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Anti-Claudin-1 Conjugated to a Near-Infrared Fluorophore Targets Colon Cancer in PDOX Mouse Models

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Abstract

Introduction: Claudins are tight-junction proteins, which maintain an epithelial barrier in normal colon cells. Overexpression of Claudin-1 has been implicated for development of colon cancer. We postulated that Claudin-1 may be a useful target in near-infrared imaging and fluorescence-guided surgery.

Methods: We conjugated Claudin-1 antibody to LI-COR IR800DyeCW (Claudin-1-IRDye800CW). Western blotting of 9 human colon cancer cell lysates was performed. Animal imaging was performed with the LI-COR Pearl Trilogy Fluorescence Imaging System. A dose-response study was carried out with subcutaneous LS174T colon cancer cell line models. Increasing doses of Claudin-1-IRDye800CW via tail vein injection were administered to three groups of mice. Two groups of mice were used as controls (antibody alone, and dye alone). *In vivo* imaging was performed at 24, 48, and 72 h after administration of the conjugated dye. Orthotopic implantation of patient-derived tumors and cell lines was performed and peritoneal carcinomatosis models were created. After tumor growth, mice were administered Claudin-1-IRDye800CW and imaged *in vivo* 48 h later. The mice were euthanized and laparotomy was performed to assess internal organs and toxicity.

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Authors' contributions: H.M.H. wrote the manuscript and was actively involved in each step of the experimental process. T.M.L. was involved in the initial testing of Claudin-1 antibody and imaging. S.A. was actively involved in each step of the experimental process and contributed to the materials and methods portion of the article. F.F. was involved in much of the preparation and execution of experiments. S.K.B. was involved in preliminary claudin research and in contribution of anti-Claudin antibody. R.M.H. was involved in revision and editing of the article. P.D. contributed to information of claudin and provided anti-Claudin-1 antibody for initial experiments. She was also involved in editing the manuscript and oversight of the research design. M.B. was involved in oversight of the research design, implementation, and editing of the manuscript.

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Disclosure

R.M.H. is an unsalaried affiliate of AntiCancer, Inc that offers PDOX models for contract research. The other authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

Results: Western blotting revealed that all colon cancer cell lysates expressed varying amounts of Claudin-1. All tumors demonstrated strong and specific fluorescence labeling at 800 nm, even with the lowest dose of 12.5 μg of Claudin-1-IRDye800CW.

Conclusions: Claudin-1 is a useful target for near-infrared antibody-based imaging for visualization of colorectal tumors for future use in fluorescence-guided surgery.

Keywords

Claudin; Near infrared dye; IRDye800CW; Colon cancer; Fluorescence guided surgery

Introduction

Colorectal cancer remains one of the most prevalent cancers in the United States and is the second leading cause of cancer-related death.[1] Although many technical advances have improved surgical options, surgical resection of colorectal cancer remains a challenge in part because of the difficulty in achieving negative margins in relatively small surgical fields. Positive margin rates of colorectal cancers have been previously reported at 6.83%, which may contribute to higher rates of local recurrence and subsequent metastases.[2]

In current surgical practice, indocyanine green (ICG) is commonly used for visualization of biliary structures and to confirm adequate perfusion of tissues. Although ICG is generally not used in clinical practice techniques for colorectal cancer, the use of ICG creates a technological platform for novel fluorophores to be introduced to the operating room for various surgical fields. In comparison with ICG, tumor-specific fluorescent antibodies could provide clearer margins between normal tissue and cells overexpressing specific antigens.[3] This has been demonstrated in previous studies comparing ICG fluorescence with anti-carcinoembryonic antigen (CEA) antibodies in colon cancer.[3,4] Anti-CEA antibodies have also been successful at labeling metastases in patient-derived pancreatic cancer mouse models.[5] However, not all colon cancers express CEA and therefore other targets are needed.

The claudin family of proteins has been linked to tumorigenesis and development of colon cancer.[6] Claudin proteins are tight-junction proteins, which help to maintain an epithelial barrier in normal colon cells.[6,7] The deregulation of claudin expression leads to a loss of epithelial integrity and promotes tumorigenesis.[8] Overexpression of Claudin-1 has been implicated in the development of colon cancer and cancer cells express a significantly higher amount of claudin when compared with normal colonic mucosa.[8] Adenomatous popysosis coli (APC) mutations, which are implicated in development of colon cancer, cause an increase in Claudin-1 expression.[9]

The use of claudin as a target for tumor-specific imaging is beginning to gain interest. A recent publication utilized CpC-APC mice (mice in which APC allele has been deleted and polyps spontaneously form).[10] A cyanine5.5 fluorophore that fluoresces around 700 nm was tagged to a peptide that competitively binds to Claudin-1. This was administered via enema and imaging was performed on the IVIS small animal system. This demonstrated enhancement of polyp visualization as well as flat lesions that were not detectable with bright-light imaging.[10]

In the present report, we aimed to develop clinically translatable claudin-targeted tumor-specific fluorescent antibodies. We used a near-infrared (NIR) 800 nm fluorophore that has compatibility with many of the fluorescent imaging systems currently used for ICG imaging.[5] We show that Claudin-1 is a useful target for NIR imaging of colorectal cancer and metastases on patient-derived orthotopic xenografts (PDOX) as well as orthotopic colon cancer cell line models in nude mice.

Methods

Animals

All studies were approved by the San Diego Veterans Administration Medical Center Institutional Animal Care and Use Committee (animal use protocol A17-020). Male nude (nu/nu) mice, aged 4-6 wk, purchased from the Jackson Laboratory (Bar Harbor, ME), were used for this study. Only male mice were used to prevent colonization and breeding. The animals were fed an autoclaved laboratory diet. All surgical procedures were performed with anesthesia by intramuscular injection of ketamine, xylazine and acepromazine reconstituted in phosphate buffered saline (PBS). Mice were treated with bupre-norphine for pain control after surgical procedures. At the conclusion of the study, or if tumor burden became too large, mice were euthanized with CO_2 inhalation, which was confirmed with cervical dislocation.

Claudin conjugation

Claudin-1 rabbit polyclonal antibody (lLS-C382732 CLDN1; LifeSpan BioSciences Inc, Seattle, WA) was conjugated with IR800CW NHS ester (LI-COR Biosciences Inc, Lincoln, NE) under basic conditions. Excess dye was removed using a Zeba desalting column (Thermo Fisher Scientific, Waltham, MA) and the product suspended in PBS. Protein concentration was measured using the Lowry assay on a microplate reader (BioRad Laboratories Inc, Hercules, CA)

Western blotting

Tissue lysates of patient-derived and cell line-derived xenograft colon tumor samples were established and samples were placed on ice. The membrane was then incubated with Claudin-1 antibody conjugated with IRDye800CW at 4°C overnight. Removal of excess primary antibody was achieved by washing the membranes in tris-buffered saline and poly-sorbate 20 three times. The membrane was scanned with the LI-COR Odyssey Infrared Imaging System (LI-COR, model 9120) and detection and quantification of band intensities was conducted using Image Studio Lite Ver 5.2 software (LI-COR). Bands were normalized to total protein by dividing the intensity of the band by the intensity of the total protein from the same sample on the same blot. Beta-actin was utilized as a control.

Establishment of colon cancer cell line tumors

The human colon cancer cell line LS174T, obtained from the American Type Culture Collection (Manassas, VA) was utilized for establishment of subcutaneous tumors. Nude male mice were anesthetized using 0.02 mL of ketamine solution. The mice were prepped

with ethanol solution and cells were injected into the bilateral flanks. The tumors were allowed to grow until they reached 50 mm^3.

Establishment of subcutaneous patient-derived xenografts with tumors from the operating room

Human patient colon cancers were excised from patients under standard sterile conditions in the operating room at UCSD Thornton Hospital under UCSD IRB protocol 140046. After dividing the tumor in the pathology laboratory, a portion of the tumor was placed in PBS on ice and properly transferred to the laboratory. Under standard protocols with IRB approval and informed consent from the patient, these samples were used for establishment of xenograft models. Initially, tumor fragments were implanted subcutaneously within 30 min of patient surgery over the right and left upper and lower flanks in male nude mice between 4 and 6 wk of age. Subcutaneous tumors were allowed to grow for 2-4 wk until they reached at least 50 mm^3.

Colon cancer PDOX models

Mice were anesthetized by intramuscular injection of 0.02 mL of ketamine solution. The abdomen was prepped with 70% ethanol solution. An incision was then made vertically in the midline of the abdomen through the skin and peritoneum. The cecum was carefully exposed and a 1 mm^3 tumor fragment was surgically implanted onto the serosal layer of the cecum using 8-0 surgical sutures (Ethicon Inc, NJ). The bowel was then returned into the peritoneal cavity, and the abdominal wall and skin was closed with 6-0 nylon surgical sutures (Ethicon Inc, NJ).[11]

Mouse imaging

For noninvasive and intravital imaging, the Pearl Trilogy Small Animal Fluorescence Imaging system was used (LI-COR). The Pearl Trilogy is equipped for sensitive imaging of NIR fluorophores at 700 nm and 800 nm. Claudin-1 antibody conjugated to LI-COR IRdye800CW (Claudin-1-IRDye800CW) was used to image subcutaneous cell line tumors and PDOX colon tumors. Images were assessed for dose response, signal to background comparison, signal duration and phototoxicity.

A time and dose-response study was completed on subcutaneous cell line models, established as described above. Three groups of three mice were administered increasing doses of Claudin-1-IRDye800CW: 12.5, 25, and 50 mg reconstituted in 100 mL PBS, via tail vein injection. *In vivo* imaging of the mice was performed at 24, 48, and 72 h after intravenous injection. Two mice were used as controls (antibody alone, and dye alone). After imaging, all mice were euthanized and laparotomy was performed to assess internal organs.

Once the optimal dose and timing for imaging was determined, orthotopic models of cell line tumors and PDOX tumor models were established using LS174T and two patient-derived tumors. 5 mice were used in each group for a total of 15 mice. After tumors grew to at least 50 mm^3, the mice were administered 25 μg Claudin-1-IRDye800CW via tail vein injection. Imaging was performed 48 h after administration of Claudin-1-IRDye800CW.

Establishment of peritoneal carcinomatosis models

Intraperitoneal injection of one million LS174T cells reconstituted in 100 mL of PBS was performed with three Nu/Nu male mice. After 3 wk, mice received 25 μg of Claudin-1-IRDye800CW reconstituted in 100 μg of PBS via tail vein injection. Images were obtained 48 h after intravenous injection on the Pearl Trilogy Small Animal Imaging System.

Results

Claudin-1 expression is expressed in patient-derived tumors

Immunoblotting using nine patient-derived tumors and LS174T colon cancer cell line revealed that eight of nine patient-derived tumors expressed varying amounts of Claudin-1 and LS174T cell lysate overexpressed Claudin-1 (Fig. 1). The cell lysates with the highest signal intensity were all patient-derived colon cancer metastases, including lung, liver, and distant colon metastases (Fig. 1).

Optimal schedule of administration of Claudin-1-IRDye800CW

An initial time response study demonstrated no enhancement of tumor visualization compared with the background signal when mice were imaged 24 h after administration of the antibody-fluorophore conjugate. At 48 h, tumor margins were clearly defined with no surrounding background signal. 72 h after administrations of Claudin-1-IRDye800CW, there was no longer a tumor signal, suggesting metabolism of the dye at this time point (Fig. 2). Increasing amounts of Claudin-1-IRDye800CW were administered to mice (12.5, 25 and 50 μg), and demonstrated clear tumor margins at each dose when imaged 48 h after administration. For subsequent studies, mice received 25 μg Claudin-1-IRDye800CW via tail vein injection and mice were imaged 48 h after administration.

In vivo fluorescence labeling of Claudin-1 expression in orthotopic and carcinomatosis models

All orthotopic mouse models, including cell line and PDOXs, demonstrated fluorescence of tumors with clear margins after administration of the fluorescent anti-Claudin antibody (Fig. 3). There was a minimal background signal as seen on fluorescence images. All models demonstrated fluorescence of the liver due to metabolism of the dye in the liver (Fig. 3). Three weeks after intraperitoneal injection of LS174T human colon cancer cells, mice were imaged to assess for peritoneal implantation and tumor burden. Each mouse demonstrated multiple small fluorescent tumors on the peritoneal surface of the abdominal wall and various organs (Fig. 4). Regional fluorescent metastases to the cecum and colon were identified in multiple PDOX models. Claudin-1-IRDye800CW was able to detect these small local metastases that were not visible under bright light (Fig. 5). The mouse that received Claudin-1 antibody alone without conjugation to dye did not demonstrate any fluorescent signal on imaging with the Pearl Trilogy. The mouse that received IR-800CW dye alone without antibody did not demonstrate tumor specificity and had a significant background signal throughout the mouse.

Toxicity and side effects

After imaging was performed, mice were euthanized using techniques described previously and necropsy was performed. Internal organs were removed and examined to determine if any gross toxicity was identified. In all mice involved in the study, there were no gross defects of internal organs to suggest toxicity. All mice survived tail vein injection and administration of all doses. No mice had to be euthanized because of toxicity or side effects.

Discussion

The results of the present study show that Claudin-1 is a useful target for NIR antibody-based imaging for visualization of colorectal tumors. Western blotting demonstrated overexpression of claudin to varying degrees in eight of the nine cell line and patient-derived colon cancer samples studied. Although not all samples overexpressed Claudin-1, it can be extrapolated that most colorectal tumors overexpress Claudin-1. The cell lysates with the highest signal intensity on Western blot were all derived from patient samples of colon cancer metastases. This is consistent with previous studies, which demonstrated a higher expression of Claudin-1 in liver metastases.[7] Claudin has also been implicated in promotion of metastases, which is consistent with the higher expression of claudin in patient-derived colon cancer metastases.[12]

Claudin-1-IRDye800CW was the most effective 48 h after administration, as there was minimal background signal and there were clear tumor margins. When mice were imaged at 24 and 72 h, there was minimal tumor signal compared with surrounding tissue. In orthotopic models, imaging 48 h after administration of Claudin-1-IRDye800CW demonstrated clear tumor margins with no fluorescence of other organs of the gastrointestinal tract. The control mouse that received IR800 dye alone demonstrated fluorescence of the entire mouse and was not localized in any specific tissue due to the nonspecific hematogenous distribution of the dye. When Claudin-1 antibody is conjugated to the NIR dye, the antibody binds to Claudin-1 in the tumor tissue and delivers the dye specifically to the tumor. The other control mouse that was imaged after administration of Claudin-1 alone did not have any fluorescence signal, which validates that the tumor does not have any interfering autofluorescence.

A recent study was conducted to determine the clinical impact of NIR fluorophore–labeled antibodies on surgical decision-making during resection of head and neck squamous cell carcinoma.[13] The authors concluded that antibody-guided fluorescence imaging aided surgical decision-making and improved tumor margin detection. The use of cancer-specific antibodies labeled with NIR fluorophores allows for visualization of tumor margins and tumors otherwise not visible under bright light. Another recent clinical study demonstrated the utility of an anti-CEA antibody tagged to a 700 nm fluorophore (SGM-101) for fluorescence-guided surgery of colorectal cancer.[14] In the present study, we demonstrate that Claudin-1 detects tumor margins in murine models of primary and metastatic colon tumors. Therefore Claudin-1 may be a useful target for use in fluorescence-guided surgery, enhanced imaging, and improved surgical decision-making for colorectal cancer. While this application could be applied to all colorectal malignancies, this method would likely be the most clinically relevant in patients with rectal cancer, for improved visualization of tumor

burden and for subclinical metastases not visualized with standard imaging techniques. Further studies need to be performed to determine the clinical application in patients with colorectal cancer.

Studies continue to be conducted to determine the most useful clinical applications for fluorescence imaging.[15] Fluorescence imaging offers an advantage in debulking cancer operations as it allows for visualization and detection of subclinical disease at the time of surgery. Fluorescence imaging also has been demonstrated to improve detection of hepatic metastases derived from colorectal cancers at the time of surgery. In the present study, we have determined that Claudin-1-IRDye800CW detects peritoneal carcinomatosis and metastatic lesions of colon origin in PDOXs, and that Claudin-1-IRDye800CW may be useful for detection and improved visualization of metastases and peritoneal implants at the time of surgery.

One limitation to the use of Claudin-1-IRDye800CW is the significant fluorescent liver signal that was identifiable at all doses. This liver signal would make it difficult to visualize liver metastases with fluorescence imaging. Future studies will be performed with conjugation of Claudin-1 to various NIR dyes to identify a dye that does not have significant liver signal. This conjugate can then be used to study fluorescence imaging of liver metastases using Claudin-1 antibody.

Claudin-1-IRDye800CW may be useful for early detection given its implication in tumorigenesis and further studies regarding early detection of polyps may be indicated. Previous studies have utilized a claudin-binding peptide to visualize polyps endoscopically.[10] The probe in this study may be useful in the visualization of precancerous lesions during endoscopic evaluation. Further studies should be performed to evaluate the use of Claudin-1-IRDye800CW in early detection of polyps and precancerous lesions. In addition, Claudin-1-IRDye800CW may be useful for fluorescence-guided surgery, as we have visualized clear margins and small tumor implants in the present study with this probe.

Conclusion

Claudin-1 is a useful target for NIR antibody-based imaging for visualization of primary and metastatic colorectal tumors for future use in fluorescence-guided surgery.

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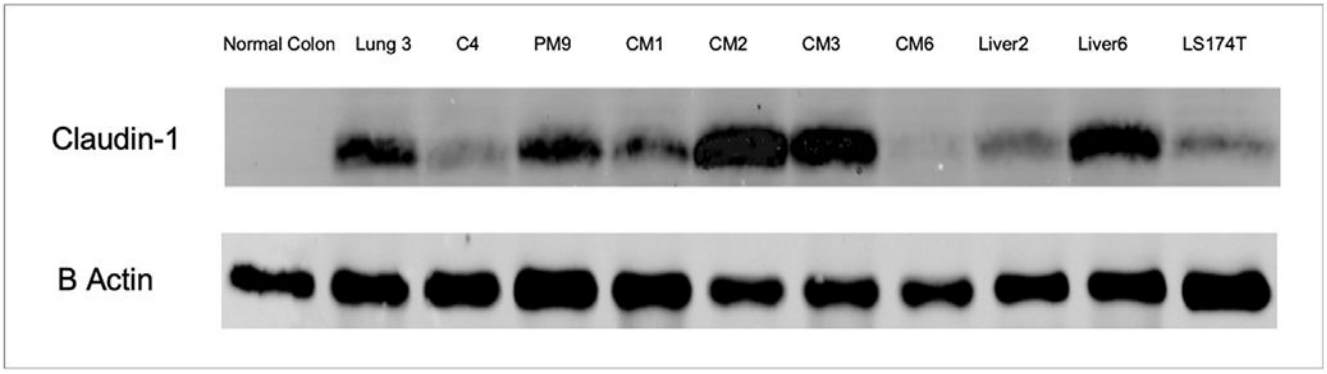

Fig. 1 –.

Western blot of multiple cell-line and patient-derived primary and metastatic colon cancer cell lysates. The bottom line represents the control with B actin. LS174T is a colon cancer cell line. CM 1, 2, 3, and 6, liver 2, liver 6, lung 3, and PM9 are patient-derived colon cancer metastases. C4 is a patient-derived colon cancer primary tumor. Eight of nine tumor lysates demonstrated overexpression of Claudin-1 to varying degrees.

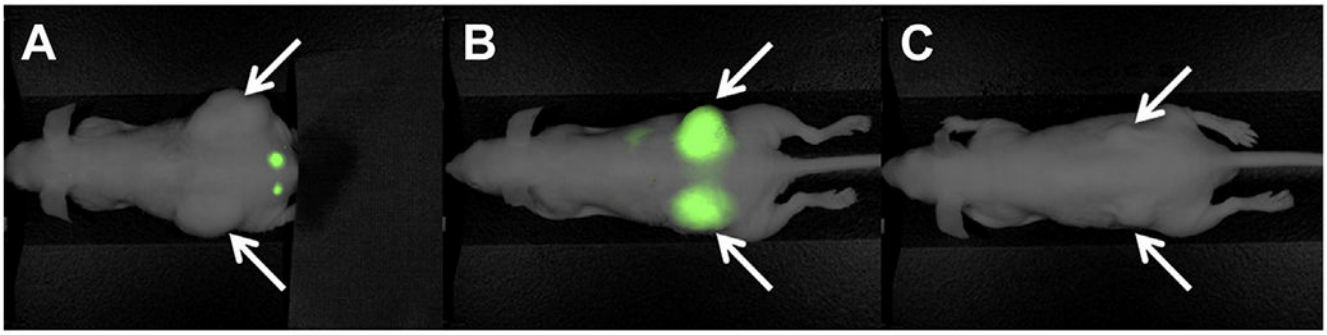

Fig. 2 –.

Nine mice were imaged at different time points to determine optimal time of imaging after administration of Claudin-1-IRDye800CW (A) 24 h, (B) 48 h, and (C) 72 h. Each mouse in the panel was administered 25 μg Claudin-1-IRDye800CW, and different mice were imaged at the various time points. The best tumor visibility with minimal background was 48 h after injection of Claudin-1-IRDye800CW. Arrows point to tumors on the bilateral flanks. (Color version of figure is available online.)

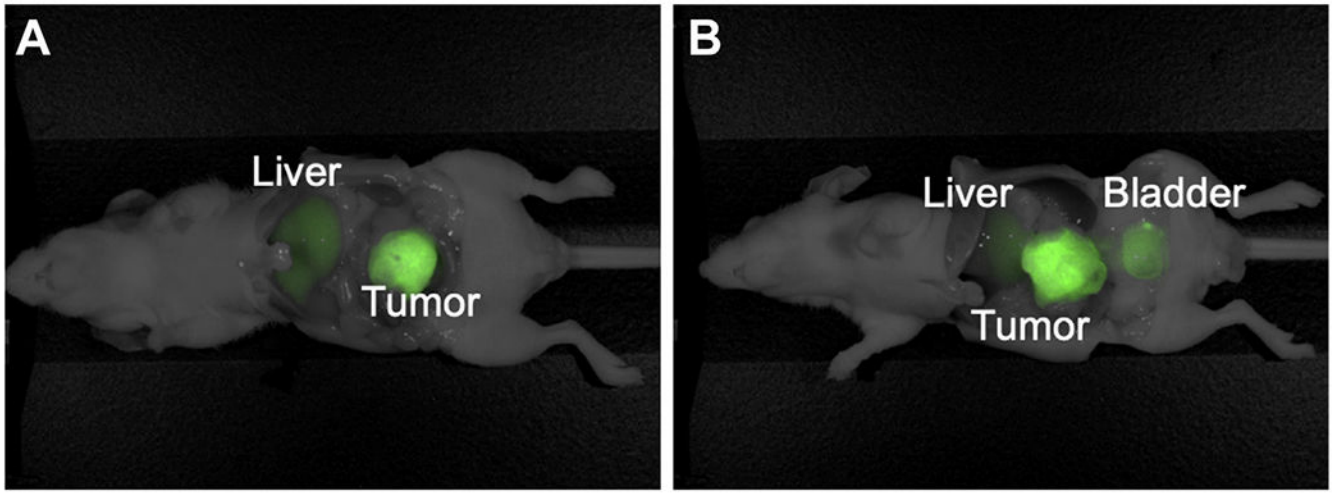

Fig. 3 –.

(A) Orthotopic model of colon cancer cell line LS174T. (B) PDOX model of C4, a patient-derived primary colon cancer. The tumor is brightly labeled with the fluorophore-antibody conjugate and the tumor margins are clearly defined in both mouse models, suggesting potential for use in surgical practice. Liver fluorescence is noted in both mice because of metabolism of the IR800 dye. The bladder is fluorescent due to excretion of the dye in the urine in panel (B). (Color version of figure is available online.)

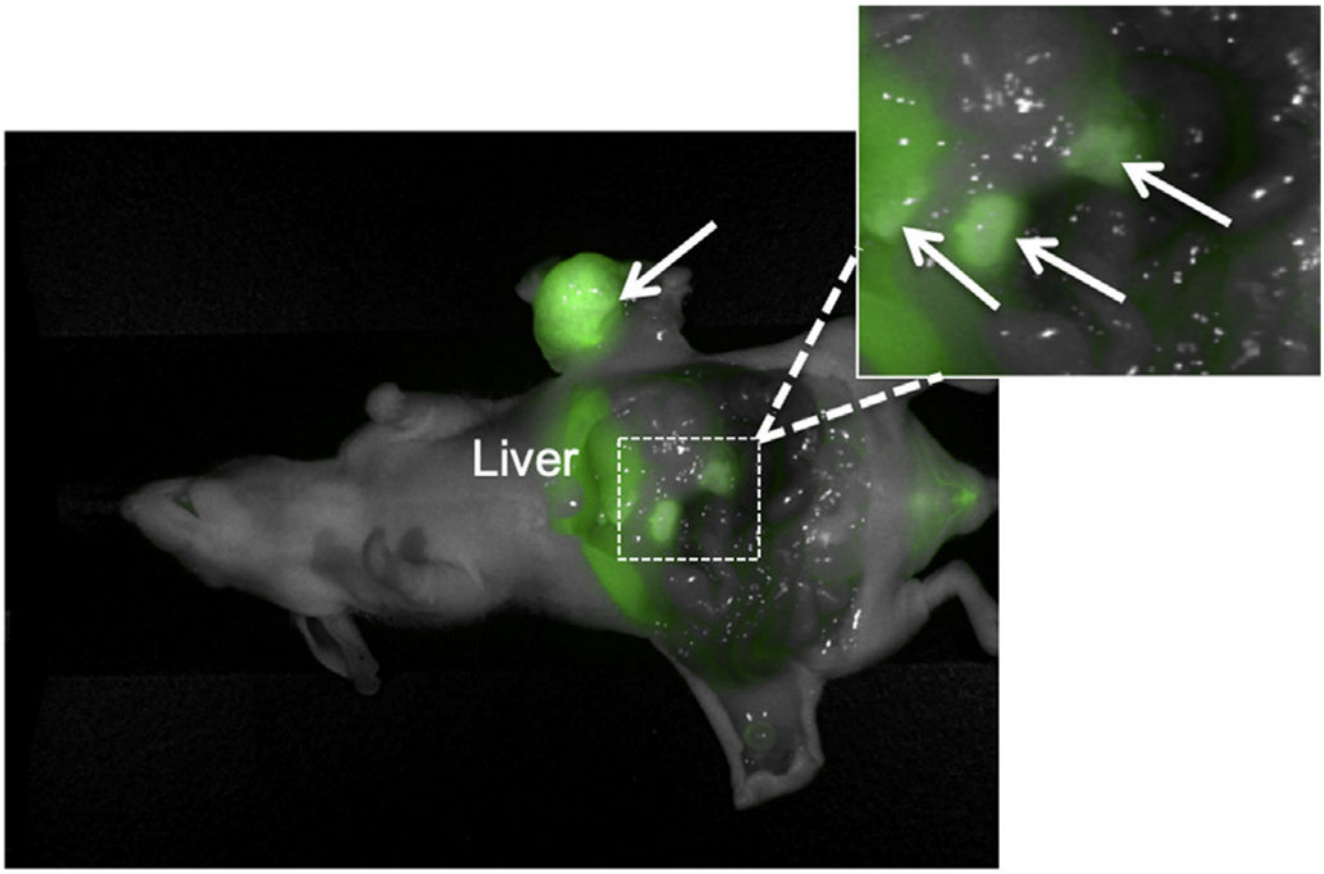

Fig. 4 –.

LS174T colon cancer cell line carcinomatosis model. The mouse had multiple tumor implants on the abdominal wall peritoneum and peritoneal surface of abdominal organs. Arrows point to tumors. (Color version of figure is available online.)

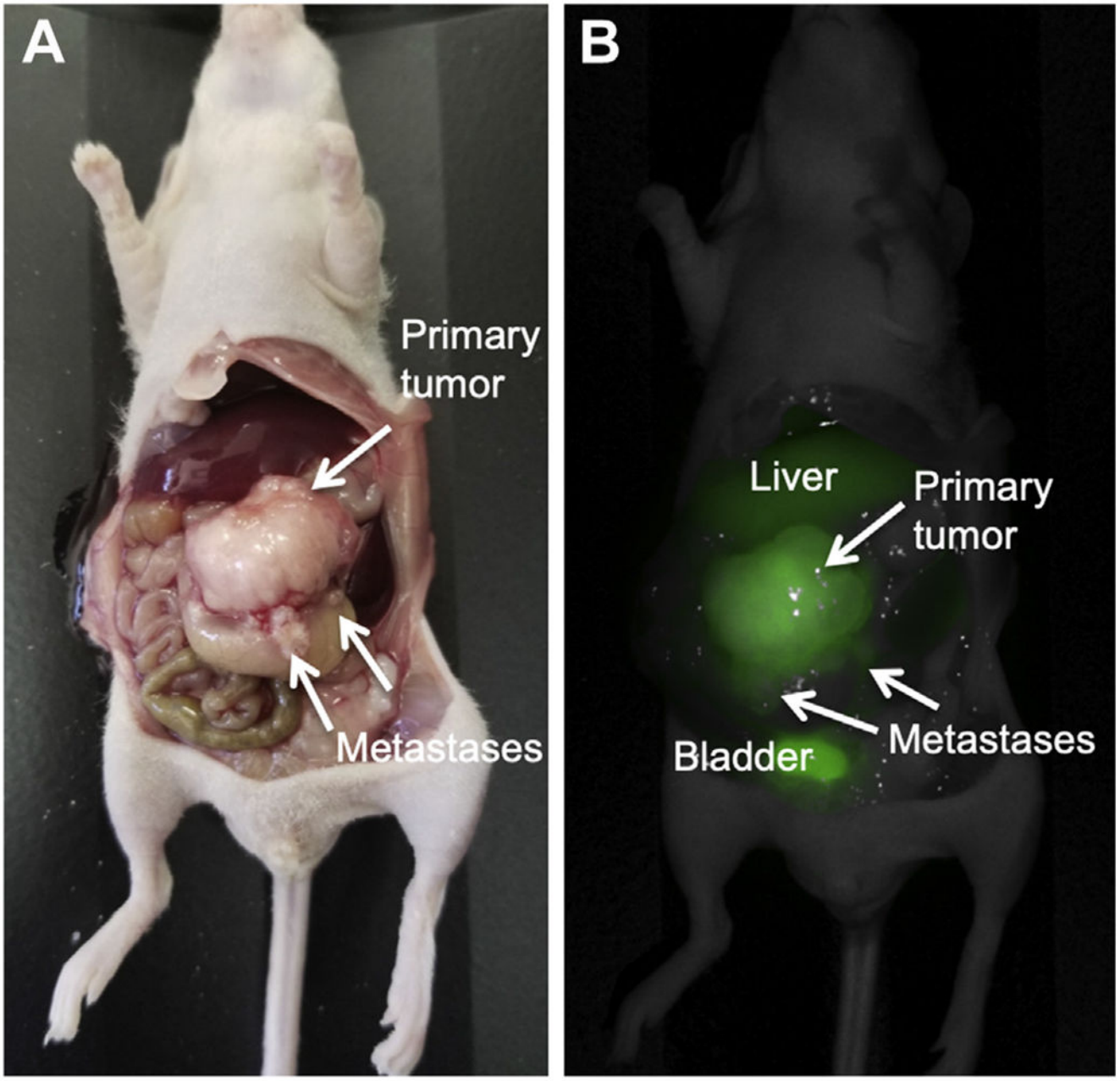

Fig. 5 –.

Orthotopic LS174T tumor. (A) Bright-light imaging demonstrates visible local metastases. (B) Fluorescence imaging shows identifiable margins of the tumor and nearby metastases. (Color version of figure is available online.)