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Advantages of fluorescence-guided laparoscopic surgery of pancreatic cancer labeled with fluorescent anti-CEA antibodies in an orthotopic mouse model

Cristina A Metildi, M.D.¹, Sharmeela Kaushal, PhD¹, George A Luiken, M.D.², Robert M. Hoffman, PhD³, and Michael Bouvet, M.D., FACS¹

¹University of California San Diego, Department of Surgery, San Diego, CA

²OncoFluor, Inc., San Diego, CA

³AntiCancer, Inc., San Diego, CA

Abstract

Background—Our laboratory has previously developed fluorescence-guided (FGS) of pancreatic and other cancers in orthotopic mouse models. Laparoscopic surgery is being used more extensively in surgical oncology. The present report describes the efficacy of laparoscopic FGS of pancreatic cancer in an orthotopic mouse model.

Study Design—Mouse models of human pancreatic cancer were established with fragments of the BxPC-3 RFP human pancreatic cancer using surgical orthotopic implantation (SOI). Mice were randomized to bright light laparoscopic surgery (BLLS) or to fluorescence guided laparoscopic surgery (FGLS). FGLS was performed with an LED light source through a 495-nm emission filter in order to remove the primary tumors and any additional separate sub-millimeter deposits within the pancreas, the latter of which was not possible with BLLS. Tumors were labeled with anti-CEA-Alexa 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.

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Address correspondence to: Michael Bouvet, MD, Department of Surgery, Moores UCSD Cancer Center, 3855 Health Science Drive #0987, La Jolla, CA 92093-0987, Phone: 858-822-6191, Fax: 858-822-6192, mbouvet@ucsd.edu.

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Disclosure Information:

Dr Hoffman is a stockholder in AntiCancer, Inc. and Dr Luiken is a stockholder in OncoFluor, Inc. Other authors have nothing to disclose.

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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, Bouvet, Luiken, Hoffman

Critical revision: Metildi, Hoffman, Bouvet

Results—At termination, the FGLS group had less pancreatic tumor volume than the BLLS group (5.75 mm² vs 28.43 mm², respectively; p=0.012) and lower tumor weight (21.1 mg vs 174.4 mg, respectively; p=0.033). FGLS compared to 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 than the BLLS group were free of tumor at termination (25% vs 5%, respectively). The median disease free survival (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).

Conclusions—FGLS is more effective than BLLS, and therefore has important potential for surgical oncology.

Keywords

pancreatic cancer; orthotopic mouse models; fluorescence-guided laparoscopic surgery; fluorophore-conjugated chimeric antibodies

Introduction

Refinements in laparoscopic instruments and advances in robotic platforms have improved movement precision and dexterity allowing for more complex laparoscopic procedures to be undertaken. 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 prior studies, we have demonstrated improved resection rates and surgical outcomes in mouse models of cancer when resected under fluorescence-guided surgery (FGS).³⁻⁹ Under the guidance of fluorescent probes, fluorescence-guided surgery led to more complete resections and lengthened disease-free survival 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-Alexa 488-labeled metastatic lesions of pancreatic cancer.¹⁰ The aim of this study was to evaluate the advantages of fluorescence-guided laparoscopic surgery (FGLS) of a CEA-expressing pancreatic tumor labeled with a chimeric fluorescently-labeled anti-CEA antibody compared to 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, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). The cell culture medium was supplemented with penicillin/streptomycin (Gibco-BRL), sodium pyruvate (Gibco-BRL), sodium bicarbonate (Cellgro, Manassas, VA), 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 carcino-embryonic antigen (CEA) were obtained from Aragen Biosciences (Morgan Hill, CA).^{9, 11} The antibody was labeled with the AlexaFluor 488 Protein Labeling Kit (Molecular Probes Inc., Eugene, OR) according to the manufacturer's instructions.^{9, 11} Briefly, the monoclonal antibody was reconstituted at 2 mg/mL in PBS. 500 L of the 2 mg/mL solution plus 50 µL of 1M sodium bicarbonate was 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 by Nanodrop ND 1000 spectrophotometer.

Animal care

Female athymic nu/nu nude mice (AntiCancer, Inc., San Diego, CA) 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 ResearchProducts, Orange, CA). All surgical procedures were performed under anesthesia with an intramuscular injection of 100 L of a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine. For each procedure, 20 L of 1 mg/kg buprenorphine was 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 National Institutes of Health (NIH) 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 greater than 95% using the Vi-Cell XR automated cell viability analyzer (Beckman Coulter, Brea, CA). Cells (2×10^6 in 100 L serum-free media) were injected subcutaneously within 30 min 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 2-4 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 mm³ tumor fragments from BxPC-3-RFP tumors growing subcutaneously as described above. The tail of the pancreas was delivered through a small 6 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 two layers using 6.0 Ethibond non-absorbable sutures (Ethicon Inc., Somerville, NJ).¹²⁻¹⁵

Our laboratory developed the technique of surgical orthotopic implantation (SOI) of intact cancer tissues in immunodeficient mice including pancreatic cancer.^{13, 15} A metastatic nude-mouse model of human pancreatic cancer constructed orthotopically from histologically

intact patient specimens.¹³ Although orthotopic injection of cells give rise to tumors, the rate of tumor growth and metastasis is less than compared to SOI and cell leakage may occur.¹⁵

Fluorescence Laparoscopy

A standard laparoscopic tower provided by Stryker (Stryker, San Jose, California) was slightly modified as previously described.^{10, 16} The excitation light source, a Stryker L9000 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 (L9Calibration0.03DOT3), 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 fluorescence-guided laparoscopic resection (FGLS), while 22 underwent bright light laparoscopic resection (BLLS). Two weeks following 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 of anti-CEA-Alexa 488 24 hours prior to 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 intraabdominal pressure of 2-4 mm Hg. The 2.7 mm 0° 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 three 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, Tokyo, Japan)¹⁷ under both standard bright field and fluorescence illumination to assess completeness of surgical resection in both groups and to evaluate the size of 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 ten weeks after resection, the mice were sacrificed and intravital and ex vivo images were obtained in order to evaluate primary pancreatic and metastatic tumor burden. All images were analyzed with Image J v1.440 (National Institutes of Health, Bethesda, MD) by two

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 ten weeks until deemed premonitory or until one-year survival postoperatively was achieved, whichever came first. To determine premonitory, 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 previously described.

Tissue histology

Samples at the time of the initial surgery and at necropsy were collected when possible for histologic preparation with hematoxylin and eosin (H&E) staining. Fresh tissue samples were fixed in Bouin's solution and regions of interest embedded in paraffin prior to sectioning and staining with H&E for standard light microscopy. H&E-stained permanent sections were examined using an Olympus BX41 microscope equipped with a Micropublisher 3.3 RTV camera (QImaging, Surrey, B.C., Canada). All images were acquired using QCapture software (QImaging) without post-acquisition processing.

Data processing & statistical analysis

SAS v9.2 (The SAS Institute, Inc) was used for statistical analyses. Continuous variables (tumor size, tumor weight, and area of primary and metastatic tumor burden) are described using means $\pm$ 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's correlation was used to explore the association between two continuous variables. Categorical variables (local and distant recurrence, cure, and one-year survival) were expressed as counts and percentages, and tests of significance used Fisher's exact test. To adjust for a factor that may 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% confidence intervals were calculated using the linear confidence interval method. We reported $+\infty$ for upper bound of the interval if it could not be estimated. A two-sided *p*-value of 0.05 was considered statistically significant for all comparisons.

Results

Efficacy of Anti-CEA Labeling of Pancreatic Tumors

The first objective of this project was to confirm accuracy of the chimeric anti-CEA antibody conjugated to Alexa 488 (anti-CEA-488) in labeling the CEA-expressing pancreatic tumor (Figure 1). The average area of the red fluorescence was not significantly different than the Alexa 488 green fluorescence (6 mm² vs 7 mm²). Thus, the chimeric antibody was highly accurate in binding to and thus labeling CEA-expressing pancreatic tumor as indicated by the high correlation between red and green fluorescence (Pearson's 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 to standard bright light (Figure 2a). As a result, all 24 mice in the FGLS group underwent a complete resection as evident by the lack of fluorescence signal on whole body postoperative images taken with the OV-100. In contrast, two mice (out of 22) in the BLLS group had evidence of residual fluorescence in postoperative images (Figure 2b), indicating incomplete resection.

Overall, the average specimen size resected was not significantly different between both laparoscopic resection groups ($12.15 \pm \text{SE } 0.9 \text{ mm}^2$ vs. $14.36 \pm \text{SE } 1.5 \text{ mm}^2$, $p=0.213$). Tumor burden was assessed by measuring area of fluorescence using ImageJ software. Again, there was no significant difference in average tumor burden between the two groups ($5.7 \pm \text{SE } 0.6 \text{ mm}^2$ vs. $6.2 \pm \text{SE } 0.9 \text{ mm}^2$, $p=0.657$). However, under FGLS, there was significantly less pancreatic tumor burden at termination than with BLLS ($p=0.012$) (Figure 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 (DFS) was defined as the point in the postoperative period in which fluorescence was first detected in weekly whole body images. FGLS afforded mice significantly longer DFS by more than doubling the average time (in weeks) as demonstrated by the Kaplan Meier Survival Curve in Figure 4. The 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 to BLLS (hazard ratio = 0.405, 95% CI (0.194, 0.846), $p=0.016$).

In addition to lengthening disease-free survival, laparoscopic resection of primary pancreatic cancer under fluorescence-guidance significantly reduced local and distant recurrence rates (Figure 5). At termination, the abdomen of all mice in the termination groups were exposed and the organs were harvested for intravital and ex-vivo images. 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 to BLLS, respectively ($p=0.048$) and distant recurrence rates were reduced to 42.4% with FGLS from 85.7% with BLLS, respectively ($p=0.046$).

Cure Rates

Forty-two mice randomized to either BLLS or FGLS were terminated at either 8 or 10 weeks postoperatively. Cure was defined as the complete absence of fluorescence 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 paper 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. Orthotopic mouse models of human pancreatic cancer were established using the BxPC-3 pancreatic cancer cell line expressing red fluorescent protein (RFP). A more complete resection of pancreatic cancer was achieved using FGS compared with BLS. FGS resulted in significantly longer disease-free survival than BLS.³

In our next paper, mouse models of human pancreatic cancer with surgical orthotopic implantation of the human BxPC-3 pancreatic cancer were injected iv with anti-CEA-Alexa Fluor 488. Complete resection was achieved in 92 % of mice in the FGS group compared to 45.5 % in the BLS group. Cure rates with FGS compared to 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 then improved fluorescence laparoscopy of pancreatic cancer in an orthotopic mouse model with the use of a light-emitting diode (LED) light source and optimal fluorophore combinations. 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 Alexa 488 or Alexa 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 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 surgical orthotopic implantation (SOI). FGLS was performed with an LED light source through a 495-nm emission filter. Tumors were labeled with anti-CEA-Alexa 488 antibodies 24 hours before surgery with intravenous injection. Bright light laparoscopic surgery (BLLS) was performed with a xenon light source. At termination, the FGLS group had significantly less pancreatic tumor volume than the group and lower tumor weight. FGLS compared to BLLS also significantly decreased local and distant recurrence (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 termination (25% vs 5%, respectively). The median disease free survival (DFS) was lengthened from 2 weeks with BLLS to 7 weeks with FGLS. FGLS is more effective than BLLS, and therefore has important potential for surgical oncology.

The present study enabled us to expand the utility of fluorescence laparoscopy from a purely diagnostic method to a feasible therapeutic approach. FGLS of fluorescently-labeled CEA-expressing pancreatic cancer permitted more accurate detection and localization of the primary tumor as well as any sub-millimeter lesions separate from the primary tumor within the pancreas, for improved resection and thus better short-term and long-term outcomes. The lack of tactile sense that accompanies laparoscopic surgery was virtually a non-issue when

tumor margins were more objectively established with the fluorescent probes. The bright fluorescence achieved with the 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 fluorescent signal from the labeled pancreatic tumor.

In addition, we used a more clinically relevant antibody, a chimeric anti-CEA antibody, to further 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 while maintaining important properties from the “parent” mouse protein, eliminating most of the potential immunogenic portions of the antibody without compromising its specificity for the intended target.¹⁹ We had previously demonstrated the chimeric antibody maintained its sensitivity and specificity for labeling colon cancer in patient-derived orthotopic xenograft mouse models.⁹ In the present study, we successfully demonstrated the utility of the antibody for FGLS. Currently, several chimeric protein FDA-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 Thus in the near future, fluorescently-labeled antibodies should be available for FGLS and FGS.

The small animal model used in the present study made full laparoscopic exploration of the abdomen challenging. In our prior studies of open laparotomy, the advantage of FGS was achieved when these small satellite lesions separate from the primary tumor were identified and excised. These lesions were not detected under standard bright light. In our laparoscopic resection model, some of these lesions could have been missed. Our primary outcome of interest was local recurrence rates and to best answer this question, 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.

Regarding the 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 and similarly labeled by the near-infrared (NIR) fluorescing dye indocyanine.²³ In another study, patients with malignant gliomas were given 5-aminolevulinic acid orally three hours before undergoing either BLS or FGS. In the FGS group, 65% of 139 patients displayed complete removal of their tumors, in contrast to 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 *et al.* conjugated folate to fluorescein isothiocyanate (FITC) for targeting folate receptor- α (FR- α) in 10 ovarian cancer patients who were undergoing abdominal surgery.²⁵ FGS resection of tumor deposits less than 1 mm in size 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 patient-derived orthotopic xenografts (PDOX).²⁶ Patient colon tumors were grown orthotopically in nude mice to make PDOX models. A CEA antibody conjugated with AlexaFluor488 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 over 6 months.

PDOX models of pancreatic cancer were labeled with Alexa Fluor 488-conjugated anti-carbohydrate antigen 19-9 antibody. In the PDOX model labeled with Alexa Fluor 488-conjugated anti-carbohydrate with 19-9, the portable hand-held device could distinguish the residual tumor from the background, and complete resection of the residual tumor was achieved under fluorescence navigation.²⁷

These studies along with our current results indicate future clinical success of MIS 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 thus distinction of tumor margins to perform distal pancreatectomies laparoscopically in a safe, well-tolerated manner that improved intermediate outcomes. 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, DFS was lengthened and local and distant recurrence rates were lower with FGLS. And more mice from the FGLS group were completely free of tumor at termination. FGLS can be used for resection of metastatic nodes and other metastases as well as primary tumor resection.

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Figure 1. Labeling Efficacy of Chimeric Anti-CEA-Alexa 488 Antibodies

A representative bright field image of a resected pancreatic tumor is shown in panel (a). The RFP-expressing tumor is visualized under the RFP filter (excitation 535-555, emission 570-623) of the OV-100 Small Animal Imaging System (b). With a 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-expressing RFP 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. FGLS vs BLLS for pancreatic cancer

a) The RFP-expressing pancreatic tumor (seen in the FL image) is difficult to identify under standard bright lighting (BLL). However, when the tumor was labeled with the chimeric anti-CEA-488 antibody, identification and visualization of the tumor was significantly enhanced allowing for better resection under fluorescence-guidance (bottom images). BLL, bright light laparoscopy; FL, fluorescence laparoscopy; FGLS, fluorescence-guided laparoscopic surgery; RFP, red fluorescent protein. b) Representative postoperative images from the BLLS group and the FGLS group. The arrow identifies residual red fluorescence on postoperative whole body images indicating an incomplete resection. This occurred in two out of 22 mice in the BLLS group. All 24 mice in the FGLS group underwent a

complete resection. The preoperative image is representative of all mice undergoing resection. These images were obtained in order to confirm presence of tumor.

Figure 3. Tumor burden at termination using BLLS and FGLS

There were no significant differences with regard to average preoperative tumor burden ($p=0.657$) or average resected specimen size ($p=0.213$) between the two surgical groups. However, the improved resection achieved under fluorescence-guidance led to significantly lower pancreatic tumor burden at termination in the FGLS group compared to the BLLS group ($p=0.012$).

Figure 4. Kaplan meier curve for disease-free survival with BLLS vs FGLS

FGLS improved DFS in mice harboring pancreatic tumor by more than doubling the average time in weeks compared to BLLS. Median DFS improved from 2 weeks in the BLLS group to 7 weeks, $p=0.001$.

Figure 5. Metastatic tumor burden at termination twelve weeks postoperatively with BLLS vs FGLS

Representative intravital images obtained with the OV-100 Small Animal Imaging System demonstrating improved outcomes achieved by FGLS compared to BLLS at termination. FGLS significantly reduced the number of mice with local and distant recurrence compared to BLLS.