In vivo fluorescence imaging of hepatocellular carcinoma xenograft using near-infrared labeled epidermal growth factor receptor (EGFR) peptide

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Abstract: Minimally-invasive surgery of hepatocellular carcinoma (HCC) can be limited by poor tumor visualization with white light. We demonstrate systemic administration of a Cy5.5-labeled peptide specific for epidermal growth factor receptor (EGFR) to target HCC in vivo in a mouse xenograft model. We attached a compact imaging module to the proximal end of a medical laparoscope to collect near-infrared fluorescence and reflectance images concurrently at 15 frames/sec. We measured a mean target-to-background ratio of 2.99 ± 0.22 from 13 surgically exposed subcutaneous human HCC tumors in vivo in 5 mice. This integrated imaging methodology is promising to guide laparoscopic resection of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related deaths worldwide [1]. The prognosis for HCC is poor with a 5-year survival rate of less than 15%. Although liver transplantation is the preferred choice, this option is often limited by a shortage of donor organs. Surgery can be curative with a 5-year survival rate >50% if the tumors can be found at an early stage [2]. Accurate identification of tumor margins can minimize tumor recurrence and preserve function in cirrhotic livers.

Current methods for imaging HCC, including CT, MRI, and PET, cannot provide real-time image guidance. Ultrasound is used frequently but provides low contrast and lacks tumor specificity. Laparoscopy is minimally invasive, and can minimize morbidity to the patient [3]. However, the optics limits the field-of-view available to visualize tumors, and use of visible illumination provides low contrast. Methods that improve image contrast are needed. Indocyanine green (ICG) has been used to enhance tumor contrast [4], however, this dye is non-specific, and cannot distinguish HCC from benign cirrhotic nodules, which are frequently found during surgery. Use of near-infrared (NIR) light minimizes hemoglobin absorption, and can penetrate up to 1-2 cm in tissue [5].

Targeted methods can provide a biological basis for disease detection. Antibodies are commonly used, but they are bulky, and can get trapped in the liver and spleen [6]. By comparison, peptides are smaller in size and lower in molecular weight, and can provide improved pharmacokinetic properties for diagnostic imaging, including greater extravasation from leaky vasculature and enhanced tissue diffusion to achieve greater penetration and higher concentration in tumors [7]. Also, peptides can clear rapidly, and result in reduced biodistribution to non-target tissues, and have less potential for immunogenicity than antibodies, which allows for repeated use.

Epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase that is overexpressed in 40% to 70% of HCC [8]. High levels of EGFR expression is associated with early recurrence and reduced survival rates following surgical resection [9]. Recently, we have identified a peptide specific for EGFR [10]. Here we aim to demonstrate proof-of-concept for use of a peptide specific for EGFR to detect human HCC using a conventional surgical laparoscope by imaging xenograft tumors in vivo in mice using NIR fluorescence.

2. Methods

2.1 Imaging agent

We used standard Fmoc solid-phase synthesis to synthesize a monomeric linear 7 amino acid peptide with sequence QRHKPRE that has previously been found to bind specifically to EGFR along with a scrambled (control) peptide PEHKRRQ [10]. Both peptides were labeled with water soluble sulfo-Cy5.5-N-hydroxysuccinimide ester (Lumiprobe LLC) on the C-terminus using a GGGSK linker, hereafter QRH*-Cy5.5 and PEH*-Cy5.5. We purified the Cy5.5-labeled peptides to >95% on HPLC, and confirmed properties of the final compound with Q-TOF (Agilent Technologies) mass spec analysis and ESI (Waters Inc) detection. After completion of synthesis, the peptides were lyophilized and stored at −80°C.
2.2 Imaging system

We adapted a standard surgical laparoscope (#49003 AA, HOPKINS® II Straight Forward Telescope 0°, Karl Storz) that is ~10 mm in diameter and has a 31 cm length rigid sheath to collect reflectance and fluorescence concurrently. We used as a solid state diode laser (660-S, Toptica Photonics) to deliver excitation at $\lambda_{\text{ex}} = 660$ nm into the fiber optic light guide. Reflectance from the laparoscope is reflected by a dichroic mirror DM (FF685-Di02-25x36, Semrock) through a neutral density filter ND (Thorlabs, #ND10B), and is focused by an achromatic doublet $O_1$ (49-766, Edmund Optics) onto a color CCD camera CCD1 (#GX-FW-28S5C-C, Point Grey Research).

![Fig. 1. Schematic. Light leaving proximal end of laparoscope is split by dichroic mirror (DM). Reflectance at $\lambda_{\text{ex}} = 660$ nm is attenuated by neutral density filter (ND) and focused by objective $O_1$ onto color camera CCD1. NIR fluorescence is bandpass filtered (BPF) at center wavelength $\lambda = 716$ nm over a 40 nm band and is focused by objective $O_2$ onto monochrome camera CCD2.

Fluorescence from the laparoscope passes through a bandpass filter BPF (67-039, Edmund Optics) with 40 nm bandwidth centered at 716 nm, optical density $\OD \geq 6.0$, and 93% transmission efficiency and is focused by an achromatic doublet $O_2$ (49-766, Edmund Optics) onto a monochrome CCD camera CCD2 (Point Grey Research, #GX-FW-28S5M-C). Both cameras weigh 86 grams, and collect images with a maximum of 2.8 megapixels and 1932 $\times$ 1452 resolution. Co-registered fluorescence and reflectance images were collected at 15 frames per second. Reflectance is used to guide collection of NIR fluorescence images when the white light camera is detached.

2.3 HCC xenograft tumors

All experimental procedures were performed in accordance with University of Michigan guidelines and regulations. All animal experiments were performed with approval by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan. We used SK-Hep1 human HCC cells (ATCC) that have previously been shown to overexpress EGFR [11]. These cells were grown in culture with Eagle's Minimum Essential Medium (EMEM) at 37 °C in 5% CO$_2$ and were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. About $5 \times 10^6$ tumor cells were suspended in Matrigel (BD Bioscience) and were inoculated subcutaneously at several locations in either flank of nude athymic mice (nu/nu, Jackson Laboratory) at 6–8 weeks of age. The mice were fed with a special rodent diet (AIN-76A, Lot#180565) to minimize background from autofluorescence. Tumor dimensions were monitored weekly with a portable ultrasound (SonixTablet, Ultrasonix, Analogic Corp).

Lyophilized peptides were reconstituted in water at a concentration of 150 $\mu$M in a volume of 200 $\mu$L, and were injected via tail vein in 5 mice at ~3 weeks after inoculation of the tumor cells when the HCC xenografts were ~1-2 cm in size. Fluorescence images from the HCC xenograft tumors in living mice were collected over time to evaluate the pharmacokinetics for tumor uptake of the peptide imaging agent.
The skin overlying the tumors was then surgically exposed. The mice were first anesthetized via a nose cone with inhaled isoflurane (Fluriso, MWI Veterinary Supply Co.) mixed with oxygen at a concentration of 2 to 4% and flow rate of ~0.5 L/min. Imaging was performed first using a standard color camera (S3 3-chip camera head, Karl Storz) with white light illumination. Then, the laser input was directed into the light guide, and the imaging module containing the color and monochrome cameras was attached to collect reflectance and fluorescence concurrently.

After completion of imaging, the fluorescence images were evaluated by identifying 3 regions of interest (ROI) with dimensions of 25 × 25 pixels from representative areas of high and low intensities within the tumor (target) and from surrounding regions of non-tumor (background). The target-to-background (T/B) ratio was then calculated by dividing the mean values of the two ROIs identified. All measurements were performed using custom software written in Matlab (Mathworks, Inc).

Tumors from 5 mice were cut in 5 μm thick sections, and mounted onto glass slides (Superfrost Plus, Fischer Scientific) with ProLong Gold reagent using #1 cover glass (1.5 μm thickness). Confocal fluorescence images were collected with a confocal microscope (Leica TCS SP5 Microsystems) using a Cy5.5 filter set with a 63X oil immersion objective.

2.4 Statistical analysis

For the in vivo fluorescence images, the T/B ratios for HCC with the EGFR and control peptides were log-transformed to improve normality and stabilize variance. The fold-change between classification pairs was estimated using the anti-log of the difference in the log-transformed data. All results are shown as mean ± SD, and all statistical analyses were processed with SPSS (V 17.0) software.

3. Results

3.1 Imaging system

Our laparoscope is used routinely by surgeons to perform radical resection of HCC. On the proximal end, we attached a conventional color camera to collect white light images and can be quickly removed and replaced with the lightweight, compact imaging module to collect co-registered reflectance and fluorescence images, Fig. 2. Excitation at \( \lambda_{\text{ex}} = 660 \) nm was delivered into the fiber optic light guide attached to a side port.

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3.2 Imaging agent

We used the fluorescently-labeled NIR peptide imaging agent QRH*-Cy5.5 that binds specifically to domain 2 of EGFR and a scrambled peptide PEH*-Cy5.5 for control. For both peptides, we measured a molecular weight of 2232.88 g/mol, which agrees with the expected value, and found peak fluorescence emission at $\lambda_{em} = 710$ nm.

3.3 HCC xenograft tumors

![Graph showing the target-to-background (T/B) ratio over time for QRH*-Cy5.5 and PEH*-Cy5.5.]

We measured the NIR fluorescence intensity from 13 tumors in 5 living mice with either QRH*-Cy5.5 or PEH*-Cy5.5 over time to evaluate uptake, Fig. 3. After the peptide injection, the T/B ratios were measured every hour until the maximum value was observed. For QRH*-Cy5.5, the TBR exceeded 2.0 after the first hour, and reached a peak mean ± std value of 2.53 ± 0.20 at 6 hours. After ~24 hours, the T/B ratio returned to ~1. The T/B ratio for PEH*-Cy5.5 was significantly less than that for QRH*-Cy5.5 at each time point.

![Images showing white light, reflectance, and fluorescence images of tumors.]

Fig. 4. In vivo images of HCC xenograft tumors. Representative a) white light, b) reflectance, and c) fluorescence images were collected with the NIR laparoscope ~6 hours after intravenous injection of the EGFR peptide QRH*-Cy5.5 are shown. d-f) The same set of images were collected with scrambled peptide PEH*-Cy5.5 in a different tumor.
About 6 hours after peptide injection, a wide excision was performed in the skin overlying the xenograft tumors for direct laparoscopic visualization. Live video streams were collected at 15 frames per second. Representative white light (Visualization 1), reflectance (Visualization 2), and fluorescence (Visualization 3) videos are shown, along with representative images with either QRH*-Cy5.5, Fig. 4(a)-4(c), and PEH*-Cy5.5, Fig. 4(d)-4(f). For QRH*-Cy5.5, we observed strong fluorescence signal from the HCC xenograft tumors with high contrast and clear tumor margins. For PEH*-Cy5.5, we found only minimal fluorescence intensity.

Fig. 5. EGFR peptide binding to xenograft tumors. a) We quantified T/B ratios from in vivo images collected from 13 tumors in 5 mice for either QRH*-Cy5.5 or PEH*-Cy5.5 and found mean ± std of 2.99 ± 0.22 versus 1.81 ± 0.16, respectively, \( P < 0.0001 \) by unpaired t-test. Representative confocal fluorescence microscopy of excised tumor sections are shown for b) QRH*-Cy5.5 and c) PEH*-Cy5.5 at 63X magnification. Note intense staining of QRH*-Cy5.5 to surface of SK-Hep1 human HCC cells.

We measured the T/B ratio from 13 HCC xenograft tumors found in a total of 5 mice. Individual frames with minimal motion artifacts and no saturated regions were identified. We quantifyed one representative image from each tumor. We found a significantly greater result for QRH*-Cy5.5 compared with that for PEH*-Cy5.5 of 2.99 ± 0.22 versus 1.81 ± 0.16, \( P<0.0001 \) by unpaired t-test, Fig. 5(a).

After imaging was completed, the mice were euthanized, and the tumors were excised and sectioned for examination with confocal fluorescence microscopy at 63X magnification. We observed intense staining of QRH*-Cy5.5 to surface of SK-Hep1 human HCC cells but not for PEH*-Cy5.5, Fig. 5(b), 5(c) to further confirm selective uptake of the EGFR peptide in the HCC xenograft tumor.

4. Discussion

Here, we demonstrate use of a standard surgical laparoscope attached to a compact imaging module to collect NIR fluorescence images in vivo of EGFR overexpressed in HCC xenograft tumors. We measured a T/B ratio that exceeded 2 after the first hour, and reached a peak value of 2.53 at 6 hours. This duration is adequate to guide minimally invasive resection of HCC tumors in the operating theater. We imaged xenografts with dimensions of ∼1-2 cm that are comparable to early stage human HCC tumors. These results demonstrate feasibility for clinical translation of this integrated approach to target tumors with imaging. Previously, we demonstrated topical administration of QRH*-Cy5.5 to bind EGFR overexpressed in mouse colonic adenomas that arise from the epithelial surface. We now show that this peptide can also be systemically administered with good uptake in solid tumors as well.

Xenograft tumors of colorectal cancer have been imaged using a handheld confocal laser endomicroscope following injection of a FITC-labeled antibody specific for EGFR in living mice [12]. The images collected have a field-of-view that is microscopic in scale, thus would be cumbersome for guiding surgical resection. The use of FITC as a label results in
significantly reduced tumor penetration depth compared with that of Cy5.5, a NIR fluorophore. Antibodies are large and bulky in size, and can be easily trapped in tumor vasculature and the reticuloendothelial system. Moreover, they are much more costly to mass manufacture for widespread clinical use.

Use of NIR fluorescence to guide liver surgery has recently been demonstrated using intravenous administration of indocyanine green (ICG) [4]. ICG is FDA approved, and clinical results have shown that enhanced contrast from ICG fluorescence can be helpful in identifying additional HCC tumors and to better characterize tumor margins during resection. ICG enhances tumors based on a non-specific contrast mechanisms, and tumor uptake is achieved primarily by the enhanced permeability and retention (EPR) effect. The peak fluorescence emission can shift depending on the extent of complex formation with serum proteins. Use of targeting moieties, such as peptides, can potentially improve imaging performance compared with ICG by increasing specificity for disease detection.

Clinical detection of HCC has been demonstrated with intravenously administered ICG using a bulky, handheld photodynamic eye (PDE) imaging system (Hamamatsu Photonics, Hamamatsu, Japan) during open laparotomy [13]. Excitation is provided from 750 to 830 nm, and NIR fluorescence at 845 nm is detected with a CCD camera. Fluorescence imaging with laparoscopy has previously been demonstrated for real-time identification of HCC in one patient [14]. A CCD camera is used to collect NIR fluorescence below 810 nm, and a xenon light source was used for excitation. A footswitch was used to change the imaging mode between white light and fluorescence. Previously, a rigid 10 mm diameter endoscope has been developed for for minimally invasive surgery that simultaneously detects color and NIR fluorescence, and was used to image lymph nodes in live pigs using ICG for contrast [15].

Our compact imaging module is similar in weight (977 versus 726 grams), but has higher image resolution (1932 × 1452 versus 960 × 960) and faster speed (15 versus 6.8 frames/sec) to provide clearer images with less motion artifact. We use reflectance rather than color images to examine liver parenchyma for presence of HCC tumors when no signal is apparent on fluorescence.

5. Summary

We demonstrate a novel imaging system that uses a standard surgical laparoscope to perform targeted imaging of overexpressed EGFR in HCC xenograft tumors implanted subcutaneously in living mice. Using a Cy5.5-labeled peptide, we were able to detect lesions in vivo based on the molecular expression of the tumor. By comparison, other fluorescence laparoscopy systems have been developed to detect ICG, a non-specific contrast agent. We used a compact imaging module to collect real time NIR fluorescence videos at 15 frames/sec. We observed high fluorescence intensity from tumors and minimal background from surrounding non-tumor over a period of several hours. This approach is promising for image-guided surgery of HCC by improving visualization while being minimally invasive.

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