Chapter 12

Identification and image-guided resection of occult superficial liver metastases using indocyanine green and near-infrared fluorescence imaging

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ABSTRACT

Background

Near-infrared (NIR) fluorescence imaging using indocyanine green (ICG) is a promising technique for identifying and resecting colorectal liver metastases, however, optimal dosage and timing is not known. The current study was performed to assess feasibility of NIR fluorescence in liver surgery and to assess the optimal dose of ICG and timing of ICG administration.

Methods

The Mini-FLARE™ imaging system was used for real-time identification of colorectal liver metastases in 22 patients undergoing liver resection. NIR fluorescence imaging was performed 24 or 48 h after systemic administration of 10 or 20 mg ICG. Resected specimens were prepared for ex vivo macroscopic and microscopic evaluation of fluorescent patterns.

Results

A total of 40 superficially located colorectal liver metastases were identified and resected using NIR fluorescence imaging and ICG. In all patients, ICG fluorescence was seen as a rim around the tumor, located microscopically in the transition zone between tumor and normal liver tissue. Median tumor-to-liver ratio (TLR) was 7.4 (range 1.9 – 18.7) and no significant differences between time-points or doses were found. Four metastases detected using NIR fluorescence were occult and not visible using preoperative CT, palpation, or intraoperative ultrasound (IOUS). NIR fluorescence also distinguished benign liver lesions from metastases. Preoperative CT, IOUS, and/or palpation, however, found seven lesions, all deeper than 8 mm, which were not seen using NIR fluorescence.

Conclusions

This study suggests that NIR fluorescence imaging is complementary to conventional imaging for liver metastasectomies, and has the potential to improve surgical care.
INTRODUCTION

Prognosis and survival of colorectal cancer patients depends primarily on the occurrence of distance metastases, which occur most frequently in the liver. A resection with curative intent can offer patients with colorectal liver metastases a 5-year survival rate of 36% to 60%. Despite improvements in preoperative imaging modalities, surgical techniques, and chemotherapy regimens, intrahepatic recurrence rates vary from 11% to 37.5%, and 65% to 85% of these recurrences appear within 2 years after resection. A possible explanation for this high intrahepatic recurrence rate is that some hepatic metastases are already present at time of liver resection, but were undetected by preoperative imaging, intraoperative ultrasound (IOUS), and inspection by the surgeon. It is known that small and superficially located liver metastases are difficult to identify using conventional imaging modalities such as preoperative computed tomography (CT) and IOUS.

Near-infrared (NIR) fluorescence imaging using indocyanine green (ICG) is a promising technique to intraoperatively visualize the contrast between liver metastases and normal liver tissue in real time. ICG is excreted exclusively into the bile after intravenous injection. It has been hypothesized that colorectal liver metastases can be visualized due to passive accumulation of ICG caused by hampered biliary excretion, which results in a fluorescent rim around the metastasis. Recently, Ishizawa et al. described the intraoperative detection of colorectal liver metastasis using NIR fluorescence imaging after intravenous ICG injection 1 to 14 days prior to surgery. The interval between ICG injection and surgery is one of the key determinants of the remaining background fluorescence signal of the liver and the fluorescent rim surrounding the tumor. In a preclinical study in rats, performed by our group, the influence of injection time prior to surgery and dose of ICG on the contrast between the fluorescent rim around the hepatic metastases and normal liver tissue (tumor-to-liver ratio) was examined. In this preclinical study, the highest tumor-to-liver ratio was reached when ICG was injected 72 hours prior to surgery. Furthermore, this study demonstrated that even small liver metastases (1 mm) could be identified using NIR fluorescence. In the current study, these preclinical results were translated to a clinical trial in colorectal cancer liver metastases patients in order to optimize intraoperative identification of liver metastases using ICG.

MATERIALS AND METHODS

Intraoperative Near-Infrared Imaging System

Intraoperative NIR fluorescence imaging of the liver was performed using the Mini-FLARE™ image-guided surgery system as described in Chapter 6. Briefly, the system
consists of 2 wavelength isolated light sources: a “white” light source, generating 26,600 lx of 400 to 650 nm light and a “near-infrared” light source, generating 7.7 mW/cm² of 760 nm light. Color video and NIR fluorescence images are simultaneously acquired and displayed in real-time using custom optics and software that separate the color video and 800 nm NIR fluorescence images. A pseudo-colored (lime green) merged image of the color video and NIR fluorescence images is also displayed. The imaging head is attached to a flexible gooseneck arm, which permits positioning of the imaging head virtually anywhere over the surgical field, and at extreme angles. For intraoperative use, the imaging head and imaging system pole stand are wrapped in a sterile shield and drape (Medical Technique Inc., Tucson, USA).

**Preparation and administration of Indocyanine Green**

ICG (25 mg vials) was purchased from Pulsion Medical Systems (Munich, Germany) and resuspended in 10 cc of sterile water for injection to yield a 2.5-mg/ml (3.2 mM) stock solution. Of this stock solution 4 or 8 mL, corresponding to doses of 10 or 20 mg, was administered intravenously.

**Clinical trial**

The study was approved by the Local Medical Ethics Committee of the Leiden University Medical Center and was performed in accordance with the ethical standards of the Helsinki Declaration of 1975. A total of 22 consecutive patients with suspected colorectal liver metastases who were planning to undergo curative intended liver resection were included. All patients provided informed consent. Exclusion criteria were pregnancy, lactation or an allergy to iodine, shellfish or indocyanine green.

Patients received 10 mg or 20 mg of ICG diluted in a total volume of 4 mL and 8 mL, respectively, as an intravenous bolus at 24 or 48 hours prior to surgery on an inpatient base. This resulted in 4 groups of 4 patients per group (N = 16 patients). Subsequently, six patients were included at the optimal combination of ICG dose and injection time. During surgery and after mobilization of the liver, NIR fluorescence signal of metastases and normal liver were measured using the Mini-FLARE™ imaging system. Directly following liver resection, liver resection specimens were immediately delivered to the Department of Pathology, where the specimens were sliced into 5 to 7 mm thick slices and examined for NIR fluorescence using the Mini-FLARE™ imaging system.

**Fluorescence Microscopy**

Based on macroscopic evaluation and *ex vivo* fluorescence imaging, the transition area between tumor and normal liver tissue was identified whereupon these areas were snap-frozen on dry ice. Frozen tissue sections of 20 μm were measured for fluorescence
using the Nuance multispectral imager (CRi, Woburn, USA) mounted on a Leica DM IRE2 inverted microscope (Leica, Wetzlar, Germany) and subsequently stained with hematoxylin and eosin. White light images where created using the same microscope and subsequently merged with fluorescence images.

**Statistical Analysis**

For statistical analysis, SPSS statistical software package (Version 17.0, Chicago, USA) was used. Graphs were generated using GraphPad Prism Software (Version 5.01, La Jolla, USA). Tumor-to-liver (TLR) signal, rim fluorescence, and background fluorescence were reported as median and range. Tumor size was reported as mean and standard deviation. To test differences between groups, the Kruskal-Wallis one-way analysis of variance test and the Dunn's Multiple Comparison Test were used to test for differences between time and dose groups. Statistical tests were two-tailed and $P < 0.05$ was considered significant.

**RESULTS**

**Study Subjects**

Patient and tumor characteristics of the 22 patients included are listed in Table 1. In five patients, no liver resection was performed due to invasion of tumor into the portal vein ($N = 3$), the presence of additional irresectable liver metastases ($N = 1$), or the appearance of lymph node metastases ($N = 1$). These patients were included for TLR, rim fluorescence and background fluorescence analysis.

**Intraoperative detection of colorectal liver metastases**

Results of liver metastases detection are summarized in Table 1. Using a combination of preoperative CT scanning, IOUS, visual inspection, and/or palpation, a total of 49 lesions were identified as suspected colorectal liver metastases. After resection, six of these lesions were histologically proven to be benign, for a net detection of 43 metastatic lesions by conventional imaging. NIR fluorescence imaging (Fig. 1) detected a total of 40 lesions proven histologically to be metastases (Table 1), all of which were $\leq 6.2$ mm from the surface of the liver capsule.

However, only 36 of the 40 lesions identified using NIR fluorescence overlapped with conventional imaging. In four patients, superficially located, occult liver metastases were detected using NIR fluorescence but not by conventional imaging (Fig 2). After resection, these lesions were found to be 2, 4, 6, and 9 mm in diameter. Histopathological examination confirmed these lesions to be colorectal liver
metastases. One of these 4 occult lesions was labeled as a complicated cyst based on IOUS and CT, whereas the clear NIR fluorescent ring around the lesion suggested that it was a liver metastasis. Seven liver metastases identified by conventional imaging could not be identified using NIR fluorescence and were located 8, 13, 13, 16, 24, 30, and 32 mm beneath the liver surface.

In addition to liver metastases, a total of four hemangiomas and four cysts were identified in four patients. These hemangiomas and cysts did not show a NIR fluorescent signal or rim (Fig. 2). Thus, NIR fluorescence imaging appeared to differentiate benign from malignant lesions.

**Optimization of ICG dose and injection timing**

To determine the effect of ICG dosage and post-injection imaging time, patients were allocated to two dose groups and imaged at two time-points after ICG administration, resulting in four groups containing four patients per group. Subsequently six more patients were included at the most favorable combination of ICG dose and time of

<table>
<thead>
<tr>
<th>Table 1. Patient Characteristics</th>
<th>Median (Range) / N (%)</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>63 (49 - 77)</td>
</tr>
<tr>
<td>BMI</td>
<td>25 (19 - 38)</td>
</tr>
<tr>
<td>Sex (M / F)</td>
<td>10 (45%) / 12 (55%)</td>
</tr>
<tr>
<td>Primary tumor location</td>
<td></td>
</tr>
<tr>
<td>- Colon</td>
<td>11 (50)</td>
</tr>
<tr>
<td>- Sigmoid</td>
<td>5 (22.7)</td>
</tr>
<tr>
<td>- Rectum</td>
<td>5 (22.7)</td>
</tr>
<tr>
<td>- Anus</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>Primary Tumor type</td>
<td></td>
</tr>
<tr>
<td>- Adenocarcinoma</td>
<td>14 (63.6)</td>
</tr>
<tr>
<td>- No vital tumor cells</td>
<td>2 (9.1)</td>
</tr>
<tr>
<td>- No resection</td>
<td>6 (27.3)</td>
</tr>
<tr>
<td>Primary Tumor size (mm)</td>
<td>20 (1.7 - 70)</td>
</tr>
<tr>
<td>Type of resection</td>
<td></td>
</tr>
<tr>
<td>- Right hemihepatectomy</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>- Left hemihepatectomy</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>- Left lateral hepatic resection + metastasectomy</td>
<td>3 (13.6)</td>
</tr>
<tr>
<td>- Metastasectomy</td>
<td>9 (40.9)</td>
</tr>
<tr>
<td>- Metastasectomy + Radiofrequency ablation</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>- Radiofrequency ablation</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>- No resection</td>
<td>6 (27.3)</td>
</tr>
<tr>
<td>Number of liver metastases identified A</td>
<td></td>
</tr>
<tr>
<td>- Preoperative CT scan, IOUS, and/or palpation</td>
<td>43 B</td>
</tr>
<tr>
<td>- Intraoperative (NIR fluorescence)</td>
<td>40 C</td>
</tr>
</tbody>
</table>

*A Liver metastases were confirmed by histology or in the case of non-resected lesions by clinical appearance, IOUS, and CT.*

*B 7 of these lesions were not seen by NIR fluorescence imaging.*

*C 4 of these lesions were not seen by preoperative CT scan, IOUS, and/or palpation.*
Intraoperative imaging of colorectal liver metastases

Intraoperative imaging of colorectal liver metastases

Intraoperative imaging of colorectal liver metastases

Figure 1 – NIR fluorescence imaging of colorectal liver metastases: A colorectal liver metastasis (arrow) is clearly identified by a rim around the tumor in vivo (top row), 24 h after injection of 10 mg ICG. Normal liver tissue (arrowhead) shows minimal background uptake of ICG. After resection and slicing of the specimen, the rim around the tumor can be visualized ex vivo (bottom row).

Figure 2 – Identification of occult metastases and differentiation from benign lesions: In 4 patients, small superficial metastases (top row, arrow) were identified by NIR fluorescence imaging that were otherwise occult. Benign lesions (bottom row, arrow) could be differentiated from malignant lesions by a lack of a fluorescent rim around the lesion.

Injection. Fluorescence intensity of the rim around the liver metastases was significantly higher than the fluorescent signal in the liver ($P < 0.001$). Median tumor-to-liver ratio (TLR) in all patients was 7.4 (range: 1.9 – 18.7). Median TLRs were 6.4 (range: 2.2 – 15.4), 6.7 (range: 2.7 – 9.2), 10.5 (range: 1.9 – 18.7), 8.0 (range: 7.0 – 9.3) for the 10 mg at 24 hr, 20 mg at 24 hr, 10 mg at 48 hr and 20 mg at 48 hr patient group, respectively (Fig. 4). Median rim fluorescence (normalized pixel value) was 700.1 (range: 220.7 – 1144.5), 938.4 (range: 902.3 – 1239.1), 648.6 (range: 137.1 – 1929.36), 608.5 (range: 507.6 – 688.1) for the 10 mg at 24 hr, 20 mg at 24 hr, 10 mg at 48 hr and 20 mg at 48 hr patient group, respectively (Fig. 4). Median background fluorescence (normalized pixel value) was 97.9 (range: 53.6 – 165.9), 209.1 (range: 96.1 – 356.5), 64.6 (range: 53.4
- 112.4), and 77.4 (range: 67.5 – 96.2) for the 10 mg at 24 hr, 20 mg at 24 hr, 10 mg at 48 hr, and 20 mg at 48 hr patient group, respectively (Fig. 4). Using the independent samples Kruskal-Wallis Test no significant differences in signal-to-background ratios \((P = 0.72)\) and rim fluorescence \((P = 0.38)\) were observed. Using the independent samples Kruskal-Wallis Test, a significant difference in background fluorescence was observed \((P = 0.038)\). Post tests using Dunn’s Multiple Comparison Test showed a significant difference between the 20 mg at 24 hr and the 10 mg at 48 hr groups. No differences were observed between other separate groups. Because no differences in TLRs were observed between the various groups, the optimal dose was determined by clinical and logistical preferences (the minimal dose of 10 mg of ICG administered 24 h prior to surgery).

**Ex vivo detection of colorectal liver metastases and fluorescence microscopy**

Liver resection specimens were sliced in 5-7 mm slices and subsequently the slices were imaged with the Mini-FLARE imaging system. In all patients for whom a liver resection was performed \((N = 17)\), ex vivo NIR fluorescence imaging was performed. All known metastases were identified ex vivo by a clear fluorescent ring around the lesion. A tissue section containing both tumor tissue and normal liver tissue was then snap frozen and sectioned at 20 μm for fluorescence microscopy. After fluorescence microscopy, tissue sections were stained with hematoxylin and eosin and overlay images of NIR fluorescence were created (Fig. 3). Fluorescence signal was located in liver transition tissue surrounding the tumor and appeared to be located in the vicinity of blood vessels.

**DISCUSSION**

The current study investigated the use of intraoperative NIR fluorescence imaging in patients undergoing liver surgery for colorectal cancer liver metastases. The aim of this study was to assess the effect of timing of ICG administration and dose of ICG. Furthermore, ex vivo imaging of the liver resection specimen was performed. All superficially located (≤ 6.2 mm beneath the liver surface) metastases were identified using NIR fluorescence. Additionally, in four patients occult metastases were detected using NIR fluorescence, which were missed by conventional detection methods.

A major problem with NIR fluorescence imaging, though, is the limited penetration depth of ≈ 6 - 8 mm. Indeed, in the current study, seven metastases that were located 8 mm or more beneath the liver capsule could not be identified using NIR fluorescence. Preoperative CT scanning and IOUS are more appropriate for deeper located lesions and did successfully identify these seven lesions. However, superficially located, small occult metastases are known to be difficult to detect using IOUS, inspection and palpation. Indeed, in the current study, four superficially located,
malignant lesions were detected by NIR fluorescence that were otherwise missed and would not have been resected. Although IOUS is still required to identify deep (≥ 6 mm) metastases in the liver, our results suggest that NIR fluorescence imaging is complementary and helps find small, superficially located liver metastases. However, to prove clinical outcome and patient benefit, larger clinical trials must be performed.

The use of NIR fluorescence imaging to detect liver metastases is dependent on the clearance of ICG by the liver. To optimize the use of this technique, it is necessary to examine the influence of ICG dose and timing of ICG administration prior to surgery. In the current study, differences in dose and timing did not significantly influence the TLR. A previously performed study in rats by our group showed an optimal TLR in the group where ICG was administered at 72 hours prior to surgery.15 In the current clinical study, liver signal was comparable to pre-injection baseline level (data from Hutteman and van der Vorst et al, manuscript submitted) at 24 to 48 hours post-injection of 10 mg ICG, eliminating the need to test other time-points. Therefore, NIR fluorescence imaging at 72 hours after ICG administration was not performed. Other clinical work performed by Ishizawa et al. suggested an interval between administration of ICG and liver surgery of at least 2 days to lower background fluorescence and to obtain adequate TLRs.13 However, in that study a substantially higher dose of ICG (0.5 mg/kg; ≈ 35 mg per subject) was administered. In the current study, a relatively low dose of ICG (0.13 – 0.26 mg/kg) was used and it was therefore possible to reach acceptable TLRs and sufficiently low background liver fluorescence at 24 hours after administration of 10 mg ICG, which is safe and desirable from a logistical point of view.

The liver can be a challenging organ for optical imaging, as liver tissue has a relatively high light absorptivity and scatter, and many fluorescent probes show hepatic accumulation.17, 18 In a previously reported preclinical study by our group, using a tumor-targeted NIR fluorescent probe in a rat model of colorectal liver metastases, TLRs of approximately 2 were observed.19 Preclinical work using ICG for NIR fluorescence imaging in the same tumor model showed TLRs of approximately 4,15 whereas in the current clinical study, a median TLR of 7.4 was observed. These results demonstrate excellent performance of ICG as a non-targeted NIR fluorescent probe for intraoperative imaging of colorectal metastases. The development of novel, tumor-targeted probes with minimal background uptake is, however, essential to be able to detect other tumor types or extrahepatic colorectal tumors.20

Liver function, and in particular liver clearance capacity, affects the clearance rate of ICG and its biliary secretion and thereby retention of ICG fluorescence in the liver. However, the liver has a large reserve capacity, as has been shown in patients with cirrhosis and patients undergoing hepatic resection, potentially enabling the use of NIR fluorescence imaging after ICG administration even in patients with reduced liver function.21 In these patients, however, the optimal time interval between ICG
administration and imaging could differ from patients with a normal liver function. Future clinical studies must clarify this issue.

In addition to tumor detection, NIR fluorescence imaging appears capable of differentiating between benign and malignant tumors. In three patients, additional benign liver lesions were intraoperatively identified. These lesions could be clearly differentiated from malignant lesions due to the lack of fluorescent signal in the lesion and the lack of a fluorescent rim around them. Using fluorescence microscopy, we observed accumulation ICG in the transition area between tumor and normal liver tissue, in the vicinity of blood vessels. This phenomenon of rim enhancement is observed in arterial phase CT-scans of patients with liver metastases. This peripheral enhancement seen in metastases may be caused by disrupted vasculature surrounding the tumor tissue, causing delayed drainage of ICG from the tumor surroundings.
As in other areas of surgery, the use of laparoscopy is expanding to liver surgery. Minor liver resections such as the left lateral hepatic resections are being performed laparoscopically as standard-of-care in several centers.\textsuperscript{23} NIR fluorescence may also be of great value in laparoscopic surgery because palpation of the liver is not possible and the surgeon can only rely on visual inspection, IOUS, and preoperative imaging. To implement NIR fluorescence in laparoscopic liver surgery, laparoscopic NIR fluorescence camera systems are currently being developed and tested.\textsuperscript{24, 25} In conclusion, this study suggests that NIR fluorescence imaging is complementary to conventional imaging for liver metastasectomies, and has the potential to improve surgical care.

ACKNOWLEDGEMENTS

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REFERENCES

