer D

- Comment 1: Firstly, the language has to be checked by a native speaker.
- Reply 1: Thank you for the important comment. I checked the language in this paper with a native speaker.
- Changes in the text: There are many changes overalls with words or grammar.

- Comment 2: The title has to be changed. Now it is a sentence.
- Reply 2: Thank you for your suggestion. I changed the title (please see Page 1, line 1-2).
- Changes in the text: Near-infrared fluorescent imaging with indocyanine green in rabbit and patient specimens of esophageal cancer

- Comment 3: The citation in the text is done in a wrong manner.
- Reply 3: Thank you for pointing this out and I'm very sorry that I didn't check in advance. I changed the font of the citation using same manner with the main text in references section (please see Page 14-15, line 271-319)
- Changes in the text: Font changing of References.

- Comment 4: An identification number of the animal and patient study is missing.
- Reply 4: Thank you for your comment. I added the identification number of the animal and patient study in methods section (please see Page 4, line 84, and Page 6, line 129).
- Changes in the text: All animal experiments and protocols were approved by the institutional animal care and received committee approval from Korea University (KOREA-2016-0224). This study was approved by the ethics committee of the Korea

University Guro Hospital (No. 2020GR0181).

- Comment 5: The link to the figures is wrong e.g. page 4 line 113.
- Reply 5: Thank you for pointing this out. I deleted “(Figure 2A and 2B)” from results section (please see Page 6, line 115).
- Changes in the text: The fluorescence intensity in freshly excised esophageal tumor specimens was assessed using a custom-manufactured intraoperative color and fluorescence-merged imaging system (ICFIS), as previously described (13).

- Comment 6: How you calculate the TNR? Please describe in detail. Furthermore, if you not taken the same distance from object to camera for all recordings, the grey value can be adapted automatically by the software of the imaging system. How you consider this issue? You didn't describe the distance between object and camera system. Please add this information.
- Reply 6: Thank you for pointing out. We measured the fluorescence intensity of a region of interest on the normal tissue and tumor tissue each and calculated the signal of tumor to normal ratio. If the signal is greater than 1, which means the intensity of tumor is higher than the normal, we thought that the tumor has signal. However, the more signal is close to 1, the less difference is in between cancer and normal tissue. And we also considered that it can be easily distinguished the cancer and normal tissue by the fluorescent when the signal of TNR is higher than 2 (JAMA Surg. 2020 Aug 1;155(8):732-740.). Therefore, it is difficult to identify a tumor with only fluorescent signal, if the signal is less than 2.
The distance from specimens to camera for all recordings was same. We used a 30cm ruler to measure the distance between the object and the camera, and each time we recorded, we kept the same distance from specimens to camera. I added these methods in methods section (please see Page 6, line 115-122).
- Changes in the text: The distance from specimens and camera was kept the same with 30cm for all recordings. The ICFIS images of the esophageal tumor were used to measure a fluorescent intensity. Each intensity of a region of interest on the tumor and normal was measured using Image J software (64-bit Java 1.8.0_172, National Institute of Healthcare, Maryland, U.S.A.), and the signal of tumor-to-normal tissue ratio (TNR) was calculated. The signal greater than 1 means the intensity of tumor is higher than

the normal, and the tumor has signal. Also, it can be easily distinguished the cancer and normal tissue by the fluorescent when the signal of TNR is higher than 2 (14).

- Comment 7: You have written in page 6 line 158 that both fluorescence signals was sufficient to detect tumors. Do you have a threshold for the TNR?
- Reply 7: Thank you for your important question. As I explained in the Reply 6, we measured the fluorescence intensity of a region of interest on the normal tissue and tumor tissue each and calculated the signal of tumor to normal ratio. If the signal is greater than 1, which means the intensity of tumor is higher than the normal, we thought that the tumor has signal. As you can see in figure 2C, all specimens have value greater than 1, so they all have fluorescent signal of the tumor. However, the more signal is close to 1, the less difference is in between cancer and normal tissue. And we also considered that it can be easily distinguished the cancer and normal tissue by the fluorescent when the signal of TNR is higher than 2 (JAMA Surg. 2020 Aug 1;155(8):732-740.). Therefore, it is difficult to identify a tumor with only fluorescent signal, if the signal is less than 2. We hope this concept could make you understand our results easier. I added these methods in methods section (please see Page 6, line 116-122).
- Changes in the text: Each intensity of a region of interest on the tumor and normal was measured using Image J software (64-bit Java 1.8.0_172, National Institute of Healthcare, Maryland, U.S.A.), and the signal of tumor-to-normal tissue ratio (TNR) was calculated. The signal greater than 1 means the intensity of tumor is higher than the normal, and the tumor has signal. Also, it can be easily distinguished the cancer and normal tissue by the fluorescent when the signal of TNR is higher than 2 (14).

- Comment 8: In the discussion, you have written that you have tested the probability. How you have calculated these measurements?
- Reply 8: Thank you for your question. We think we used the wrong word. We're very sorry to confuse you. We changed the probability to possibility (please see Page 9, line 191). What we're trying to say is that other cancer, especially chest cancers, are actively researching on detecting cancer during surgery using NIR fluorescent imaging with ICG, while esophageal cancer is not being studied. Therefore, we're trying to show the possibility if NIR imaging with ICG can detect esophageal cancer. We confirmed the

possibility that NIR imaging with ICG could also be applied to cancer detection in esophageal cancer patients because fluorescent signal could be identified in cancer when ICG was injected into patients. However, more research is required to apply ICG during the surgery in real time as we mentioned in discussion sections.
- Changes in the text: In this proof of concept study, the possibility of esophageal tumor detection with intravenous injection of ICG was evaluated for the first time.

- Comment 9: In the discussion, you have written with all doses you can detect tumor tissue. But in the results, you have mentioned that 1 mg/kg is not enough. Why?
- Reply 9: Thank you for your important question. As I explained in the Reply 6 and 7, all specimens have the signal of TNR greater than 1, so we thought they all have fluorescent signal of the tumor. However, the more signal is close to 1, the less difference is in between cancer and normal tissue. Therefore, it is difficult to identify a tumor, which have low signal, with only fluorescent signal. And we also considered that it can be easily distinguished the cancer and normal tissue by the fluorescent when the signal of TNR is higher than 2 (JAMA Surg. 2020 Aug 1;155(8):732-740.). We added these in methods sections to make the results easier to understand (please see Page 6, line 116-122).
- Changes in the text: The ICFIS images of the esophageal tumor were used to measure a fluorescent intensity. Each intensity of a region of interest on the tumor and normal was measured using Image J software (64-bit Java 1.8.0_172, National Institute of Healthcare, Maryland, U.S.A.), and the signal of tumor-to-normal tissue ratio (TNR) was calculated. The signal greater than 1 means the intensity of tumor is higher than the normal, and the tumor has signal. Also, it can be easily distinguished the cancer and normal tissue by the fluorescent when the signal of TNR is higher than 2 (14).

- Comment 10: Please add to your discussion a comparison to other studies and why the TNR has its peak after 12 h.
- Reply 10: Thank you for your kind comment. Other studies have suggested that the cancer is detected when 5mg/kg is injected into the patient 24 hours before the surgery. From animal data, our results also show that cancer is still detected when ICG with concentration of 5mg/kg is injected at 24 hours before the surgical resection. However, we're trying to show the best time and concentration to detect cancer with ICG. Also,

since the ICG injected intravenously, the ICG will be transferred to each internal organ through the blood stream, which we think it can be detected well at different times because of the different blood circulation times of each organ. I added this in discussion section (please see Page 10, line 207-214)

- Changes in the text: In the different thoracic cancer surgery except esophageal cancer, they can be detected using 5 mg/kg of ICG intravenously injected 24 h prior to surgery using NIR fluorescent imaging (8-10). Also, our previous research of pulmonary neoplasms can be detected with 1 mg/kg of ICG at 24 hours before (11). From animal data, our results also show that cancer is still detected when ICG with concentration of 5mg/kg is injected at 24 hours before the surgical resection. However, the ICG will be transferred to each internal organ through the blood stream, which it can be detected well at different times because of the different blood circulation times of each organ.