Advantages of Fluorescence-Guided Laparoscopic Surgery of Pancreatic Cancer Labeled with Fluorescent Anti–Carcinoembryonic Antigen Antibodies in an Orthotopic Mouse Model

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BACKGROUND: Our laboratory has previously developed fluorescence-guided surgery of pancreatic and other cancers in orthotopic mouse models. Laparoscopic surgery is being used more extensively in surgical oncology. This report describes the efficacy of laparoscopic fluorescence-guided surgery of pancreatic cancer in an orthotopic mouse model.

STUDY DESIGN: Mouse models of human pancreatic cancer were established with fragments of the BxPC-3 red fluorescent protein–expressing human pancreatic cancer using surgical orthotopic implantation. Mice were randomized to bright-light laparoscopic surgery (BLLS) or to fluorescence-guided laparoscopic surgery (FGLS). Fluorescence-guided laparoscopic surgery was performed with a light-emitting diode light source through a 495-nm emission filter in order to resect the primary tumors and any additional separate submillimeter tumor deposits within the pancreas, the latter of which was not possible with BLLS. Tumors were labeled with anti-CEA AlexaFluor 488 antibodies 24 hours before surgery with intravenous injection. Perioperative fluorescence images were obtained to evaluate tumor size. Mice were followed postoperatively to assess for recurrence and at termination to evaluate tumor burden.

RESULTS: At termination, the FGLS–treated mice had less pancreatic tumor volume than the BLLS–treated mice (5.75 mm^2 vs 28.43 mm^2, respectively; $p = 0.012$) and lower tumor weight (21.1 mg vs 174.4 mg, respectively; $p = 0.033$). Fluorescence-guided laparoscopic surgery compared with BLLS also decreased local recurrence (50% vs 80%, respectively; $p = 0.048$) and distant recurrence (70% vs 95%, respectively; $p = 0.046$). More mice in the FGLS group than the BLLS group were free of tumor at termination (25% vs 5%, respectively). Median disease-free survival was lengthened from 2 weeks with BLLS (95% CI, 1.635–2.365) to 7 weeks with FGLS (95% CI, 5.955–8.045; $p = 0.001$).

CONCLUSIONS: Fluorescence-guided laparoscopic surgery is more effective than BLLS and, therefore, has important potential for surgical oncology. (J Am Coll Surg 2014;219:132–142. © 2014 by the American College of Surgeons)

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Refinements in laparoscopic instruments and advances in robotic platforms have improved movement precision and dexterity, allowing for more complex laparoscopic procedures to be performed. More specifically, laparoscopic pancreatectomy has recently emerged as one of the most advanced and complex procedures performed.[1,2] However, this procedure, as its open counterpart, has its challenges. In addition to the retroperitoneal location of the pancreas and its complicated surrounding anatomy, the lack of tactile sense that accompanies laparoscopic surgery can make a complete resection even more challenging.

In earlier studies, we have demonstrated improved resection rates and surgical outcomes in mouse models of cancer

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Abbreviations and Acronyms
BLLS = bright-light laparoscopic surgery
BLS = bright-light surgery
DFS = disease-free survival
FGLS = fluorescence-guided laparoscopic surgery
FGS = fluorescence-guided surgery
LED = light-emitting diode
PDOX = patient-derived orthotopic xenograft
RFP = red fluorescent protein
SOI = surgical orthotopic implantation

when resected under fluorescence-guided surgery (FGS).[3-9] Under the guidance of fluorescent probes, FGS led to more complete resections and lengthened disease-free survival (DFS) and overall survival in mice. Recurrence rates were decreased and cure rates improved.[3,4,9] We also described a successful diagnostic role of fluorescence laparoscopy in detecting and localizing anti-CEA–conjugated AlexaFluor 488–labeled metastatic lesions of pancreatic cancer.[10] The aim of this study was to demonstrate the advantages of fluorescence-guided laparoscopic surgery (FGLS) of a CEA-expressing pancreatic tumor labeled with a chimeric fluorescently labeled anti-CEA antibody compared with standard bright-light laparoscopic surgery (BLLS) in orthotopic mouse models.

METHODS

Cell culture

Human BxPC-3 pancreatic cancer cells expressing red fluorescent protein (RFP) were maintained in RPMI (Gibco-BRL) supplemented with 10% fetal bovine serum (Hyclone).[3] The cell culture medium was supplemented with penicillin/streptomycin (Gibco-BRL), sodium pyruvate (Gibco-BRL), sodium bicarbonate (Cellgro), L-glutamine (Gibco-BRL), and minimal essential medium nonessential amino acids (Gibco-BRL). Cells were incubated at 37°C with 5% carbon dioxide.

Antibody conjugation

Chimeric monoclonal antibodies specific for CEA were obtained from Aragen Biosciences.[9,11] The antibody was labeled with the AlexaFluor 488 Protein Labeling Kit (Molecular Probes Inc.), according to the manufacturer's instructions.[9,11] Briefly, the monoclonal antibody was reconstituted at 2 mg/mL in phosphate-buffered saline. Five hundred microliters of the 2 mg/mL solution plus 50 μL of 1M sodium bicarbonate were added to the reactive dye and allowed to incubate for 1 hour at room temperature, then overnight at 4°C. The conjugated antibody was then separated from the remaining unconjugated dye on a purification column by centrifugation. Antibody and dye concentrations in the final sample were determined using spectrophotometric absorbance with a Nanodrop ND 1000 spectrophotometer.

Animal care

Female athymic nu/nu nude mice (AntiCancer, Inc.) were maintained in a barrier facility on high-efficiency particulate air filtered racks. The animals were fed with autoclaved laboratory rodent diet (Teckland LM-485; Western Research Products). All surgical procedures were performed under anesthesia with an intramuscular injection of 100 μL of a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine. For each procedure, 20 μL of 1 mg/kg buprenorphine were administered for pain control. Euthanasia was achieved by 100% carbon dioxide inhalation, followed by cervical dislocation. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH's *Guide for the Care and Use of Animals* under assurance number A3873-01.

Subcutaneous tumor cell implantation

Human BxPC-3-RFP pancreatic cancer cells were harvested from monolayer in vitro culture by trypsinization and washed twice with serum-free medium. Viability was verified to be >95% using the Vi-Cell XR automated cell viability analyzer (Beckman Coulter). Cells (2×10^6 in 100 μL serum-free media) were injected subcutaneously within 30 minutes of harvesting over the right and left flanks in female nu/nu mice between 4 and 6 weeks of age. Subcutaneous tumors were allowed to grow for 2 to 4 weeks until large enough to supply adequate tumor for orthotopic implantation.

Orthotopic tumor implantation

Orthotopic human pancreatic cancer xenografts were established in nude mice by direct surgical orthotopic implantation (SOI) of single 1-mm^3 tumor fragments harvested from BxPC-3-RFP tumors growing subcutaneously, as described here. The tail of the pancreas was delivered through a small 6-mm to 10-mm transverse incision made on the left flank of the mouse. The tumor fragment was sutured to the tail of the pancreas using 8-0 nylon surgical sutures. Upon completion, the pancreas was returned to the abdomen and the incision was closed in 2 layers using 6.0 Ethibond nonabsorbable sutures (Ethicon Inc.).[12-15]

Our laboratory previously developed the technique of SOI of intact cancer tissues in immunodeficient mice, including pancreatic cancer.[12-15] Metastatic nude-mouse models of human pancreatic cancer were constructed orthotopically from histologically intact patient specimens.[13] Although orthotopic injection of cells gives rise

to tumors, the rate of tumor growth and metastasis is less than with SOI, and cell leakage can occur.[15]

Fluorescence laparoscopy

A standard laparoscopic tower provided by Stryker was slightly modified as described previously.[10,16] The excitation light source, a Stryker L9000 light-emitting diode (LED) lamp, was filtered through a glass emission filter (Schott GG495) placed between the laparoscope and the Stryker 1288 HD camera. Using the computer software system provided by Stryker (L9Calibration 0.03DOT3), adjustments to the red, blue, and green components of the Stryker L9000 LED light source were made to allow visualization of the fluorescent tumors. A Stryker X8000 Xenon light source was used for bright field laparoscopy.[10,16]

Laparoscopic tumor resection

A total of 46 mice were used in this experiment, 24 of the mice underwent FGLS, and 22 underwent BLLS. Two weeks after orthotopic implantation of human pancreatic cancer, mice bearing BxPC-3-RFP tumors were randomly assigned to BLLS or FGLS. The mice in the FGLS group received tail vein injection of 75 μg anti-CEA AlexaFluor 488 24 hours before planned resection of the pancreatic tumor. Surgery was performed 2 weeks after surgical implantation of tumor fragments, at which time there was no gross evidence of invasion of the primary tumor to surrounding organs or peritoneum.

For both FGLS and BLLS groups, at the time of surgery, mice were anesthetized as described, and their abdomens were sterilized. A 3-mm trocar was placed mid-abdomen and secured with a 6-0 nylon purse-string suture. Pneumoperitoneum was established to maintain an intra-abdominal pressure of 2 to 4 mm Hg. The 2.7-mm, 0-degree laparoscope was inserted and the primary pancreatic tumor was identified either under standard lighting or fluorescence lighting. A pediatric laparoscopic grasper inserted into the lower abdomen just right to the midline was used to grasp and elevate the tumor. A pediatric laparoscopic scissor, inserted through the left lower quadrant, was then used to sharply dissect the primary tumor as well as any lesions separate from the primary tumor within the pancreas, thereby performing a distal pancreatectomy. The excised tumor lesions were then delivered through the larger laparoscopic incision, the mid-abdominal trocar site. All 3 incisions were closed with a single interrupted 6-0 Vicryl suture. Postoperatively, the mouse and the excised tumor were imaged with the OV-100 Small Animal Imaging System (Olympus Corp.)[17] under both standard bright field and fluorescence illumination to assess completeness of surgical resection in both groups and to evaluate the size of the tumor excised along with surgical margins.

Animal imaging

To assess for recurrence and to follow tumor progression postoperatively, weekly whole-body imaging of the mice was obtained with the OV-100 Imaging System. Eight to 10 weeks after resection, the mice were sacrificed and intravital and ex vivo images were obtained to evaluate primary pancreatic and metastatic tumor burden. All images were analyzed with Image J v1.440 (NIH) by 2 separate reviewers who were blinded to the treatment group to avoid any potential bias. All primary pancreatic tumors were excised and weighed. Two randomly selected mice from each group, determined at the time of randomization, were followed beyond 10 weeks until deemed premorbid or until 1-year survival postoperatively was achieved, whichever came first. To determine premorbidity, mice were evaluated for the degree of ascites, cachexia, and mobility on a scale of 0 to 4 (4 being the highest grade). When ascites and cachexia or mobility reached a grade of 4, the mouse was terminated. At termination, mice were examined as described previously.

Tissue histology

Samples at the time of the initial surgery and at necropsy were collected when possible for histologic preparation with hematoxylin and eosin staining. Fresh tissue samples were fixed in Bouin's solution and regions of interest embedded in paraffin before sectioning and staining with hematoxylin and eosin for standard light microscopy. Hematoxylin and 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) without post-acquisition processing.

Data processing and statistical analysis

SAS software (version 9.2, SAS Institute) was used for statistical analyses. Continuous variables (ie, tumor size, tumor weight, and area of primary and metastatic tumor burden) are described using mean ± SE. The normality of the variables was assessed by visual inspection of histograms and normal Q-Q plots. A Welch's *t*-test or Wilcoxon rank sum test was used to compare groups, as appropriate. Pearson correlation was used to explore the association between 2 continuous variables. Categorical variables (local and distant recurrence, cure, and 1-year survival) were expressed as counts and percentages, and tests of significance used Fisher's exact test. To adjust for a factor that can affect the binary outcomes, logistic regression analysis was performed. We compared overall survival and disease-free survival between treatment groups

using a log rank test. Median survival time and their 95% CIs were calculated using the linear CI method. We reported $+\infty$ for upper bound of the interval if it could not be estimated. A 2-sided p value of ≤ 0.05 was considered statistically significant for all comparisons.

RESULTS

Efficacy of anti–carcinoembryonic antigen labeling of pancreatic tumors

The first objective of this study was to confirm the accuracy of the chimeric anti-CEA antibody conjugated to AlexaFluor 488 (anti–CEA-488) in labeling the CEA-expressing pancreatic tumor that also expressed RFP (Fig. 1). The mean area of the red fluorescence was not significantly different than the AlexaFluor 488 green fluorescence (6 mm^2 vs 7 mm^2). The chimeric antibody was highly accurate in binding to and labeling the CEA-expressing pancreatic tumor, as indicated by the high correlation between red and green fluorescence (Pearson correlation 0.899; $p < 0.001$).

Fluorescence laparoscopy vs bright-light laparoscopy in identifying and resecting the primary tumor

The primary pancreatic tumor was better visualized under fluorescence compared with standard bright light (Fig. 2A). As a result, all 24 mice in the FGLS group underwent a complete resection, evidenced by the lack of fluorescence signal upon whole-body postoperative images obtained with the OV-100. In contrast, 2 mice (of 22) in the BLLS group had evidence of residual fluorescence in postoperative images (Fig. 2B), indicating incomplete resection.

Overall, mean specimen size resected was not significantly different between both laparoscopic resection groups (12.15 ± 0.9 mm^2 vs 14.36 ± 1.5 mm^2; $p = 0.213$). Tumor burden was assessed by measuring area of fluorescence using ImageJ software. Again, there was no significant difference in mean tumor burden between the 2 groups (5.7 ± 0.6 mm^2 vs 6.2 ± 0.9 mm^2; $p = 0.657$). However, under FGLS, there was significantly less pancreatic tumor burden at the time of termination than with BLLS ($p = 0.012$) (Fig. 3).

Disease-free survival and recurrence rates

All mice in the termination groups were followed postoperatively for 8 or 10 weeks with weekly imaging obtained with the OV-100. Disease-free survival was defined as the point in the postoperative period in which fluorescence was first detected in weekly whole-body images. Fluorescence-guided laparoscopic surgery afforded mice significantly longer DFS by more than doubling the mean time (in weeks) as demonstrated by the Kaplan-Meier Survival Curve in Figure 4. Median DFS was lengthened from 2 weeks with BLLS (95% CI, 1.635–2.365) to 7 weeks with FGLS (95% CI, 5.955–8.045; $p = 0.001$). A Cox proportional hazards model, adjusting for preoperative tumor burden and margins (specimen size minus preoperative tumor burden), also showed reduced risk of recurrence in the FGLS group compared with BLLS (hazard ratio = 0.405; 95% CI, 0.194–0.846; $p = 0.016$).

In addition to lengthening DFS, laparoscopic resection of primary pancreatic cancer under fluorescence guidance substantially reduced local and distant recurrence rates (Fig. 5). At the time of termination, the abdomen of all mice in the termination groups was exposed and the organs were harvested for intravital and ex vivo images.

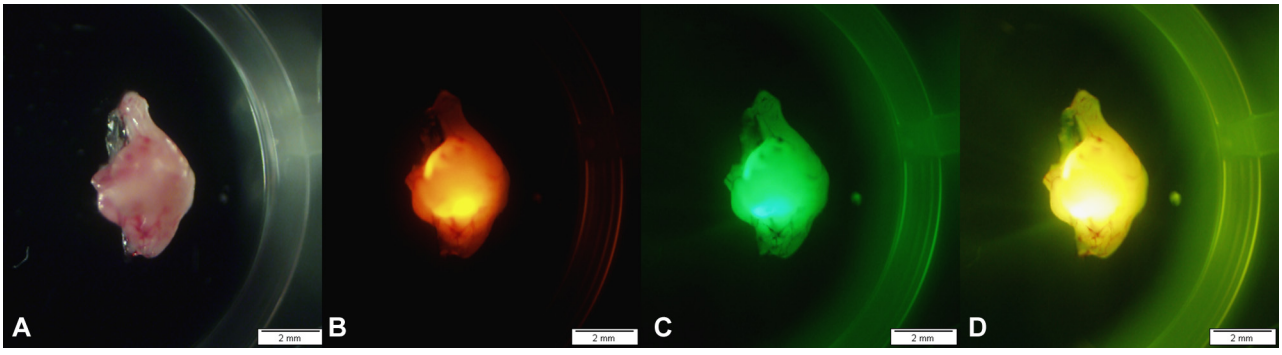

Figure 1. Labeling efficacy of chimeric anti-CEA AlexaFluor 488–conjugated antibodies. A representative bright field image of a resected pancreatic tumor is shown in (A). The red fluorescent protein (RFP)–expressing tumor is visualized under an RFP filter (excitation 535–555, emission 570–623) of the OV-100 Small Animal Imaging System (B). With a green fluorescent protein (GFP) filter (excitation 460–490, emission 510–550) (C), only the tumor labeled with the green fluorescent antibody is visualized. The accuracy of the green fluorescent antibody in labeling the CEA-and RFP–expressing pancreatic tumor results in a yellow image under the GFP filter (excitation 460–490, emission 510F), which can visualize both green and red fluorescence (D).

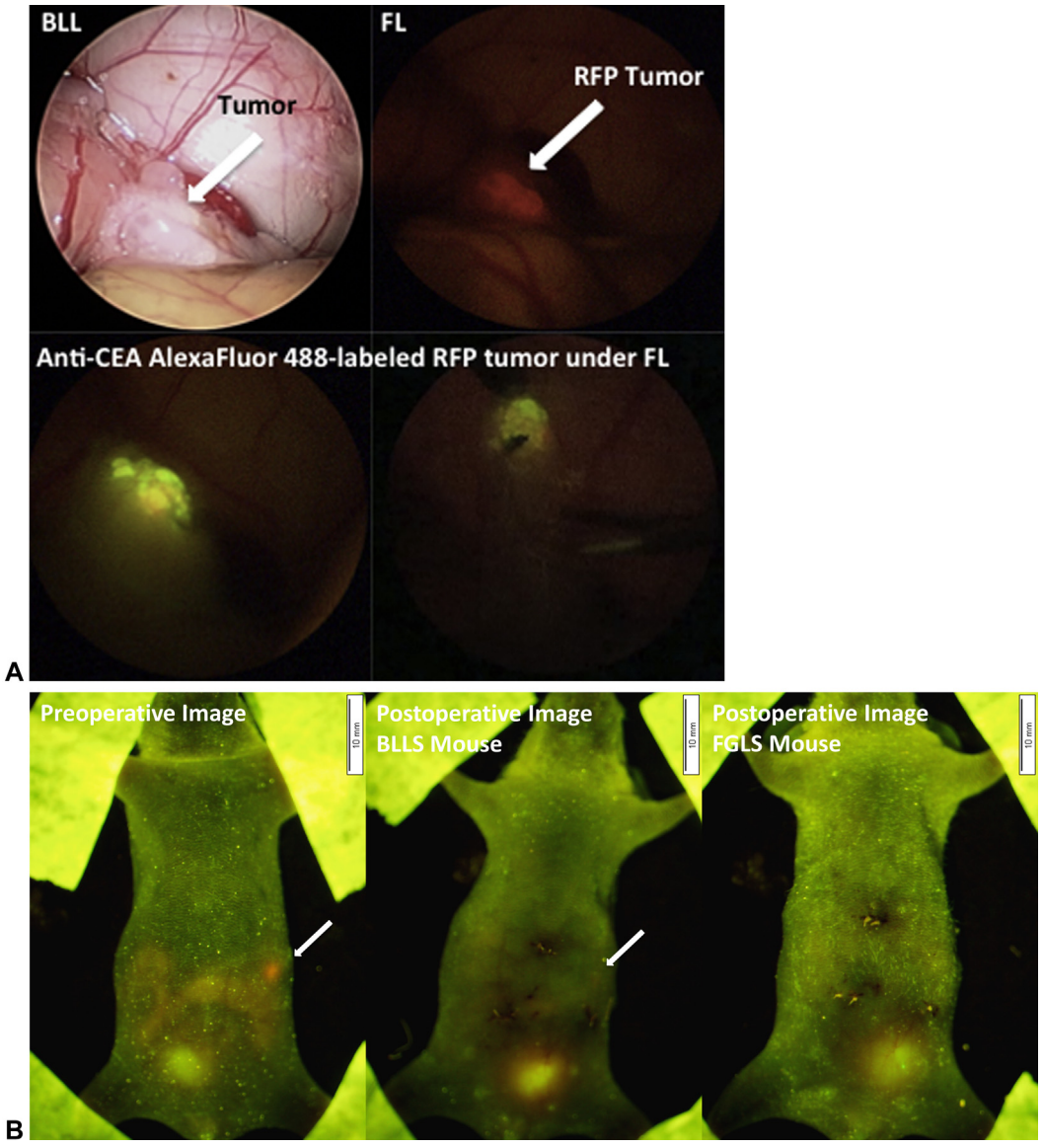

Figure 2. Fluorescence-guided laparoscopic surgery (FGLS) vs bright-light laparoscopic surgery (BLLS) for pancreatic cancer. (A) The red fluorescent protein (RFP)–expressing pancreatic tumor (seen in the fluorescence laparoscopy [FL] image) is difficult to identify under standard bright lighting. However, when the tumor was labeled with the chimeric anti-CEA AlexaFluor 488–conjugated antibody, identification and visualization of the tumor was significantly enhanced, allowing for subsequent better resection under fluorescence guidance (bottom images). BLL, bright-light laparoscopy. (B) Representative postoperative images from the BLLS group and the FGLS group compared to a preoperative mouse. The arrow identifies residual red fluorescence on postoperative whole-body images indicating an incomplete resection by BLLS. This occurred in 2 of 22 mice in the BLLS group. All 24 mice in the FGLS group underwent a complete resection. A typical image is shown. The preoperative image is representative of all mice undergoing resection. These images were obtained to confirm presence of tumor.

Local recurrence was defined as the presence of fluorescent tumor identified within the pancreas. Distant recurrence was defined as the presence of fluorescent tumor in any organ other than the pancreas. Local recurrence rates decreased from 71.4% to 38.5% with FGLS compared with BLLS, respectively ($p = 0.048$). Distant recurrence rates were reduced to 42.4% with FGLS from 85.7% with BLLS ($p = 0.046$).

Cure rates

Forty-two mice randomized to either BLLS or FGLS were terminated at either 8 or 10 weeks postoperatively.

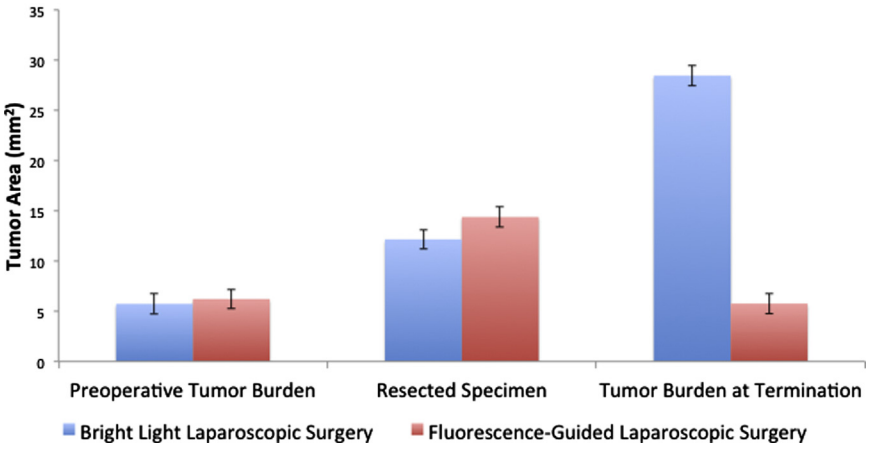

Figure 3. Tumor burden at termination using bright-light laparoscopic surgery (BLLS) and fluorescence-guided laparoscopic surgery (FGLS). There were no significant differences with regard to mean preoperative tumor burden ($p = 0.657$) or mean resected specimen size ($p = 0.213$) between the 2 surgical groups. However, the improved resection achieved under fluorescence guidance led to significantly lower pancreatic tumor burden at the time of termination in the FGLS group compared with the BLLS group ($p = 0.012$).

Cure was defined as the complete absence of fluorescent tumor on either intravital or ex vivo images. Although statistical significance was not reached, cure rates were 44.1% in the BLLS group and 83.3% in the FGLS group ($p = 0.091$).

DISCUSSION

In our original article on FGS of pancreatic cancer, we inquired if FGS could improve surgical outcomes and reduce recurrence rates in orthotopic mouse models of human pancreatic cancer.[3] Orthotopic mouse models of human pancreatic cancer were established using the BxPC-3 pancreatic cancer cell line expressing RFP. A more complete resection of pancreatic cancer was achieved using FGS compared with bright light surgery (BLS). Fluorescence-guided surgery resulted in a significantly longer DFS than BLS.[3]

In our next study, mouse models of human pancreatic cancer, with surgical orthotopic implantation of the human BxPC-3 pancreatic cancer, were injected intravenously with anti-CEA AlexaFluor 488.[18] Complete resection was achieved in 92% of mice in the FGS group compared with 45.5% in the BLS group. Cure rates with FGS compared with BLS improved from 4.5% to 40%, respectively, and 1-year postoperative survival rates increased from 0% with BLS to 28% with FGS.[18]

In another previous study, we improved fluorescence laparoscopy of pancreatic cancer in an orthotopic mouse model of pancreatic cancer with the use of an LED light source and optimal fluorophore combinations. Orthotopic human pancreatic cancer nude mouse models were established with pancreatic cancer cells lines. Diagnostic laparoscopy was performed after tail-vein injection of CEA antibodies conjugated with AlexaFluor 488 or AlexaFluor 555. Fluorescence laparoscopy with a 495-nm emission filter and an LED light source enabled real-time visualization of the fluorescence-labeled tumor deposits in the peritoneal cavity, thereby enhancing detection of submillimeter lesions without compromising background illumination.[10]

In the current study, we describe the efficacy of laparoscopic FGS of pancreatic cancer in an orthotopic mouse model. Mouse models of human pancreatic cancer were established with fragments of the BxPC-3-RFP human pancreatic cancer using SOI. Fluorescence-guided laparoscopic surgery was performed with an LED light source through a 495-nm emission filter. Tumors were labeled with anti-CEA AlexaFluor 488 antibodies 24 hours before surgery with intravenous injection. Bright-light laparoscopic surgery was performed with a xenon light source. At the time of termination, the FGLS group had significantly less pancreatic tumor volume than the BLLS group and lower tumor weight. Fluorescence-guided laparoscopic surgery compared with BLLS also significantly decreased local (50% vs 80%, respectively; $p = 0.048$) and distant recurrence (70% vs 95%, respectively; $p = 0.046$). More mice in the FGLS than the BLLS group were free of tumor at the time of termination (25% vs 5%, respectively).

Median DFS was lengthened from 2 weeks with BLLS to 7 weeks with FGLS. Our results thus demonstrate that fluorescence-guided laparoscopic surgery is more effective

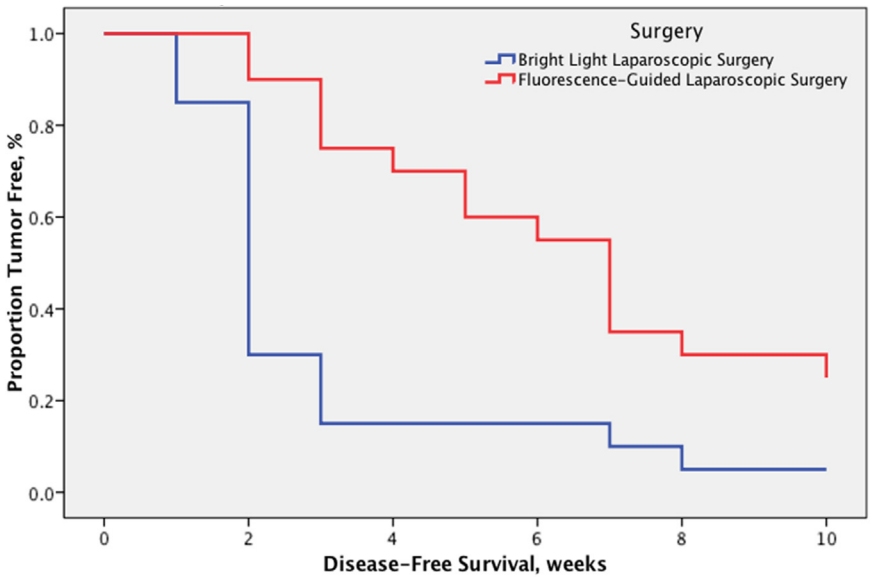

Figure 4. Kaplan-Meier curve for disease-free survival with bright-light laparoscopic surgery (BLLS) and fluorescence-guided laparoscopic surgery (FGLS). Fluorescence-guided laparoscopic surgery improved disease-free survival (DFS) in mice harboring pancreatic tumors by more than doubling the mean time in weeks compared with BLLS. Median DFS improved from 2 weeks in the BLLS group to 7 weeks ($p = 0.001$).

than BLLS and therefore has important potential for improving the outcomes of surgical oncology.

The current study enabled us to expand the use of fluorescence laparoscopy from a purely diagnostic method to a feasible therapeutic approach. Fluorescence-guided laparoscopic surgery of fluorescently-labeled CEA-expressing pancreatic cancer permitted more accurate detection and localization of the primary tumor, as well as detection and localization of any submillimeter lesions separate from the primary tumor within the pancreas. This improved resection and resulted in better short-term and long-term outcomes. The lack of tactile sense that accompanies laparoscopic surgery became less critical when tumor margins were more objectively established with the fluorescent probes. The bright fluorescence achieved with the conjugated chimeric anti-CEA antibody permitted enough light leakage for background illumination that was adequate for surgical navigation and precise resection. The appropriate tumor to background fluorescent ratio was maintained so as not to compromise the contrasting fluorescence signal from the labeled pancreatic tumor.

In addition, we used a more clinically-relevant antibody, a chimeric anti-CEA antibody, to test its feasibility for future use in human patient trials of FGS or FGLS. This "fusion" protein allows the introduction of segments of human constant domains and maintains important properties from the "parent" protein, eliminating most of the potential immunogenic portions of the antibody without compromising its specificity for the intended target.[19] We had previously demonstrated that the chimeric antibody maintained its sensitivity and specificity for labeling colon cancer in patient-derived orthotopic xenograft (PDOX) mouse models.[9] In the current study, we successfully demonstrated the use of the chimeric antibody for FGLS. Currently, several chimeric protein US Food and Drug—approved drugs are available for clinical use, such as rituximab (Rituxan), basiliximab (Simulect), and infliximab (Remicade), with many more new developments on the way for cancer therapy.[20-22] In the near future, fluorescently-labeled antibodies should be available for FGLS and FGS.

The small-animal model used in the current study made full laparoscopic exploration of the abdomen challenging. In our earlier studies of open laparotomy, the advantage of FGS was achieved when small satellite lesions separate from the primary tumor were identified and excised. These lesions were not detected under standard bright light. In our FGLS model, some of these lesions could have been missed. Our primary outcome of interest included local recurrence rates. Timed termination was determined to be the most appropriate study design. Future studies will address whether larger, more invasive tumors can be resected with FGLS.

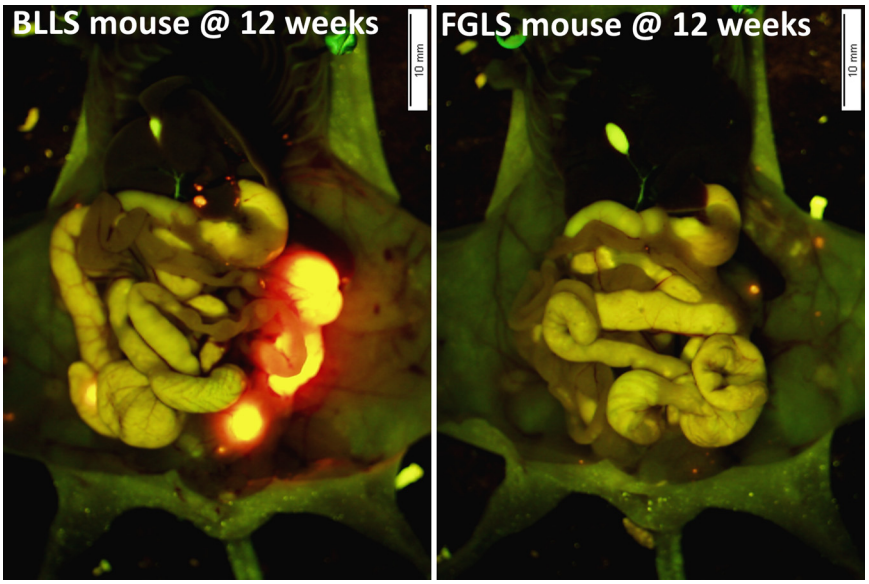

Figure 5. Metastatic tumor burden at termination 12 weeks postoperatively with bright-light laparoscopic surgery (BLLS) vs fluorescence-guided laparoscopic surgery (FGLS). Representative intravital images obtained with the OV-100 Small Animal Imaging System demonstrating improved outcomes achieved by FGLS compared with BLLS at termination, as can be seen by the extensive red fluorescent pancreatic tumor remaining in the BLLS–treated mouse and its absence in the FGLS—treated mouse. Fluorescence-guided laparoscopic surgery substantially reduced the number of mice with local and distant recurrence compared with BLLS.

For labeling of tumors, there are a number of approaches currently used in the clinic for FGS. For example, sentinel lymph nodes in breast cancer patients were detected by labeling with the near-infrared fluorescing dye indocyanine.[23] In another study, patients with malignant gliomas were given 5-aminolevulinic acid orally 3 hours before undergoing either BLS or FGS. In the FGS group, 65% of 139 patients displayed complete removal of their tumors. In contrast, in the BLS group only 36% of 131 patients showed complete tumor resection. Patients who underwent FGS had higher 6-month progression-free survival rates than did those who had surgery under white light.[24] Van Dam and colleagues conjugated folate to fluorescein isothiocyanate for targeting folate receptor—α in 10 ovarian cancer patients who were undergoing abdominal surgery.[25] Fluorescence-guided surgical resection of tumor deposits <1 mm was able to be performed.

We believe a humanized anti-CEA antibody has great potential to label tumors in patients, as suggested by our recent results with PDOX.[26] Patient colon tumors were grown orthotopically in nude mice to make PDOX models. A CEA antibody conjugated with AlexaFluor 488 was delivered to the PDOX models as a single intravenous dose before laparotomy. The tumors were completely resected under fluorescence navigation. Histologic evaluation of the resected specimen demonstrated that cancer cells were not present in the margins, indicating successful tumor resection. The FGS animals remained tumor free for >6 months.

The PDOX models of pancreatic cancer were labeled with AlexaFluor 488—conjugated anti-carbohydrate antigen 19-9 antibody. In the PDOX model labeled with AlexaFluor 488—conjugated anti-carbohydrate 19-9 antibody, a portable hand-held imaging device could distinguish the residual tumor from the background, enabling complete resection of the residual tumor to be achieved under fluorescence navigation.[27]

These studies along with our current results indicate future clinical success of minimally invasive surgery for cancer using FGLS as well as FGS.

CONCLUSIONS

In this study, we demonstrated the feasibility of performing laparoscopic resection of primary pancreatic cancer under fluorescence guidance using chimeric anti-CEA antibodies conjugated to a fluorophore. The accuracy of the fluorescent probe permitted adequate labeling and distinction of tumor margins to perform distal pancreatectomies laparoscopically in a safe, well-tolerated manner that improved outcomes. The improved resection under fluorescence guidance

substantially reduced the primary and metastatic tumor burden observed at the time of termination of our mouse models. Tumors were considerably smaller, DFS was lengthened, and local and distant recurrence rates were lower with FGLS than BLLS, and more mice from the FGLS group were completely free of tumor at the time of termination than mice in the BLLS group. Fluorescence-guided laparoscopic surgery can be used for resection of metastases as well as primary tumor resection.

Author Contributions

Study conception and design: Metildi, Kaushal, Luiken, Hoffman, Bouvet

Acquisition of data: Metildi, Kaushal

Analysis and interpretation of data: Metildi, Kaushal, Luiken, Hoffman, Bouvet

Drafting of manuscript: Metildi, Luiken, Hoffman, Bouvet

Critical revision: Metildi, Hoffman, Bouvet

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Discussion

INVITED DISCUSSANT: DR KELLY MCMASTERS (Louisville, KY): In this elegant study, Drs Metildi, Bouvet, and colleagues describe use of fluorescence-guided laparoscopic surgery (FGLS) using anti-CEA antibody in a mouse model of pancreatic cancer. Fluorescent-guided surgery resulted in more complete resection, decreased local and distant recurrence, and improved disease-free survival.

I have a few questions:

1. In my experience, gross tumor at the transected pancreatic margin is not usually the problem with laparoscopic distal pancreatectomy. Do you think there are often discontiguous nests of tumor within the pancreas, remote from the main tumor mass, that we miss with conventional open or laparoscopic surgery?
2. How sensitive is the fluorescence-guided approach for detecting microscopically positive margins? If not very sensitive, are there other methods for detection of nonvisible fluorescence at the cut margin that would be helpful rather than waiting for frozen sections?
3. Do you think the main application of this technique is to achieve negative radial margins, perform more complete resection of involved lymph nodes, or perhaps to assess for occult peritoneal implants? What is the major clinical advantage in your opinion?
4. Finally, I believe that the major problem with adenocarcinoma of the pancreas is not that we don't do adequate surgical resection; the biology of the disease is such that peritoneal and liver metastases occur early and we have poorly effective adjuvant therapy. You saw this in your mice: although you performed more complete resection with fluorescence-guided surgery, and improved disease-free survival, the vast majority of the mice eventually recurred. Do you therefore think that fluorescence-guided surgery will cure more patients, prolong survival, or perhaps delay the time to recurrence? What would you do in the case of fluorescence-detected occult peritoneal metastases, for example? Resect the pancreas or close?

DR MICHAEL BOUVET: In this study, we demonstrated the feasibility of performing FGLS of primary pancreatic cancer using chimeric anti-CEA antibodies conjugated to a fluorophore. The improved resection under fluorescence guidance significantly reduced the primary and metastatic tumor burden observed at termination of our mouse models. Tumors were significantly smaller, disease-free survival was lengthened, and local and distant recurrence rates were lower with FGLS.

More mice from the FGLS group were completely free of tumor at termination. Because CEA is overexpressed in a number of gastrointestinal cancers, there is potential for this technology to be used for resection and detection of cancers other than pancreatic cancer, such as colorectal cancer, as we have shown in mouse models.

Although these studies are still preclinical in nature, we are beginning to see some fluorescence imaging techniques in the clinic now. For instance, fluorescence cholangiography using indocyanine green to help identify the biliary anatomy during laparoscopic cholecystectomy is being trialed at several centers in the US and in Japan.

Fluorescence-guided surgery for glioblastoma using the metabolite 5-aminolevulinic acid, a precursor of hemoglobin, can drive the accumulation of porphyrins within malignant glioma and was shown in one study to improve complete resection rates. In another study, folate was conjugated to fluorescein isothiocyanate for targeting folate receptor, which is often overexpressed in ovarian cancers, in 10 ovarian cancer patients who were undergoing abdominal surgery. The surgeons used a real-time multispectral intraoperative fluorescence imaging system for tumor detection and achieved fluorescence-guided resection of tumor deposits less than 1 mm in size. New hand-held, high-resolution, fluorescence imaging systems for fluorescence-guided surgery are being described and may have clinical utility.

Your question regarding discontiguous nests of tumor with the pancreas is pertinent. As you know, pancreatic ductal adenocarcinoma (PDAC) is a common gastrointestinal malignancy characterized by rapid progression, resulting in poor outcome and a 5-year survival rate of less than 5%. Like in most adenocarcinomas, PDAC has a massive fibrotic stoma, that is, desmoplasia, which contributes to the local inflammatory environment at the tumor site as well as systemically. The microenvironment found in PDAC supports tumor growth, progression, and the recruitment of leukocytes, such as macrophages, dendritic cells, T cells, and neutrophils. Therefore, it can be difficult to distinguish between tumor and fibrosis and pancreatitis. This makes it difficult to obtain a truly negative margin during pancreatic surgery, and therefore, the high rate of positive microscopic margins.

We have also witnessed discontiguous nests of tumor within the pancreas in our orthotopic mouse models. In our open model, with fluorescence-guided surgery we could identify these small isolated lesions separate from the primary and resect them without having to remove a significant amount of normal pancreatic tissue.

In a previous study, we reported that the R0 resection rate increased from 45% with bright light surgery to 92% with fluorescence-guided surgery when using fluorescently conjugated antibodies in orthotopic mouse models of pancreatic cancer. Additionally, another potential benefit of fluorescence-guided surgery is that the pathologist and surgeon can quickly examine the cut margin of the pancreas using fluorescence microscopy to assess