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Original Article

Evaluation of Near-infrared Fluorescence-conjugated Peptides for Visualization of Human Epidermal Receptor 2-overexpressed Gastric Cancer

Kyoungyun Jeong [1], Seong-Ho Kong [2,3], Seong-Woo Bae [1], Cho Rong Park[1,4,5], Felix Berlth [6], Jae Hwan Shin[7], Yun-Sang Lee [1,7], Hyewon Youn [4,5], Eunhee Koo[1], Yun-Suhk Suh [2,3,8], Do Joong Park [1,2,3], Hyuk-Joon Lee [1,2,3], Han-Kwang Yang [1,2,3]

[1]Cancer Research Institute, Seoul National University, Seoul, Korea
[2]Department of Surgery, Seoul National University Hospital, Seoul, Korea
[3]Department of Surgery, Seoul National University College of Medicine, Seoul, Korea
[4]Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Korea
[5]Department of Nuclear Medicine, Seoul National University College of Medicine, Seoul, Korea
[6]Department of General, Visceral and Transplant Surgery, University of Mainz, Mainz, Germany
[7]Institute of Radiation Medicine, Medical Research Center, Seoul National University College of Medicine Seoul, Korea
[8]Department of Surgery, Seoul National University Bundang Hospital, Seongnam, Korea

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Correspondence to
Seong-Ho Kong
Department of Surgery, Seoul National University Hospital, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 03080, Korea.
E-mail: seongho.kong@snu.ac.kr

ORCID iDs
Kyoungyun Jeong
https://orcid.org/0000-0002-5249-1142
Seong-Ho Kong
https://orcid.org/0000-0002-3929-796X
Seong-Woo Bae
https://orcid.org/0000-0001-6530-3983
Felix Berlth
https://orcid.org/0000-0002-3780-0728
Yun-Sang Lee
https://orcid.org/0000-0003-1640-2090
Hyewon Youn
https://orcid.org/0000-0003-1006-2689
Yun-Suhk Suh
https://orcid.org/0000-0003-3319-8482
Do Joong Park
https://orcid.org/0000-0001-9644-6127
Hyuk-Joon Lee
https://orcid.org/0000-0002-9530-647X

ABSTRACT

Purpose: A near-infrared (NIR) fluorescence imaging is a promising tool for cancer-specific image guided surgery. Human epidermal receptor 2 (HER2) is one of the candidate markers for gastric cancer. In this study, we aimed to synthesize HER2-specific NIR fluorescence probes and evaluate their applicability in cancer-specific image-guided surgeries using an animal model.
Materials and Methods: An NIR dye emitting light at 800 nm (IRDye800CW; Li-COR) was conjugated to trastuzumab and an HER2-specific affibody using a click mechanism. HER2 affinity was assessed using surface plasmon resonance. Gastric cancer cell lines (NCI-N87 and SNU-601) were subcutaneously implanted into female BALB/c nu (6–8 weeks old) mice. After intravenous injection of the probes, biodistribution and fluorescence signal intensity were measured using Lumina II (Perkin Elmer) and a laparoscopic NIR camera (InTheSmart).
Results: Trastuzumab-IRDye800CW exhibited high affinity for HER2 (K_D=2.093(3) pM). Fluorescence signals in the liver and spleen were the highest at 24 hours post injection, while the signal in HER2-positive tumor cells increased until 72 hours, as assessed using the Lumina II system. The signal corresponding to the tumor was visually identified and clearly differentiated from the liver after 72 hours using a laparoscopic NIR camera. Affibody-IRDye800CW also exhibited high affinity for HER2 (K_D=4.71 nM); however, the signal was not identified in the tumor, probably owing to rapid renal clearance.
Conclusions: Trastuzumab-IRDye800CW may be used as a potential NIR probe that can be injected 2–3 days before surgery to obtain high HER2-specific signal and contrast. Affibody-based NIR probes may require modifications to enhance mobilization to the tumor site.

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Han-Kwang Yang
https://orcid.org/0000-0003-3495-3048

Presentation
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Author Contributions
Conceptualization: K.S.H.; Data curation: J.K., B.S.W.; Formal analysis: J.K., K.E.; Methodology: K.S.H., J.K., P.C.R., Y.H., S.J.H., L.Y.S., Y.H.K., L.H.J., S.Y.S., P.D.J., K.E., B.S.W.; Project administration: K.S.H., J.K., B.S.W., B.F.; Resources: S.J.H., L.Y.S.; Software: P.C.R., J.K.; Supervision: K.S.H., Y.H.K., L.H.J., P.D.J., S.Y.S., Y.H., L.Y.S.; Validation: K.S.H.; Visualization: J.K.; Writing - original draft: J.K.; Writing - review & editing: K.S.H., J.K., P.C.R., Y.H., S.J.H., L.Y.S., Y.H.K., L.H.J., S.Y.S., P.D.J., K.E., B.S.W.

Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Keywords: Stomach neoplasms; Gastrectomy; Surgery; Fluorescence

INTRODUCTION

Near-infrared (NIR) fluorescence-guided surgery using a dedicated imaging system and tracers to visualize light at 700–900 nm has gained attention in the last several years because of its high sensitivity for visualizing functional signals with deep penetration; moreover, it is relatively inexpensive and utilizes non-toxic materials [1,2]. One of the most extensively researched applications of NIR fluorescence-guided surgery is node-navigation using indocyanine green (ICG) [3], a dye known for its ease of use and safety profile compared to radioisotopes. ICG has been used for a variety of cancers; however, its rapid diffusion characteristics and non-specificity hinder its use in sentinel node-navigation or in deciding the extent of resection.

The development of cancer-specific NIR tracers through the conjugation of NIR dyes to antibodies with affinity to specific molecular targets has been suggested to overcome and supplement non-specific fluorescence-based surgical imaging for cancer using ICG. Human epidermal receptor 2 (HER2) is a transmembrane glycoprotein of the receptor tyrosine kinase family that is overexpressed in many types of cancers, especially breast and gastric cancers [4,5]. HER2 is a target with clinical potential because its overexpression has been correlated with poor prognosis [6,7]; furthermore, monoclonal antibody-based targeted drugs may provide a good therapeutic response against cancers with high HER2 expression [8-10]. The commercial availability of targeted drugs in the form of monoclonal antibodies, such as trastuzumab (TRZ), is an added advantage in quickening the development of such probes.

Conversely, there are several concerns regarding the use of whole IgG antibodies because of the disadvantages of slow infiltration into the target tissue and delayed removal owing to the large molecular size. It can be inconvenient to administer the tracer and wait for a few days to conduct surgery; furthermore, false positivity can be caused by the retention of tracers in the tissue. To overcome these disadvantages, the use of smaller molecules with high affinities for the same target has been considered. Affibody is one such small peptide with a size of 6–7 kDa and sufficient affinity for a specific target. Owing to its fast penetration and removal from the system, surgeons may administer it just before or while performing the surgery as required and a higher signal-to-background ratio (SBR) can be obtained, lowering the risk of false positivity.

In this study, we aimed to generate a HER2-specific NIR tracer by conjugating an NIR dye, IRDye800CW® (Li-COR, Lincoln, NE, USA), to either TRZ or an HER2-specific affibody and compare biodistribution and visibility using a commercially available laparoscopic NIR camera system.

MATERIALS AND METHODS

Cell culture

NCI-N87 and SNU-601 were selected as HER2-positive and HER2-negative gastric cancer cell lines, respectively, and were obtained from the Korean Cell Line Bank (Seoul, Korea). They were maintained in Roswell Park Memorial Institute (RPMI 1640) media (Welgene,

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Gyeongsan, Korea) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Loughborough, UK) and 2% penicillin/streptomycin (Gibco) and were incubated at 37°C in a 5% CO_2 humidified environment.

Synthesis of the HER2-specific probe

The IRDye800CW azide infrared dye (Li-COR) was conjugated to TRZ (Roche, Basel, Switzerland) and an HER2-specific affibody (ABY; Affibody AB, Solna, Sweden) using a click mechanism with an azide site of the dye [11]. IRDye800CW azide in water was added to TRZ-dibenzocyclooctyne group (DBCO)-PEG4-maleimide (Futurechem. Co, Seoul, Korea) and an affibody-DBCO solution in phosphate-buffered saline (PBS). The mixture was vortexed at 38°C for 3 hours and purified using a PD-10 column (GE Healthcare, Chicago, IL, USA) with PBS to obtain TRZ-IRDye800CW (TRZ-Dye) and HER2-specific affibody-IRDye800CW (ABY-Dye).

Binding affinity assay

Surface plasmon resonance (SPR) experiments were performed using a Reichert SR7500C (Reichert Technologies, Depew, NY, USA) and a Biacore T200 instrument (GE Healthcare, Uppsala, Sweden). For HER2/FC (R&D Systems, Minneapolis, MN, USA), 1,100 relative unit proteins were captured on the protein-modified PEG sensor chip using an immobilization-capture wizard. We evaluated kinetics data using Scrubber2 (BioLogic Software Pty Ltd., Canberra, Australia) and Biacore T200 Control software version 2.0.1 and BIAevaluation software version 3.0 (GE Healthcare, Uppsala, Sweden).

Immunofluorescence

Cells (1×10^4) were plated in 6-well dishes coated with poly-L-lysine (Sigma-Aldrich, Burlington, MA, USA). When cultured cell confluency reached up to 80%, we applied a 4% paraformaldehyde solution (Thermo Fisher Scientific, Waltham, MA, USA). Both conjugates were added, and the dishes were incubated overnight. After washing the plates several times, DAPI ProLongGold (Invitrogen, Waltham, MA, USA) was added to stain the nuclei. Cultured cells were scanned and verified through the established in vitro fluorescence methods using ImageXpress (Molecular Devices, San Jose, CA, USA) and LSM800 (Zeiss, Oberkochen, Germany).

Fluorescence imaging of mice

Each cell line was harvested, and 1×10^6 cells were injected subcutaneously into the right flank of 6–8-week-old female BALB/c-nu mice (Orient Bio., Seongnam, Korea).

Tumor size was measured twice per week using calipers, and the tumor volume was evaluated as follows: 1/2×length (L)×width (W)×height (H) (mm^3).

The dose of TRZ-Dye to be used was determined to be 100 μg, which is the minimum dose for tracer detection with a fluorescence camera; the dose of ABY-Dye was determined to be 50 μg for injection into each mouse, taking into account the molecular weight and affinity. The dyes were injected into the tail vein and imaged at different timepoints post injection.

A charge-coupled device Lumina II IVIS® system (Perkin Elmer, Waltham, MA, USA) and a laparoscopic camera system for human surgery with an NIR imaging function (InTheSmart, Seoul, Korea) were used to image the tumors in living mice. The excitation laser wavelength was 798 nm and peak detection wavelength was 818 nm. We constructed a custom-made training box to prevent external light; imaging was performed by alternating the distance between the mice and the NIR camera to 3, 5, and 8 cm. All images processed with IVIS®

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were quantified using Living Image 2.50.1 (Perkin Elmer), and laparoscopic NIR camera images were analyzed using Image J software (http://rsb.info.nih.gov/ij/; National Institutes of Health, Bethesda, MD, USA). The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Clinical Research Institute at Seoul National University Hospital (SNU-180308-1-2).

Tissue histology

At each timepoint of the imaging sequences, all mice were euthanized and an autopsy was conducted to collect the primary tumors. The tumors were fixed using 10% neutral buffered formalin solution (Sigma-Aldrich) for 12–24 hours, paraffin-embedded, and pathologically examined by hematoxylin and eosin (H&E) staining. The formalin-fixed paraffin-embedded tissues were sectioned at a thickness of approximately 4 μm and stained with H&E for histological analysis using Image Scope (Aperio; Leica Biosystems, Buffalo Grove, IL, USA); immunohistochemistry (IHC) was performed using a HER2-specific antibody (Sigma-Aldrich), which binds to epitopes that bind to either TRZ-Dye or ABY-Dye.

Statistical analysis

The non-parametric Mann-Whitney U test was used to compare variables between the 2 groups. The Wilcoxon signed-rank test was used to determine differences in the regions of interest of tumors and organs. Statistical analysis was performed using GraphPad PRISM version 8.0.0 (GraphPad Software, San Diego, CA, USA) for non-parametric data. All parametric data are presented as mean ± standard deviation. Statistical significance was defined as $P<0.05$.

RESULTS

We synthesized a HER2-specific probe using NIR fluorescence dye and peptides (**Fig. 1A**), which exhibited a single peak with the same absorbance at a wavelength of approximately 800 nm (**Fig. 1B**). The binding affinity of TRZ-Dye, which was determined using SPR, was higher than that of TRZ alone (**Supplementary Fig. 1A**). The K_D value was calculated by dividing Kd (dissociation time) by Ka (association time); thus, a lower K_D value indicates higher affinity, and a K_D value lower than 10^{-9} represents high affinity to the target protein. The K_D values of TRZ-Dye and TRZ were 2.093×10^{-12} and 25.75×10^{-12}, respectively (**Table 1**). The K_D value of the affibody decreased slightly after conjugation with IRDye800CW (4.71×10^{-9} to 1.42×10^{-9}); however, ABY-Dye still exhibited sufficiently high affinity (**Supplementary Fig. 1B**).

Both TRZ-Dye and ABY-Dye were attached to the cell membrane of HER2-positive cells (NCI-N87) but not to the membrane of the HER2-negative cells (SNU-601), as assessed by confocal microscopy. Furthermore, TRZ-Dye was internalized and localized to the cytoplasm of HER2-positive cells (**Supplementary Fig. 2**).

Fluorescence signal intensity of the HER2-positive tumor in the mouse model, measured using the IVIS® system, increased gradually throughout the 72-hour period after injecting TRZ-Dye (**Fig. 2A and B**). Meanwhile, the intensities in the liver and spleen were highest at 24 hours and thereafter decreased (**Fig. 2C and D**).

Fluorescence signals were detected using a human NIR-laparoscopic camera system. Similarly, in the IVIS® system, the tumor signal gradually increased and was detectable at 24

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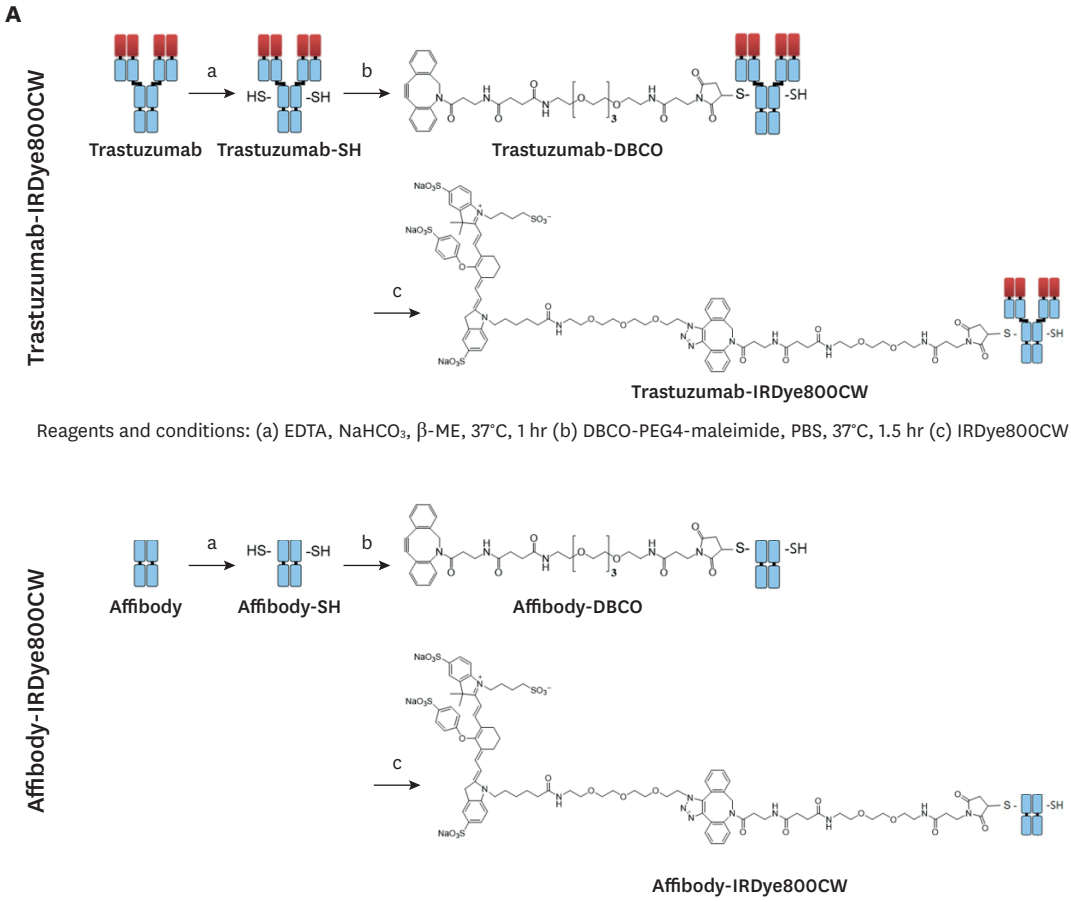

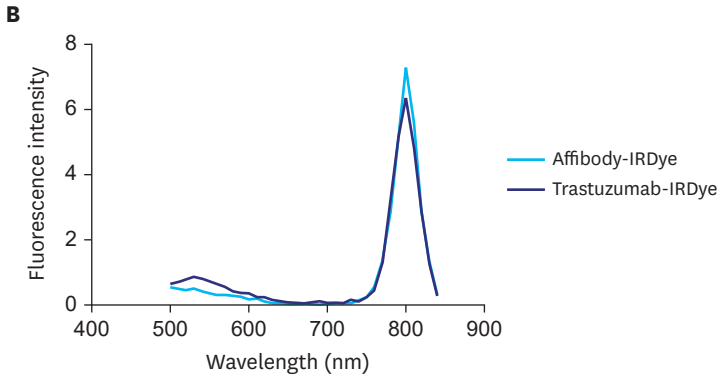

Fig. 1. (A) Schematic diagram of the synthesis of trastuzumab-IRDye800CW and affibody-IRDye800CW. Trastuzumab and affibody were first conjugated to DBCO. Then, IRDye800CW was attached via copper-free click chemistry. (B) Absorbance spectrum of trastuzumab-IRDye800CW and affibody-IRDye800CW. Both modified peptides emit light at the same peak wavelength of 800 nm detected using the microplate reader, which indicates that both the antibody and affibody were successfully conjugated to IRDye800CW.
β-ME = 2-mercaptoethanol; DBCO, dibenzocyclooctyne group.

hours post injection; however, peak signal intensity was observed at 48 hours after injecting TRZ-Dye (**Fig. 3**). The tumor signal was detectable at distances of 3, 5, and 8 cm between the tumor and camera, and the signal was distinguishable from the background signal of the liver from 24 to 72 hours post injection (**Fig. 3A**).

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Table 1. Equilibrium dissociation constants

Ligand	Analyte	Ka ($M^{-1}s^{-1}$)	Kd (s^{-1})	K_D (nM)
HER2	Unlabeled trastuzumab	4.69E+05	1.207E-05	25.75E-12
	Trastuzumab-DBCO	4.916E+05	2.720E-05	55.32E-12
	Trastuzumab-DBCO-IRDye800CW	3.725E+05	8E-07	2.093E-12
HER2	Unlabeled affibody	3.10E+04	4.38E-05	1.42E-09
	Affibody-DBCO	5.27E+04	9.57E-05	1.82E-09
	Affibody-DBCO-IRDye800CW	1.36E+04	6.40E-05	4.71E-09

Data were entered into a 1:1 kinetic binding model, and the values of Ka ($M^{-1}s^{-1}$), Kd (s^{-1}), and K_D (nM) were calculated.
Ka = association time; Kd = dissociation time; K_D = Kd/Ka; HER2 = human epidermal receptor 2; DBCO = dibenzocyclooctyne group.

After 72 hours post injection, we sacrificed the mice and compared the signals obtained using the NIR-laparoscopic camera and IVIS®. The obtained signal seemed to be higher than that of the ex vivo images, suggesting that the fluorescence probe must have accumulated in HER2-positive tumors via blood circulation (**Fig. 3A and B**). SBRs of each probe were calculated by normalizing signals from the tumor area to those from the non-tumor area (**Fig. 3C**).

Unlike that of TRZ-Dye, the signal of ABY-Dye was dispersed throughout the body and was slightly stronger in the tumor and kidney at 30 minutes post injection. Moreover, the signal in the tumor and in the body rapidly increased and accumulated in the kidney and bladder at 1 hour post injection (**Fig. 4**).

We extracted the tumor and performed IHC analysis 72 hours after injecting the probes, once the fluorescence signal faded; this was done to assess whether the lower signal could be attributed to HER2 expression in the cell lines (**Supplementary Fig. 3**).

DISCUSSION

In recent years, NIR fluorescence imaging has been widely investigated, particularly with regard to the intraoperative visualization of tumors and/or lymphatic channels [12-14]. Owing to the ability of NIR fluorescence probes to penetrate deep into tissue and the development of commercialized laparoscopic imaging systems, research has been conducted to develop probes specific to cancer surface markers.

These cancer-specific NIR probes can assist surgeons in discriminating the lesions easily and rapidly determining the required extent of dissection, which in turn allows them to make decisions in a shorter duration than possible with the use of conventional pathologic examinations during surgery [15-17].

Although a molecular target may only be expressed in smaller proportion of cancer cases—for example, HER2 amplification is shown noted in less than 20% of gastric cancer cases—to utilize antibody-based probes for a specific target, NIR probes can also be useful in indicating or evaluating the response to pharmaceutical target therapy in cases where a monoclonal antibody, such as TRZ, is available [18]. To overcome this limitation, a combination of multiple antibody-based probes targeting other clinically meaningful targets, such as other types of epidermal growth factor receptors and vascular endothelial growth factor, can be used [19]. Furthermore, the utilization of previously reported cancer-specific molecules such as carcinoembryonic antigen, which are not meaningful as treatment targets but could be

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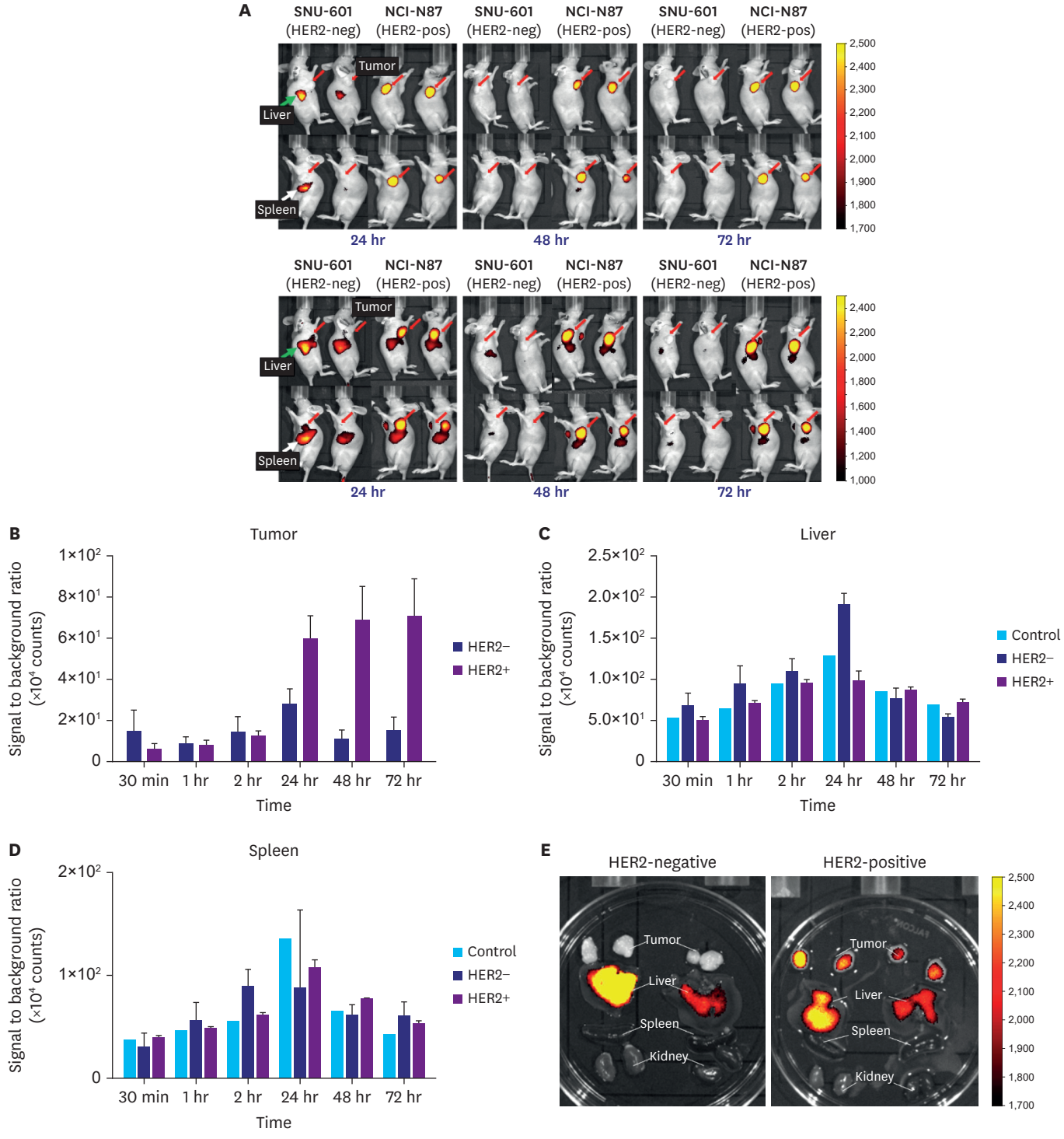

Fig. 2. (A) Images of mice at 1–3 days after injection with trastuzumab-IRDye800CW. Colored arrows indicate the fluorescence signal in the liver (green), spleen (white), and tumor (red). Both images are the same results adjusted to scale. (B) Tumor fluorescence intensity. Fluorescence signal of HER2-positive tumors appeared from 24 to 72 hours after injection of trastuzumab-IRDye800CW. The 48-hour mark appears to be the appropriate time to visualize HER2-positive tumors compared to the HER2-negative group. (C, D) Liver fluorescence intensity and spleen fluorescence intensity. Liver and spleen signals showed high intensity at 24 hours after injection. No differences were noted compared to the control. (B-D) Signal intensities were quantified for the tumor and non-tumor regions by the IVIS® system. All signal intensities were defined by SBR. (E) Ex vivo near-infrared image of sacrificed mice. After 72 hours, mice were autopsied to check the signal.
HER2 = human epidermal receptor 2; SBR = signal-to-background ratio.

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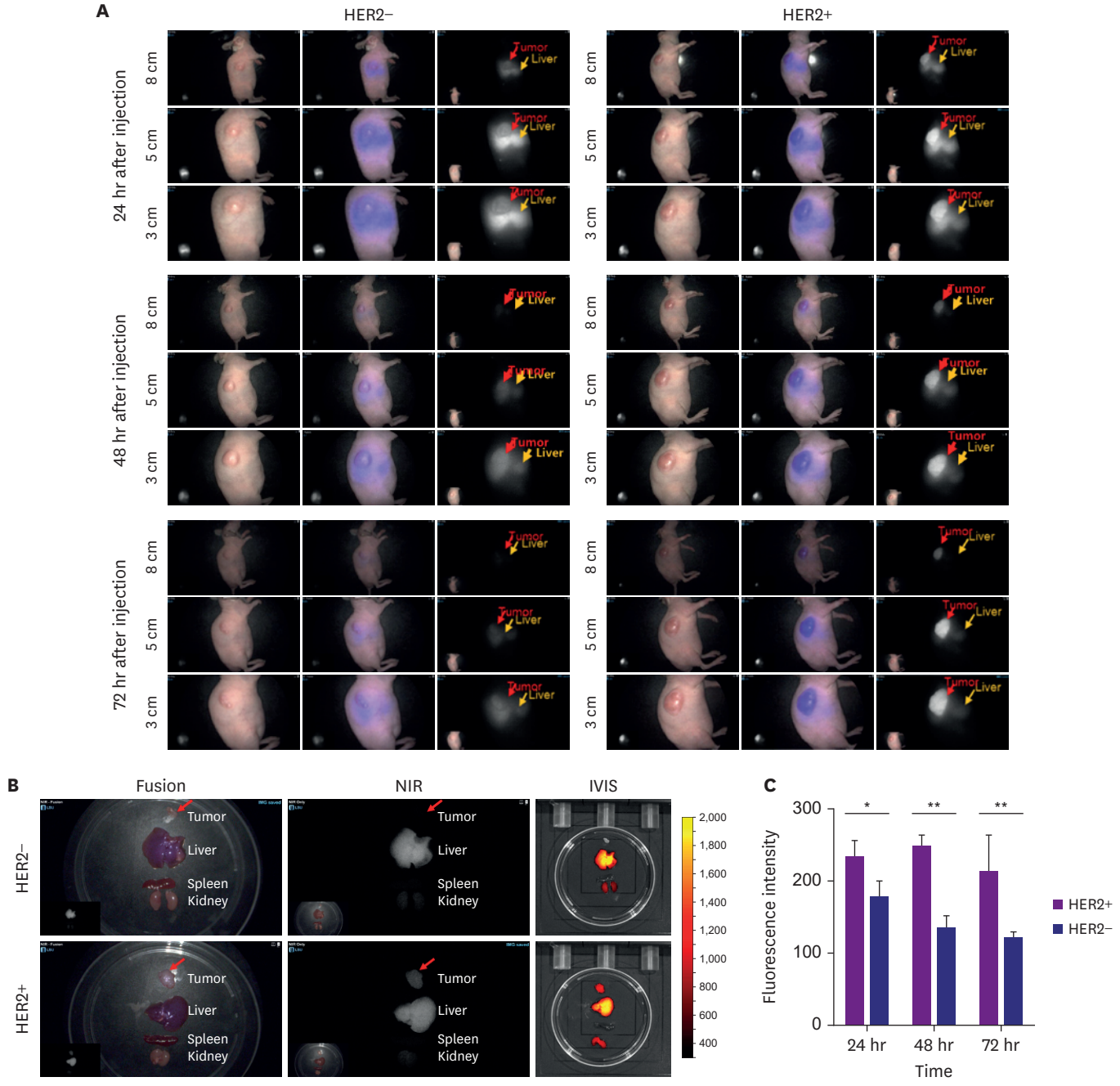

Fig. 3. (A) NIR-laparoscopic images at different distances and timepoints. Images of mice 1–3 days after injection with trastuzumab-IRDye800CW at different distances and timepoints obtained using the NIR-laparoscopic camera. Highest uptake considering background signal appears to be from 48 to 72 hours after injection. (B) Ex vivo images at 72 hours after injection. *Ex vivo* NIR images of sacrificed mice taken both by the laparoscopic camera and by the IVIS® system. Red arrow indicates tumor. (C) Graph of fluorescence intensity detected by NIR-laparoscopic camera. Signal intensity of NIR images quantified by ImageJ software indicates that the optimal timepoint would be 48 hours.
NIR = near-infrared; HER2 = human epidermal receptor 2.
*P<0.05, **P<0.001.

used as visualization targets, and exploration of new targets expressed not only from cancer cells but also from the cancer microenvironment can be considered to maximize clinical applicability [20].

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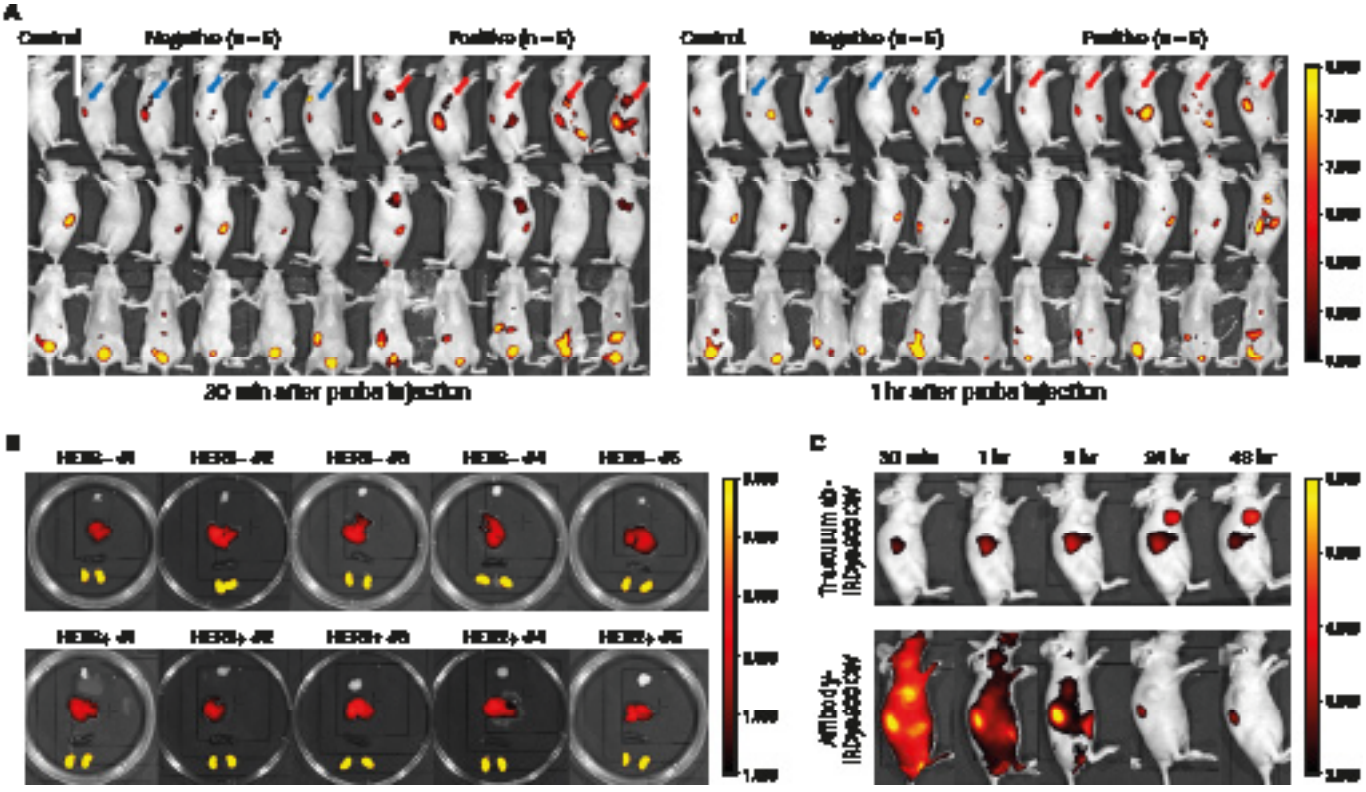

Fig. 4. (A) Fluorescence intensity of affibody-IRDye800CW. At 30 minutes and 1 hours after injection of the probe, a slight signal was observed, which washed out immediately. (B) Ex vivo images of affibody-IRDye800CW. All fluorescence accumulated to the kidney after 72 hours, suggesting that affibody-IRDye800CW cannot detect HER2-positive tumors effectively. (C) Images of trastuzumab-IRDye800CW and affibody-IRDye800CW with an adjusted scale bar. When adjusting the scale bar, fluorescence was identified n HER2-positive tumors at 30 minutes after injection.
HER2 = human epidermal receptor 2.

Several approaches for the development of NIR molecular probes have been suggested. One method is to use the phage display technique to select molecules with the highest affinities to the target from a chemically generated library of fluorescence-emitting small molecules. Alternatively, a more direct way would be to conjugate NIR dyes to proteins with a high affinity to the target.

Probes can be characterized based on their sizes. Antibodies are relatively large biomolecules, and affibody is one of the smallest antibody mimetics [21]. A HER2-target-affibody (Affibody AB) is composed of 6–7 kDa peptides, and it does not bind the same epitope as that of HER2, which is targeted by TRZ [22-24].

TRZ may be disadvantaged by slow penetration and removal caused by its large molecular size, resulting in lower SBR and high risk of false positivity; however, it is advantageous in that it has been proven to have high affinity and to be therapeutically safe [22,25]. In contrast, affibody, given its small molecular size, was expected to have the advantage of being used more liberally during surgery owing to its fast biodistribution. In the present study, both antibody and affibody labeled with IRDye800CW had high binding affinity, which was sufficient for visualizing the tumor in vivo (**Table 1**). Considering that most antibodies exhibit K_D values in the low micromolar to nanomolar range (10^{-6}–10^{-9}), those with K_D values of 10^{-9}–10^{-12} and less than 10^{-12} indicate high and very high affinities, respectively. The K_D values of TRZ-Dye and ABY-Dye were 2.09×10^{-12} and 4.71×10^{-9}, respectively, thereby representing sufficiently high affinities as probes [26].

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Contrary to our expectations, ABY-Dye was not found to be clinically applicable in vivo because of its very short retention time in circulation and filtration by the kidney within 1 hour (**Fig. 4**). Thus, we may consider further modifying the affibody by attaching additional molecules to slow down renal clearance and enhance delivery to the target organ [27]. Such a method would be an example of high-efficiency targeting of proteins in vitro; however, the applicability of this method cannot always be guaranteed in vivo for the required time. If we focus on the visualization of the lymph node metastasis only, peritumoral injection of ABY-Dye could be another option to overcome the delivery problem for the systemic injection of small molecules.

Conversely, TRZ-Dye exhibited relatively sensitive and specific NIR signals despite having a slow peak time. Because of its large size and internalization into tumor cells, as shown in **Supplementary Fig. 2**, the TRZ-Dye signal was detectable for more than 48–72 hours (**Fig. 2**). A decrease in the background signal of the liver and spleen after 24 hours of injection resulted in the highest SBR. For further application in a clinical setting, TRZ-Dye administration 2–3 days before surgery may provide useful images for the surgery.

Unlike the imaging systems previously used for small animals such as the IVIS® system, which are too sensitive to reflect signal intensities in real-time, we used an NIR-laparoscopic camera for human use to ensure that the signal would be strong enough for detection. Because the sensitivity of the laparoscopic system is highly dependent on the distance between the object being imaged and the camera, we evaluated the signal at 3, 5, and 8 cm, which are feasible distances of separation for application during surgery (**Fig. 3**). The NIR signal was detected at all distances, and the tumor signal was clearly distinguished from the liver background signal.

In conclusion, we generated probes with high affinity for HER2 in vitro through the conjugation of IRDye800CW with an HER2-specific antibody and affibody. The affibody-based probe failed to generate useful in vivo images, which was likely owing to rapid renal clearance. However, a TRZ -based probe was found to be useful for visualizing the maximal SBR at 48 hours post injection using an imaging system for small animals and a commercialized NIR-laparoscopic system for human use. Thus, TRZ -IRDye800CW could be used as a potential NIR probe for use during gastric cancer surgery to detect HER2-positive tumors and to determine the necessary treatment strategy.

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SUPPLEMENTARY MATERIALS

Supplementary Fig. 1

Binding affinities of the conjugates. Surface plasmon resonance results. (A) Trastuzumab, trastuzumab-DBCO, and trastuzumab-IRDye800CW. (B) Affibody, affibody-DBCO, and affibody-IRDye800CW.

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Supplementary Fig. 2

TRZ-Dye- and ABY-Dye-stained images of gastric cancer cell lines. Both TRZ-Dye and ABY-Dye were diluted in complete RPMI and incubated with NCI-N87 and SNU-601 cells. Confocal microscopy images showed that TRZ-Dye localized in the cytoplasm of the HER2-positive cell line, NCI-N87. (A) TRZ-Dye-stained images of gastric cancer cell lines. TRZ-Dye was not detected in the HER2 negative cell line, SNU-601. (B) ABY-Dye-stained images of gastric cancer cell lines. ABY-Dye was attached only at the cell membrane.

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Supplementary Fig. 3

Immunohistochemistry and H&E) staining (×100). Histological observations of SNU-601 and NCI-N87 tumors. NCI-N87 expressed very high levels of HER2, while SNU-601 expressed low levels of HER2.

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