Detection of Colon Cancer Metastases With Fluorescence Laparoscopy in Orthotopic Nude Mouse Models

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Objective: To improve detection of colon cancer metastases using fluorescence laparoscopy (FL).

Design: An orthotopic mouse model of human colon cancer was established by intracecal injection of HCT-116 human colon cancer cells expressing green fluorescent protein into 12 mice. One group modeled early disease and the second modeled late metastatic disease. For the early-disease model, 2 weeks after implantation, 6 mice underwent 2 modalities of laparoscopy: bright field laparoscopy (BL) and FL. The number of metastases identified within each of the 4 abdominal quadrants was recorded with both laparoscopy modalities. This process was repeated in the late-metastatic disease group 4 weeks after implantation. All animals were then humanely sacrificed and imaged using open fluorescence laparoscopy (OL) as a positive control to identify metastases.

Setting: Basic science laboratory.

Participants: Twelve female, 6-week-old nude mice.

Interventions: Detection of tumor foci by FL compared with BL.

Main Outcome Measures: Number of tumors identified in each quadrant.

Results: Fluorescence laparoscopy enabled superior visualization of colon cancer metastases compared with BL in the early ($P = .03$) and late ($P = .002$) models of colon cancer. Compared with OL, BL was significantly inferior in the early ($P = .04$) and late ($P < .001$) groups. Fluorescence laparoscopy was not significantly different from OL in the early ($P = .85$) or late ($P = .46$) group. Thus, FL allowed identification of micrometastases that could not be distinguished from surrounding tissue using BL.

Conclusions: The use of FL enables identification of metastases that could not be visualized using standard laparoscopy. This report illustrates the important clinical potential for FL in the surgical treatment of cancer.


DIAGNOSTIC AND STAGING laparoscopy is a minimally invasive surgical procedure that allows for more precise visual examination of intra-abdominal organs. Patients with liver metastases from colorectal cancer may be candidates for curative resection provided no other extrahepatic disease exists and all hepatic lesions are resectable. Laparoscopy in these patients can enable more accuracy than noninvasive imaging for identification of hepatic lesions, including size, number, and location. The combination of staging laparoscopy and laparoscopic ultrasonography has better sensitivity than noninvasive imaging studies in the detection of nodal metastases (94% laparoscopic ultrasonography vs 18% preoperative noninvasive imaging). Indeed, the use of both laparoscopic modalities detects unresectable disease in 25% to 42% of patients in whom preoperative radiologic testing demonstrated potentially curable disease. Thus, diagnostic laparoscopy can visualize peritoneal and liver metastases in colon cancer that would otherwise go undetected by preoperative imaging. Patients with cancer deemed unresectable by diagnostic laparoscopy are spared the pain, morbidity and expense of surgery, as well as delay to start of chemotherapy.

The superior ability of fluorescence laparoscopy (FL) to visualize pancreatic tumors not seen with standard laparoscopy has been previously reported. The aim of the present study was to improve detection of colon cancer with the use of FL in an orthotopic nude mouse model of human colon cancer, stably expressing green fluorescent protein (GFP).
METHODS

CELL CULTURE

Human HCT-116 colon cancer cells were stably transduced to express GFP, as previously described.10 These cells were maintained in Dulbecco modified Eagle medium (Gibco-BRL) supplemented with 10% fetal bovine serum (Hyclone), penicillin-streptomycin (Gibco-BRL), sodium pyruvate (Gibco-BRL), sodium bicarbonate (Cellgro), L-glutamine (Gibco-BRL), and minimal essential medium supplemented with nonessential amino acids (Gibco-BRL). Cells were incubated at 37°C with a 5% carbon dioxide air mixture.

ANIMAL CARE

Female, athymic, nu/nu nude mice were maintained in a barrier facility in high-efficiency particulate air-filtered racks. The animals were fed an autoclaved, laboratory rodent diet (Teckland LM-485; Western Research Products). All surgical procedures were performed with the mice under anesthesia with intramuscular injection of a 100-µL mixture of ketamine, 100 mg/kg, and xylazine, 10 mg/kg. For each procedure, 20 µL buprenorphine, 1 mg/kg, was administered for pain control.

Euthanasia was achieved by 100% carbon dioxide inhalation, followed by cervical dislocation. All animal studies were approved by the University of California, San Diego, Institutional Animal Care and Use Committee and conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals.

COLON CANCER ORTHOTOPIC NUDE MOUSE MODEL

The HCT-116 GFP cells were harvested by trypsinization and washed 3 times with serum-free medium. Viability was verified to be greater than 95% using the Vi-Cell XR automated cell viability analyzer (Beckman Coulter). The cells were resuspended at 10^6 cells per 10 µL of serum-free medium and placed on ice before orthotopic intracecal injection was performed in 12 female, 6-week-old nude mice.

LAPAROSCOPY

The methods for achieving maximal fluorescence signals and differentiation from background and surrounding tissues, by modifying a standard laparoscopic system, have been previously described (Stryker).7,8,11 In the present study, a Stryker L9000 light-emitting diode (LED) light source allowed adjustment of blue, green, and red LEDs using L9000 calibration software to achieve optimal background and GFP illumination (Figure 1). An emission filter (GG495 Schott glass) was placed between the scope and Stryker Camera 1288 (Figure 2). Mice were divided into 2 groups: early and late cancer. A small incision was made in the abdominal midline through which a 3-mm-diameter 0° Karl-Storz laparoscope (Karl-Storz GmbH & Co) was introduced and connected to standard insufflation tubing on the laparoscopic tower. A purse-string suture was placed around this incision to prevent its widening and to maintain proper insufflation at 1 mm Hg insufflation pressure. First, laparoscopic inspection was performed using standard bright light, and tumor foci were identified and recorded by quadrant. Second, laparoscopy was performed and tumor burden was recorded in the same manner using fluorescence light and filters. Animals were then euthanized, and open fluorescence laparoscopy (OL) was performed by quadrant as the positive standard for tumor detection. Tumor samples were reserved and processed for histologic analysis.

TISSUE HISTOLOGIC ANALYSIS

At necropsy, fresh tissues were fixed in Bouin solution and embedded in paraffin before sectioning and staining with hema-

Figure 1. Photograph of a representative mouse undergoing fluorescence laparoscopy. The Stryker L9000 light-emitting diode (LED) light source allowed adjustment of blue, green, and red LEDs by L9000 calibration software to achieve optimal background and green fluorescent protein illumination.

Figure 2. Schematic illustration of the integrated bright-field and fluorescence laparoscopic setup adapted to a standard Stryker laparoscopic tower. Through a 495 emission filter, the light-emitting diode (LED) allows visualization of fluorescent tumors.11 The xenon light source is used for bright-light laparoscopy.
toxylin-eosin for standard light microscopy. Hematoxylin-eosin–stained permanent sections were examined using an Olympus BX41 microscope equipped with a Micropublisher 3.3 RTV camera (QImaging). All images were acquired using QCapture software (QImaging) and adjusted for brightness and color balance. The slides were reviewed by a pathologist (C.S.S.) to confirm the identity of the fluorescent lesions.

DATA PROCESSING

Images obtained during laparoscopy were not processed in any way. Representative frames were selected and presented in this report. Histologic images were processed for brightness and contrast using Photoshop Element 4 (Adobe Systems Inc).

RESULTS

Two weeks after cecal implantation of HCT-116 for human colon cancer cells, 6 mice were designated for the early colon cancer model. Laparoscopic examination of all 4 quadrants was performed initially under bright-field laparoscopy (BL) and then under FL. The number of tumor foci for each quadrant was recorded for each modality. Next, mice were humanely sacrificed and OL was performed as the positive control. This process was repeated in 6 mice 4 weeks after cecal implant and designated the late model of colon cancer.

Figure 3. Comparison of bright-field laparoscopy (BL) and fluorescence laparoscopy (FL). A, BL of the left upper quadrant in a mouse with early cancer. Arrow identifies tumor. B, FL of same mouse. C, BL of the left lower quadrant in a mouse with late cancer. D, FL of the same mouse. Arrows indicate tumor foci (black in BL and white in FL). Note the difficulty distinguishing tumor foci from surrounding tissue using BL compared with FL.

Figure 4. Histologic appearance of tumor deposit identified using fluorescence laparoscopy. This hematoxylin-eosin–stained tissue section shows a small tumor (Tu) implant growing on the peritoneal surface of the abdominal wall. m indicates muscle; s, skin.

Figure 5. Early colon tumor detection by bright-field laparoscopy (BL) vs fluorescence laparoscopy (FL). Open fluorescence laparoscopy (OL) was used as the positive control. Results represent the total number of metastases identified in all 6 animals and all 4 quadrants examined. Using OL, 46 metastases were identified in the early tumor group of mice, depicted here as 100%. Using BL, only 6 metastases, or 12%, were identified compared with OL (P=.04); compared with 44 metastases, or 90%, using FL (P=.03). Compared with OL, FL was not significantly different (P=.85).

In the early cancer group, when totaling all 6 mice, no tumors were detected in the right upper quadrant or left upper quadrant using any modality. In the right lower quadrant, BL was unable to detect any tumor foci, although FL detected 18 tumors. Twenty-two individual tumor foci were detected by OL. In the left lower quadrant, BL detected 6 tumor foci, whereas FL detected 26 tumor foci and OL detected 27 tumor foci. Overall, in the early cancer group, FL detected 44 metastatic foci, or 90% of the total metastases detected by OL, compared with only 6, or 12% of the total metastases detected by OL, in the BL group (P=.03) (Figure 5). In the early cancer group, the positive-control OL detected 46 metastatic foci (P=.04 compared with BL). No significant difference was found between FL and OL (P=.85).
In the late cancer group, in all 6 mice, extensive tumor burden was encountered in all quadrants (Figure 3). In the right upper quadrant, BL detected 41 tumor foci, whereas FL detected 122 tumor foci and OL detected 137 tumor foci. In the left upper quadrant, BL detected 62 tumor foci, whereas FL detected 130 tumor foci and OL detected 162 tumor foci. In the right lower quadrant, BL detected 60 tumor foci, whereas FL detected 141 tumor foci and OL detected 173 tumor foci. In the left lower quadrant, BL detected 44 total tumor foci, whereas FL detected 109 tumor foci and OL detected 115 tumor foci. Overall, in the late group, FL detected 302 metastatic foci, or 86% of the total metastases detected by OL, compared with 207, or 35% of the total metastases detected by OL, in the BL late group (P=.002). FL compared with OL was not significantly different (P=.46).

![Figure 6](image_url)

**Figure 6.** Late colon cancer detection by bright-field laparoscopy (BL) vs fluorescence laparoscopy (FL). Open fluorescence laparoscopy (OL) was used as the positive control in this setting. Results represent the total number of metastatic foci identified in all 6 animals and 4 quadrants examined in each animal. Using OL, 587 metastatic foci were identified in the late tumor group of mice, depicted here as 100%. Using BL, only 207 metastatic foci, or 35%, were identified compared with OL (P<.001) and compared with 502 metastatic foci, or 86%, using FL (P=.002). FL compared with OL was not significantly different (P=.46).

Although GFP-labeled cancer cells used in this study to implant in the mice, provide a convenient tool, in situ labeling of tumors must be accomplished before translation to human patients. In their review of the current techniques for fluorescence-guided surgery, Bouvet and Hoffman compared several approaches for in vivo tumor labeling. In one set of studies, a monoclonal antibody, directed against either cancer antigen 19-9 (CA19-9) or carcinoembryonic antigen (CEA), was conjugated to a green fluorophore and delivered intravenously into nude mice with orthotopic human pancreatic or colon tumors. Once the fluorescent antibodies adhered to the CEA or CA19-9 antigens of CEA or CA19-9–expressing cancer cells, respectively, the tumors become fluorescent. Tumors that were invisible with standard bright field imaging demonstrated clear fluorescence and under fluorescence guidance resulted in more complete resection. Kishimoto et al reported the use of a telomerase-dependent adenovirus with the GFP gene to label tumors.

The concept of FL is new and expanding. Several groups are exploring near-infrared (NIR) FL to improve surgical navigation. For instance, Ankersmit et al described the technique of sentinel lymph node mapping using NIR dyes visualized with a laparoscopic NIR fluorescence imaging system for colorectal cancer. Similarly, Cahill and Mortensen used NIR imaging, along with more conventional laparoscopic imaging, to better distinguish tissue architecture and lymphatic and vascular-channel anatomy.

Wavelengths in the NIR range (700-900 nm) have the advantage of penetrating deeply into living tissue compared with visible light that is used in sentinel lymph node mapping. However, NIR fluoroscopy cannot be seen with the naked eye and therefore requires a specialized laparoscope to visualize the fluorophore. In contrast, the fluorescence equipment used in the present study uses an LED L9000 light source that is already standard in most operating rooms and therefore needs minimal adaptation for translation to clinical practice.

In the current study, GFP-labeled cancer cells were implanted into the cecum of 12 mice to establish early and late models of colon cancer. Fluorescence laparoscopy enabled superior visualization of colon cancer metastatic foci not visualized with standard BL in the early and late cancer models when compared with the positive-control OL. Open fluorescence laparoscopy may appear more accurate than FL, although not significantly so, simply because of limitations in delineating quadrant location. In addition, placement of a single port at the intersection of the 4 quadrants made identification of peritoneal metastases adjacent to the scope impossible. For translation into human patients, these limitations can be easily overcome by placement of multiple port sites, as is traditionally done, thus allowing for complete inspection of the peritoneum and abdominal wall.

The FL technology described in the present report represents the way forward for cancer surgery and staging. Enhancing a surgeon’s ability to identify primary and metastatic tumors and distinguish tumors from surrounding vital structures, makes FL an exciting and important development. Chimerization of antibodies for use in humans has precedent in monoclonal antibody drugs, such as rituximab, which is routinely used for patients with non-Hodgkin lymphoma and rheumatoid arthritis, trastuzumab, which is used in breast cancer, and cetuximab, which is used in colon cancer. Thus, fluorescent chimeric antibodies are promising for clinical application of FL. Alternatively, the bright fluorescence of GFP can be selectively delivered to tumors via a cancer cell–selective telomerase-dependent adenovirus. Such advances make the use of FL a real possibility for clinical applicability in the near future. The use of FL enables the identification of metastases that would otherwise be missed using standard laparoscopy. Fluorescence laparoscopy could then be used not only in staging laparoscopy but also to improve the localization of liver metastases for resection or radiofrequency ablative techniques.
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REFERENCES


