Interventional Optical Molecular Imaging Guidance during Percutaneous Biopsy¹

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Purpose:

To investigate indocyanine green (ICG) as a molecular beacon for malignant lesions within the liver and evaluate the ability of a developed handheld imaging system to allow measurement of ICG fluorescence within focal hepatic lesions with high target-to-background ratios in a mouse model.

Materials and Methods:

All animal experiments were approved by the institutional animal care committee. A handheld optical molecular imaging device was constructed to pass through the introducer needle of a standard percutaneous biopsy kit. An ex vivo phantom system was constructed to quantify tissue attenuation properties of ICG in liver parenchyma. Subsequently, intrahepatic colorectal cancer metastases were generated in nude mice, and epifluorescence imaging of ICG, as well as histologic analysis of the explanted livers, was performed at 3 weeks after implantation ($n = 6$). Epifluorescence imaging with the handheld imaging device was then performed on intrahepatic colorectal metastases after the administration of ICG ($n = 15$) at 3, 6, and 24 hours after injection. Target-to-background ratios were calculated for each time point. Subsequently, a core biopsy of intrahepatic colorectal metastases was performed by using a standard clinical 18-gauge biopsy needle.

Results:

There was avid localization of ICG to the focal lesions at all time points. Similarly, fluorescence within the tumors was greater than that within normal liver, as detected with the handheld imaging system (mean target-to-background ratio $\pm$ standard deviation, 3.9 ± 0.2 at 24 hours). A core biopsy of tumor and normal adjacent liver by using a standard biopsy needle demonstrated a sharp margin of fluorescence intensity at the tumor-liver interface.

Conclusion:

The custom-designed molecular imaging device, in combination with ICG, readily allowed differentiation between normal and malignant tissue in a murine model of intrahepatic colorectal metastasis.

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Percutaneous sampling is a cornerstone in the management of focal hepatic lesions that cannot be characterized confidently by noninvasive means. Lesions smaller than 3 cm often lack specific features to allow reliable noninvasive characterization. However, biopsies are not considered a “perfect” test, and a negative biopsy finding does not exclude the diagnosis of primary hepatic malignancy (1) or metastatic disease, because of the potential for sampling error.

Multiple attempts have been made to improve biopsy accuracy. For example, some institutions perform cytologic “wet reads” while the patient is still on the procedure table; however, this additional step is not widely available in the community and adds a considerable amount of procedural and sedation time, cost, and inconvenience. In addition, it may not always provide an accurate prediction of the final biopsy result. Improved image guidance techniques with magnetic resonance imaging-guided or fusion positron emission tomography/computed tomography (CT)-guided biopsies have also been developed, but these approaches, beyond the added cost, are not widely available at present.

Alternatively, optical molecular imaging (OMI) is a real-time, high-resolution imaging discipline that holds promise for minimally invasive procedure guidance. OMI as a field encompasses a vast array of imaging modalities; one such method is the imaging of exogenously administered organic fluorochromes. Indocyanine green (ICG) is a clinically approved OMI agent that fluoresces in the near-infrared (NIR) range and localizes to hepatocellular

carcinoma and hepatic metastatic disease with high target-to-background ratios (2–6). The intraoperative imaging of ICG for the detection of intrahepatic malignancy has been demonstrated (2,3,6). Indeed, OMI may add value to standard visual inspection and intraoperative ultrasonography (US) for the detection of superficial hepatic lesions during surgical resection (7).

With features such as high spatial resolution, real-time image display, and highly sensitive imaging agents, OMI has the potential to improve image guidance during interventional radiology procedures. Given the minimally invasive nature of interventional radiology procedures, an interventional OMI system would need to be compatible with the existing clinical armamentarium of needles, sheaths, and catheters. Toward this end, we have developed a clinically translatable, handheld imaging system capable of performing OMI through the introducer needle of a standard coaxial biopsy system. We hypothesized that by using this system in conjunction with ICG, malignant hepatic lesions could be readily differentiated from hepatic parenchyma in a minimally invasive manner. The goals of this research were to investigate ICG as a molecular beacon for malignant lesions within the liver and evaluate the ability of the handheld imaging system to allow measurement of ICG fluorescence within focal hepatic lesions with high target-to-background ratios.

Materials and Methods

ICG Phantom Studies

A phantom was designed to measure ICG fluorescence signal intensity through varying thicknesses of liver tissue and at different ICG concentrations (IC-Green; Akorn, Buffalo Grove,

Ill). The phantom was composed of two separate compartments; the upper compartment consisted of a 10 × 10-mm cuvette filled with 25 mm of a homogenate of normal mouse liver tissue. The transparent bottom of the cuvette was in direct contact with a 1-mm layer of ICG mixed with liver tissue homogenate. Different concentrations of ICG mixture in liver tissue homogenate were created by using serial dilution (0.0008, 0.004, 0.02, and 0.1 mg of ICG per liter of tissue homogenate) and were placed in the compartment below the cuvette. A 1.6-mm-diameter fiber-optic endoscope was inserted into the cuvette. The endoscope was mounted on a PT1 translation stage (Thorlabs, Newton, NJ) and withdrawn by increments of 1.25 mm from the cuvette-dye interface. The light guide of the endoscope was connected to a 785-nm fiber-coupled laser (BWTEK, Lubeck, Germany), and the eyepiece was connected through an 800-nm bandpass filter (Semrock, Rochester, NY) to an NIR camera (Allied Vision Technologies, Stadroda, Germany). With each withdrawal of the endoscope, still images were recorded in triplicate with a 50-msec exposure for each ICG concentration. To reduce

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Abbreviations:

ICG = indocyanine green

NIR = near-infrared

OMI = optical molecular imaging

Author contributions:

Guarantors of integrity of entire study, R.A.S., U.M.; study concepts/study design or data acquisition or data analysis/interpretation, all authors; manuscript drafting or manuscript revision for important intellectual content, all authors; approval of final version of submitted manuscript, all authors; literature research, R.A.S., S.A.E., B.J.W.; experimental studies, R.A.S., P.H., S.A.E., U.M.; statistical analysis, R.A.S., S.A.E.; and manuscript editing, P.H., S.A.E., B.J.W., U.M.

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Conflicts of interest are listed at the end of this article.

See also Science to Practice in this issue.

Advances in Knowledge

- Indocyanine green fluorescence within focal hepatic lesions can be measured with a percutaneous, handheld optical molecular imaging (OMI) system.
- We demonstrate a path for the clinical translation of OMI combined with interventional radiology.

Implication for Patient Care

- OMI guidance for percutaneous sampling of focal hepatic lesions may help improve accuracy and reduce false-negative biopsy findings.

Figure 1: Handheld interventional OMI device. **(a)** Schematic demonstrates the optical train of the handheld device. **(b)** Photograph of the device demonstrates that the imaging endoscope slides into a standard 17-gauge biopsy introducer needle. CCD = charge-coupled device.

the variability of measurement due to charge-coupled device noise, a circular region of interest with an area of at least 20000 square pixels was placed in the center of each image by using a standard image analysis software package (ImageJ; U.S. National Institutes of Health, Bethesda, Md). Signal intensities were measured independently by two authors (R.A.S., a radiologist with 9 years of expertise in OMI experiments; and P.H., a postdoctoral fellow with 4 years of expertise in OMI experiments), and the mean of the triplicate measurements of each author was used as the mean signal intensity.

Ex Vivo Liver Metastasis Imaging with Histology and Fluorescence Microscopy Findings

All animal experiments were performed according to a protocol approved by our institutional animal care committee. A murine model of focal hepatic malignancy was generated by injecting 1×10^6 human colorectal cancer cells (HT-29; ATCC, Manassas, Va) into the livers of six athymic nude mice (nu/nu; Taconic, Germantown, NY). Three weeks later, 0.5 mg ICG per kilogram of body weight was injected intravenously via tail vein injection. The animals were then sacrificed at 24 hours after injection, and the livers were explanted. Surface reflectance NIR imaging of the excised livers was performed (In Vivo Pro; Carestream, New Haven, Conn). The tumors were then harvested and

frozen for histologic and fluorescence microscopic analysis.

Upon completion of surface reflectance imaging, the excised livers were stored at -80°C . Two serial cryostat sections were cut from the tissue samples for fluorescence microscopy (10- μm section) and hematoxylin-eosin staining (50- μm section). The fluorescence microscopy was performed by two authors (P.H. and S.A.E., a postdoctoral fellow with 3 years of expertise in OMI) by using laser scanning confocal microscopy (LSM 5 PASCAL; Zeiss, Oberkochen, Germany) with an excitation wavelength of 633 nm and 100 $\times$ magnification. The histologic specimens were evaluated qualitatively by three authors in consensus (P.H., S.A.E., and R.A.S.) to determine tumor-liver boundaries. The histologic section was then correlated visually with the serial fluorescence microscopy section to qualitatively determine fluorescence intensities within the tumor compared with liver.

Handheld OMI System Development

The hardware and software components of the custom-designed handheld OMI system are based on the design of multiple preclinical (8–11) imaging systems we have constructed previously (Fig 1). The modular design of the system allows for the seamless interchange of a variety of imaging endoscopes, expanding the depth and breadth of possible applications. The eyepiece of any

standard fiber-optic imaging endoscope connects to the video camera (Manta camera; Allied Vision Technologies, Stadtroda, Germany) that can acquire high-resolution, 12-bit images in real time. Excitation light is provided by a 450-mW, 785-nm laser (Edmund Optics, Barrington, NJ). Fluorescent light collected by the imaging endoscope is filtered to exclude reflected excitation light by a bandpass filter (Semrock, Rochester, NY). Image display and camera functions are controlled on a laptop by using a custom software program that we developed (7).

Endoscopic Imaging of ICG Uptake within Focal Hepatic Lesions

An orthotopic murine model of intrahepatic metastases was generated by injecting 1×10^6 human colorectal cancer cells (HT-29; ATCC) into the livers of 15 athymic nude mice (nu/nu; Taconic, Germantown, NY). Three weeks later, 0.5 mg/kg ICG was injected intravenously via tail vein injection. The mice were then randomized to be imaged at 3, 6, and 24 hours after the administration of ICG ($n = 5$ for each time point). At 3, 6, or 24 hours after injection, the mice were sacrificed, and a small midline abdominal incision was made to expose the liver. The liver was then delivered onto the skin surface, and epifluorescence imaging was performed (In Vivo Pro; Carestream). After this, endoscopic imaging of the liver parenchyma and the superficially

Figure 2: Graphs from a phantom experiment of ICG fluorescence as a function of concentration and distance through liver. **(a)** There is marked ICG fluorescence above background levels through 5–10 mm of homogenized liver tissue. **(b)** ICG fluorescence is measurable over two orders of magnitude of ICG concentration through liver tissue. arb = arbitrary units.

implanted focal tumors was performed by using the handheld OMI system. The endoscope tip was placed in contact initially with normal liver parenchyma distant from the focal metastasis, and baseline ICG fluorescence intensity was measured in this location. The tip was then moved and placed in contact with the surface of the focal malignant lesion, and fluorescence intensity of the tumor was measured. Imaging parameters such as field of view (1.1 mm²), focal length (1 mm), and exposure time (100 msec) were identical for all mice. Fluorescence intensity from the endoscopic data were measured by drawing a region of interest to calculate a mean pixel intensity within the circular projected 12-bit image by using a standard image analysis software package (ImageJ; U.S. National Institutes of Health) (performed by R.A.S.). A target-to-background ratio was then computed for each animal by dividing the mean fluorescence intensity from the tumor by the mean fluorescence intensity from normal liver parenchyma. The target-to-background ratios were then averaged for all five animals at each time point.

Finally, a core biopsy of the tumor was obtained by using a standard clinical 18-gauge biopsy system (Temno;

CareFusion, San Diego, Calif), and fluorescence intensity within the tissue sample was imaged by using surface reflectance imaging (In Vivo Pro; Carestream).

Statistical Analysis

The attenuation of the fluorescent signal from the ICG traveling through the liver tissue phantom was measured and plotted against distance and ICG concentration by using GraphPad Prism (GraphPad, La Jolla, Calif).

Results

Phantom Studies of ICG

ICG fluorescence was detectable through 10 mm of homogenized tissue, suggesting that lesions within approximately 1 cm of the imaging system are potentially detectable. There was a monotonically increasing relationship between ICG concentration and fluorescence intensity over a range of ICG concentrations that spanned two orders of magnitude (Fig 2).

Ex Vivo Analysis of ICG Uptake within Focal Hepatic Tumors

High-fluorescence signal intensity was detectable from the tumor, while most

of the ICG had washed out of the normal liver parenchyma. The tumors demonstrated a peripheral enhancement pattern, consistent with the description of ICG localization to human metastases from the surgical literature (3) (Fig 3).

Xenograft Fluorescence Microscopy of ICG

Histologic findings in the tumor are remarkable for densely packed tumor cells with high nuclear-cytoplasmic ratios, an appearance in contrast to the adjacent normal hepatic sinusoidal architecture (Fig 4). Fluorescence microscopy similarly shows a sharp delineation between malignant and normal tissue, with increased ICG uptake in the xenograft. Notably, focal areas of ICG pooling are present in the biliary canaliculi in the adjacent liver—an expected finding given the hepatobiliary excretion of ICG.

Handheld OMI Device Measurements of ICG within Focal Hepatic Tumors

There was significantly elevated ICG uptake within the tumors relative to the background liver parenchyma, even as early as 3 hours (Fig 5). The endoscopic data were corroborated by whole-animal surface reflectance imaging. The

Figure 3

a.

b.

Figure 3: Images demonstrate ex vivo imaging of intrahepatic colorectal metastasis xenograft. **(a)** Photograph shows an approximately 5-mm lesion implanted at the periphery of a mouse liver. **(b)** ICG fluorescence image shows that 6 hours after intravenous injection of ICG, there is avid localization of ICG to the tumor, with mild residual concentration of ICG within the hepatic parenchyma.

Figure 4

a.

b.

c.

Figure 4: Hematoxylin-eosin-stained and fluorescence microscopy images of intrahepatic colorectal tumor xenograft. **(a)** Hematoxylin-eosin staining at 40 $\times$ magnification shows colorectal tumor with densely packed cells, abnormal nuclei, and high nuclear-cytoplasmic ratios. **(b)** A magnified view (100 $\times$ magnification) of the tumor-liver margin demonstrates the sharp transition from normal to malignant cells. **(c)** The sharp tumor-liver margin is evident at ICG fluorescence imaging (100 $\times$ magnification; excitation wavelength, 633 nm), with increased ICG uptake within the tumoral cells. Of note, pools of ICG within the hepatic parenchyma reflect normal excretion of ICG into biliary canaliculi.

Figure 5

Figure 5: (a–c) Surface reflectance and (d) endoscope-based NIR fluorescence images acquired 3 hours after injection of ICG by using the handheld OMI system demonstrate substantially elevated ICG uptake within the intrahepatic colorectal tumor relative to the adjacent hepatic parenchyma, allowing for ready differentiation between normal and malignant tissue. WL = white light.

target-to-background ratio for ICG within the tumors ranged from 3.3 to 4.0 and was greatest at 24 hours (Fig 6). Moreover, the ability of OMI to show sharply demarcated tumor borders was demonstrated by imaging a core specimen that contained both tumor and normal hepatic parenchyma. Even within this thin, 18-gauge core specimen, the elevated ICG uptake within the tumor could be clearly identified.

Discussion

We have designed a custom, handheld OMI device capable of passing through a standard clinical biopsy system introducer needle. We additionally characterized ICG as a robust OMI agent that localizes to focal malignant lesions in the liver with a high target-to-background ratio. ICG is a water-soluble, tricarbo-cyanine dye used routinely in dilution studies, hepatic and cardiac function studies, and ophthalmic retinal fluoroscopy (12). In Japan, ICG is routinely administered to evaluate hepatic function prior to hepatic resection, as ICG is almost entirely excreted via the hepatobiliary system. Intravenously administered ICG has also been used in

NIR endoscopy systems to successfully differentiate superficial gastric tumors from invasive cancers (13). ICG has been shown to localize to both hepatocellular carcinoma and intrahepatic metastases 24–72 hours after injection with high target-to-background ratios (2,3,6).

The mechanism by which ICG localizes to intrahepatic metastases has recently begun to be elucidated. The observation of ICG enhancement of colorectal metastases was first described in 2009 by Ishizawa et al (2). In their description, the authors noted a peripheral or rimlike enhancement pattern of ICG for colorectal metastases, a finding that has been replicated in other studies (7,14), as well as with our data. This has led to the concept that the ICG fluorescence was due to hepatic biliary ducts that were compressed by the metastasis, causing slow flow or stasis. Given the hepatobiliary excretion of ICG, this compromise in biliary drainage would therefore result in rimlike enhancement surrounding the tumors, owing to ICG trapped within obstructed bile ducts. Other authors (7) have proposed that the typical peripheral localization of ICG is

due to immature, reactive hepatocytes that form around the metastasis. These cells may be deficient in certain cell surface transporters, including those that excrete ICG into the biliary canaliculi. Therefore, the envelope of immature hepatocytes could potentially retain intracellular ICG, resulting in the peripheral enhancement pattern.

The handheld optical system presented is amenable to clinical translation. The modular design allows for the coupling of the image collection hardware with any endoscope that has a standard eyepiece. There are multiple clinically approved endoscopes with outer diameters small enough to pass through a standard 17-gauge biopsy introducer needle. The only component of the handheld OMI system that contacts the patient is the clinically approved endoscope; the remainder of the device would be covered in sterile draping. By applying this device to percutaneous tissue sampling procedures after the administration of ICG, the tissue at the tip of the introducer needle could be interrogated for malignancy prior to obtaining the core specimen. In this way the operator could consider

Figure 6

Figure 6: (a) Graph shows time course of ICG uptake within focal hepatic tumors and demonstrates that the target-to-background ratio of ICG uptake is 3.9 ± 0.2 at 24 hours. (b) A sharp delineation between normal and malignant tissue can be seen in an 18-gauge core biopsy specimen. The image of the needle on the right is an overlay of the photograph of an 18-gauge core-biopsy needle on the left with the epifluorescence data of the core specimen. arb = arbitrary units.

repositioning the needle, were the ICG fluorescence intensity low at the initial biopsy site. Moreover, given the high temporal and spatial resolution of OMI, ICG fluorescence within a core sample could be measured by using a point-of-care epifluorescence system in the procedure suite. Samples containing certain malignancies may demonstrate high ICG uptake,

a characteristic that could be quantified by using a small footprint OMI system. Unlike point-of-care cytologic evaluation, OMI of ICG fluorescence within core specimens will be virtually instantaneous.

Although still in its nascent stage, OMI has the potential to affect interventional radiology. With numerous fluorescently labeled molecular markers in preclinical and clinical development, the opportunities to perform procedures guided by highly sensitive and specific molecular beacons of disease will continue to grow and will require sensing hardware and software. In the context of percutaneous biopsy, optical imaging probes could improve accuracy and decrease the risk of sampling error, which in turn would reduce the clinical concern of false-negative results after receiving benign biopsy findings. Moreover, by demarcating the location of malignant tissue, OMI could potentially be used to better assess tumor margins during percutaneous ablation procedures such as radiofrequency ablation and better define target ablation tissue and end points, thus ensuring there is no residual tumor target left untreated.

There are several limitations to our study. A key limitation to any nontomographic optical imaging technique is its heavily surface-weighted signal intensity, even in the NIR regimen where there is relatively better tissue penetration. All of our tumors were implanted in a superficial location; therefore, we cannot comment on utility for deeply located tumors, as opposed to the liver surface. However, when used in combination with other anatomic imaging modalities, such as CT or US in image-guided interventions, optical imaging could add molecular specificity to confirm proper needle placement. In addition, as it is common practice (ie, coaxial technique in tumors at risk for needle track seeding) to “park” the introducer needle a small distance away from the target lesion during image-guided biopsies of small lesions, our data suggest that even in these situations, ICG fluorescence from these nearby lesions should be

measurable. Another limitation is that while certain primary hepatic malignancies and intrahepatic metastases have been shown to enhance with ICG in the surgical literature, these reports also acknowledge several false-positive and false-negative findings. For example, Ishizawa et al (2) reported ICG uptake in regenerative nodules, constituting false-positive findings. They also described variable enhancement patterns for hepatocellular carcinomas, in which well-differentiated tumors exhibited uniform enhancement, while poorly differentiated tumors demonstrated peripheral enhancement. For tumors in the latter category, as well as for rim-enhancing colorectal metastases, there is a possibility of false-negative biopsy findings in our paradigm of ICG-guided interventions if the margin of the tumor is excluded in the tissue sample. The absolute amount of ICG uptake in lesions, reflected in the observed fluorescent signal intensity, and distribution pattern of the enhancement may provide additional parameters to improve lesion characterization. We have also not characterized the optimal time to image tumors with ICG, as tumor-to-background ratio changes over time, and excretion in our animal model is likely different from that of humans.

In summary, the emergence of personalized oncology has highlighted the effect of image-guided and image-correlated biopsy in the characterization of tumors. Metabolic-targeted biopsy findings affect the delivery of drugs that target those metabolic pathways and specific biomarkers. Real-time feedback from OMI during biopsy or ablation could provide the radiologist with information that directs sampling location, procedure end points, or drug selection. The imager can now image across many scales during image-guided therapies. Molecular interventional radiology is here, enabled by multiparametric imaging fusion and OMI.

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