Peer Review File

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Reviewer A

Comment 1: Figure 1 does not match the text. The authors note that the fluorescence emitted by the tumor helps the surgeons to identify tumor localization and detect microlesions intraoperatively, but the figures show cross sections of the resected specimens. Also, tumor differentiation is described in the last part of the same paragraph.

Reply 1: We appreciate your valuable comment. Figure 1 is from the study reported by Ishizawa et al. (Ishizawa T, Fukushima N, Shibahara J, et al. Real-time identification of liver cancers by using indocyanine green fluorescent imaging. Cancer 2009;115:2491-2504.). Figure 1a showed that indocyanine green (ICG) fluorescent imaging (left) delineating a fluorescing lesion at a site where no cancerous lesion had been grossly identified. Figure 1b showed that ICG fluorescent imaging detected a grossly unidentifiable lesion apart from the main tumor. Figure 1c-e depicted the fluorescence manifestations of different differentiated hepatocellular carcinomas (HCCs). However, all figures in Figure 1 are the cross section of the resected specimens rather than the intraoperative images, which does not match the text. In addition, Considering that ICG fluorescence has been widely used in surgical resection of HCC over the past decade and is well known by clinicians. Therefore, we deleted Figure 1 in the modified version (see Page 6, Line 4; Page 38, Figure Legends section). Figure 2

and Figure 3 were changed to Figure 1 and Figure 2, respectively (see Page 12, Line 22; Page 17, Line 10; Page 38, Figure Legends section).

Reviewer B

Comment 1: Figure 2 and 3 are from references 52 and 70, respectively. These sources must be mentioned in the legends of the corresponding figures.

Reply 1: We appreciate your constructive comment. Since we deleted Figure 1 in the modified version (see Reply 1 to Reviewer A's comment 1), Figure 2 and Figure 3 were changed to Figure 1 and Figure 2, respectively (see Page 12, Line 22; Page 17, Line 10; Page 38, Figure Legends section). We added the reference 52 into the figure legend of Figure 1 and the reference 70 into the figure legend of Figure 2 (see Page 38, Figure Legends section).

Comment 2: The two sentences in red concerning "narrative review" should be suppress (end of Introduction and footnote at the end of the manuscript).

Reply 2: Thank you for the helpful comment. We suppressed the two sentences in red concerning "narrative review" in the modified version (see Page 5, Line 6; Page 23, Line 1).

Comment 3: English should be verified carefully.

Reply 3: As the reviewer suggested, we invited Karen M. von Deneen, Ph.D., Associate Professor, School of Life Science and Technology, Xidian University to verify the

English of the manuscript. The wording was checked and the language was polished in the modified version.

Reviewer C

Comment 1: For hepatectomy, the timing of fluorescent dye excretion into biliary tract is critical, many of researchers have tried to estimate onset of fluorescent signals both from liver parenchyma to biliary trees & HCC. The reviewer suggests that authors add the timing of injection and fluorescence signal onsets of each dye. This information will be very helpful for hepatobiliary surgeons to evaluate each dye.

Reply 1: Thank you for the beneficial comment. The preoperative administration time of the near-infrared fluorescence (NIRF) probe is considered as one of the important factors affecting the intraoperative fluorescence imaging of hepatocellular carcinoma (HCC). Concerning hepatectomy resection of HCC, better contrast between tumor and liver background is helpful for HCC detection and localization. Compared with the timing of injection and fluorescence signal onset, we believe that the post-injection time for maximum signal-to-background ratio (SBR) of fluorescence is more instructive for liver surgery of HCC. *In vivo* fluorescence imaging experiments, most of the authors also focused on the time when the maximum SBR appeared after injection of their probes. Therefore, we added the post-injection time for maximum SBR of fluorescence for NIR-I fluorescent probes (see Page 35, Table 1). Besides, we added the column of maximum SBR of fluorescence and corresponding post-injection time for hybrid NIR-I fluorescent probes (see Page 36, Table 2) and NIR-II fluorescent probes (see Page 37,

Table 3).

Comment 2: The reviewer suggests that the authors include both positive and negative margin detection information of each probes. What are the potential benefits in positive margin over the negative margin targeting probes during hepatectomy (such as liver perfusion monitoring or ligation strategy, partial ischemic regional detection)?

Reply 2: Thank you for your insightful comment. Considering that our article focuses on the application of near-infrared fluorescence (NIRF) in hepatectomy for hepatocellular carcinoma (HCC), almost all NIR-I and NIR-II fluorescent probes were designed to light up HCC and guide resection of the illuminated area. After we carefully reviewed the published probes, only ZnGa2O4Cr0.004 (ZGC) reported by Ai et al. and NaGdF4: Nd5%@NaGdF4@Lips (Gd-REs@Lips) reported by Ren et al. were found to be positive margin targeting probes (Ai T, Shang W, Yan H, et al. Near infrared-emitting persistent luminescent nanoparticles for Hepatocellular Carcinoma imaging and luminescence-guided surgery. Biomaterials 2018;167:216-225; Ying Ren, Shuqing He, Lakshmi Huttad, et al. NIR-II/MR Dual Modal Nanoprobe for Liver Cancer Imaging. Nanoscale 2020;12:11510-11517.). We added the column of margin detection information for NIR-I fluorescent probes (see Page 35, Table 1), hybrid NIR-I fluorescent probes (see Page 36, Table 2), and NIR-II fluorescent probes (see Page 37, Table 3). Unfortunately, Ai et al. and Ren et al. did not further discuss the specificity of their probes. In fact, we agree that positive margin targeting probe may have advantages over negative margin targeting probes in liver perfusion monitoring and partial

ischemic regional detection during HCC resection. We look forward to the emergence of more relevant studies, however, there is still a lack of support from experimental results.

Comment 3: If the manuscript is entitled, "The application of near-infrared fluorescence imaging in hepatectomy for HCC", the reviewer suggests that authors include imaging device summary used both for NIR-I fluorescent probe and NIR-II Fluorescent probe imaging. Without the standard imaging equipment information, readership might find it difficult to apply clinically available probes in their practice.

Reply 3: We appreciate your valuable comment. We added the column of imaging systems for NIR-I fluorescent probes (see Page 35, Table 1) and hybrid NIR-I fluorescent probes (see Page 36, Table 2). As the reviewer mentioned, we added Table 3 to summarize the information including imaging systems of NIR-II fluorescent probes. (see Page 37, Table 3) We added the sentence "The studies of NIR-II fluorescent probes for HCC are described in Table 3." in the modified version (see Page 16, Line 19).

Comment 4: The reviewer like to see the future perspective of NIR-II fluorescent probe in aspects of penetration depth, how deep we can see through the tissues in parenchyma. What are the current limitations of NIR-I fluorescent probes in this aspect?

Reply 4: Thank you very much for the constructive comment. In terms of penetration depth, imaging in the NIR-I spectral range is not an optimal option. The application of ICG NIR-I fluorescence imaging in hepatectomy for HCCs remains to suffer the limited

tissue penetration depth. It has been reported that tumors located deeper than 10 mm from liver surface are difficult to identify as luminous nodules, presumably, their maximum detection depth is only 10 mm (Tanaka T, Takatsuki M, Hidaka M, et al. Is a fluorescence navigation system with indocyanine green effective enough to detect liver malignancies? J Hepatobiliary Pancreat Sci 2014;21:199-204.). Imaging in the NIR-II window affords significantly decreased light scattering by biological tissues as well as diminished background autofluorescence. These effects could be ascribed to the lower extinction coefficients of biological substrates in the longer electromagnetic spectral regions, especially in NIR-II window, which leads to reduced interactions between the longer-wavelength photons and the surrounding biological components. Collectively, this can result in deeper tissue penetration that may otherwise not be attainable with the current NIR-I imaging. However, NIR-II imaging is still difficult to break through the penetration depth of several centimeters for large parenchymal organs like the liver, and the intraoperative detection of deep HCC remains challenging. Intriguingly, it is worth highlighting that the high optical absorptivity of NIR-II fluorophores can also facilitate their NIR-I or NIR II photo acoustic (PA) imaging. A large number of exogenous NIR-II contrast agents could also be possibly used for dual-modal fluorescence and PA imaging. Since fluorescence imaging generally provides a higher spatiotemporal resolution while a deeper penetration depth can be intrinsically achieved with PA imaging, we believe that dual-modal NIR-II fluorescence and PA imaging will be a balancing act to maximize the desired parameters for hepatectomy of HCC. We added the discussion of penetration depth in the Challenges and Perspectives section (see Page

20, Line 17).

Comment 5: The reviewer suggests that authors add and discuss the recently published article, Luciano et al., "A biliary tract-specific near-infrared fluorescent dye for image-guided hepatobiliary surgery," Molecular Pharmaceutics 16(7), 3253-3260 (2019).

Reply 5: Thank you very much for your helpful comment. As indicated in the title of our article, “The Application of Near-Infrared Fluorescence Imaging in Hepatectomy for Hepatocellular Carcinoma”, we reviewed the development of various near-infrared fluorescence (NIRF) probes in preclinical studies and clinical applications in hepatectomy for hepatocellular carcinoma (HCC), a specific cancer. We have carefully read the article and found that the authors reported a novel NIR fluorophore, BL (Bile Label)-760, which was used for biliary tract imaging. We believe that BL-760 has highly promising properties for intraoperative navigation during hepatobiliary surgery, but there is no content about HCC in this paper. In fact, in the process of collecting relevant literatures, we excluded the studies unrelated to HCC.

Comment 6: Narrative Review Checklist needs to be completed.

Reply 6: The detailed Page Number, Line Number, Section and Paragraph have been indicated in the checklist. Narrative Review Checklist has been provided as an additional file.