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Fluorescence-guided surgery, but not bright-light surgery, prevents local recurrence in a pancreatic cancer patient derived orthotopic xenograft (PDOX) model resistant to neoadjuvant chemotherapy (NAC)

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Abstract

The aim of this study is to determine the efficacy of neoadjuvant chemotherapy (NAC) with gemcitabine (GEM) in combination with fluorescence-guided surgery (FGS) on a pancreatic cancer patient derived orthotopic xenograft (PDOX) model. A PDOX model was established from a CEA-positive tumor from a patient who had undergone a pancreaticoduodenectomy for pancreatic adenocarcinoma. Mice were randomized to 4 groups: bright light surgery (BLS) only; BLS + NAC; FGS only; and FGS + NAC. An anti-CEA antibody conjugated to DyLight 650 was administered intravenously via tail vein of mice with a pancreatic cancer PDOX 24 hours before surgery. The PDOX was clearly labeled with fluorophore-conjugated anti-CEA antibody. Only one out of 8 mice had local recurrence in the FGS only group and zero out of 8 mice had local recurrence in the FGS + NAC which was significantly lower than BLS only or BLS +NAC mice, where local disease recurred in 6 out of 8 mice in each treatment group ($p = 0.041$ and $p = 0.007$, respectively). NAC did not significantly reduce recurrence rates when combined with either FGS or BLS. These results indicate that FGS can significantly reduce local recurrence compared to BLS in pancreatic cancer resistant to NAC.

Keywords

Fluorescence-guided surgery; pancreatic cancer; patient derived orthotopic xenograft PDOX; neoadjuvant chemotherapy; CEA

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INTRODUCTION

Complete tumor resection improves overall survival of pancreatic cancer patients, which is presently 5% at five years.[1] Metastatic relapse often occurs following attempted curative resection of the primary tumor as a result of invisible microscopic tumor deposits left behind. Making tumors fluoresce offers great advantages for tumor detection during surgery in order to achieve complete resection.[2, 3] We have previously shown that fluorescence-guided surgery (FGS) for pancreatic cancer decreased the residual tumor burden and improved overall and disease-free survival in mouse models using fluorescently-labeled human pancreatic cancer cell lines.[4–6]

Patient-derived orthotopic xenografts (PDOX) recapitulate the biological characteristics of the disease of origin, including metastases and are a clinically-relevant model for fluorescence-guided surgery.[7–10]

Recently, many studies reported positive outcomes with neoadjuvant chemotherapy (NAC) of pancreatic cancer.[11–13] NAC allows for the identification of those patients with rapidly progressive metastatic disease at the time of preoperative restaging, and can increase the R0 resection rate and reduce the risk of local tumor recurrence.[11] However, a significant number of patients still develop recurrent disease immediately after NAC treatment and subsequent surgical resection.[12–14] Therefore, new strategies in addition to NAC are needed to reduce the recurrence of pancreatic cancer. In this study, we determined the efficacy FGS to illuminate pancreatic cancer PDOXs resistant to NAC.

MATERIALS AND METHODS

Animals

NOD/SCID mice and athymic *nu/nu* nude mice (AntiCancer Inc., San Diego, CA), 4–6 weeks old, were used in this study. Mice were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent diet. All mouse surgical procedures and imaging were performed with the animals anesthetized by intramuscular injection of a 0.02 ml solution of 50% ketamine, 38% xylazine, and 12% acepromazine maleate. All animal studies were conducted with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol specifically approved for this study and in accordance with the principals and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

Establishment of patient derived orthotopic xenograft (PDOX) of pancreatic cancer

Pancreatic cancer patient tumor tissues were obtained at surgery and cut into fragments (3-mm^3) and originally transplanted subcutaneously in nude mice.[15, 16] The subcutaneous tumors were then passaged in nude mice both orthotopically and subcutaneously. All patients provided written informed consent under the approval of the Institutional Review Board of the University of California San Diego.

Orthotopic tumor implantation

A small 6- to 10-mm transverse incision was made on the left flank of the mouse through the skin and peritoneum. The tail of the pancreas was exposed through this incision, and a single 3-mm^3 tumor fragment from subcutaneous tumors was sutured to the tail of the pancreas using 8-0 nylon surgical sutures (Ethilon; Ethicon Inc., NJ, USA). On completion, the tail of the pancreas was returned to the abdomen, and the incision was closed in one layer using 6-0 nylon surgical sutures (Ethilon).[15, 17]

Antibody conjugation and tumor labeling

Chimeric monoclonal antibodies specific for carcinoembryonic antigen (CEA) were obtained from Aragen Bioscience, Inc. (Morgan Hill, CA, USA).[18] The antibodies were labeled with the DyLight 650 Protein Labeling Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.[5, 7, 19] To determine if anti-CEA antibody, conjugated with DyLight650 (anti-CEA-650), could label patient pancreatic cancer in vivo, anti-CEA-650 (50 μg) was injected into the tail vein of the mice with subcutaneous tumors. Twenty-four hours later, whole body images were obtained with the OV100 Small Animal Variable Magnification Imaging System (Olympus, Tokyo, Japan).[20]

Neoadjuvant chemotherapy

After confirmation of tumor engraftment, 32 mice were randomized to 4 groups: BLS only; BLS + NAC; FGS only; and FGS + NAC. Each treatment arm involved 8 tumor-bearing mice. The mice randomized to NAC-treatment were administered gemcitabine (GEM) (80 mg/kg) (Eli Lilly and Company, Indianapolis, IN, USA). GEM was injected i.p. on day 8, 15 and 22 (Fig 2A). No significant effects on body weight, morbidity, or severe toxicities were observed in NAC-treated mice.

Fluorescence-guided surgery

For fluorescence-guided surgery (FGS), a 15-mm transverse incision was made on the left flank of the mouse through the skin and peritoneum and kept open with a retractor. The tail of the pancreas was exposed through this incision. Anti-CEA antibody conjugated to DyLight 650 (50 μg), was injected intravenously via the tail vein in the mice in the FGS group 24 hours before surgery. A MINI MAGLITE® LED PRO flashlight (MAG INSTRUMENT, Ontario, CA, USA) coupled to an excitation filter (ET 640/30X, Chroma Technology Corporation, Bellows Falls, VT, USA) was used as the excitation light source. A Canon EOS 60D digital camera with an EF–S18–55 IS lens (Canon) coupled with an emission filter (HQ700/75M-HCAR, Chroma Technology Corporation) was used as the real-time image capturing device for FGS. BLS was performed under standard bright-field using an MVX10 microscope (Olympus). After completion of surgery, the incision was closed in one layer using 6-0 nylon surgical sutures, and the mice were allowed to recover in their cages.

Tissue histology

Tumor samples were removed with surrounding normal tissues at the time of resection. Fresh tissue samples were fixed in 10 % formalin and embedded in paraffin before

sectioning and staining. Tissue sections (3 μm) were deparaffinized in xylene and rehydrated in an ethanol series. Hematoxylin and eosin (H & E) staining was performed according to standard protocols. For immunohistochemistry, the sections were then treated for 30 min with 0.3% hydrogen peroxide to block endogenous peroxidase activity. The sections were subsequently washed with PBS and unmasked in citrate antigen unmasking solution (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan) in a water bath for 40 min at 98°C. After incubation with 10% normal goat serum, the sections were incubated with anti-CA19-9 antibody (1:100) and anti-CEA antibody (1:100) at 4°C overnight. The bound primary antibodies were detected by binding with an anti-mouse secondary antibody and an avidin/biotin/horseradish peroxidase complex (DAKO Cytomation, Kyoto, Japan) for 30 min at room temperature. The labeled antigens were visualized with the DAB kit (DAKO Cytomation). The sections were counterstained with hematoxylin and observed with a BH-2 microscope (Olympus) equipped with an INFINITY1 2.0 megapixel CMOS digital camera (Lumenera Corporation, Ottawa, Canada). All images were acquired using INFINITY ANALYZE software (Lumenera Corporation) without post-acquisition processing.

Evaluation of histopathological response to NAC

Histopathological response to chemotherapy drugs was defined according to Evans's grading scheme: Grade I, little (<10%) or no tumor cell destruction is evident; Grade IIa, destruction of 10%–50% of tumor cells; Grade IIb, destruction of 51%–90% of tumor cells; Grade III, few (<10%) viable-appearing tumor cells are present; Grade IV, no viable tumor cells are present.[21]

Evaluation of tumor recurrence and progression

To assess for recurrence postoperatively, animals underwent laparotomy 12 weeks after surgery (Fig. 2A), and the tumors were imaged with the Canon EOS 60D digital camera with EF–S18–55 IS lens (Canon), excised, weighed and harvested for analysis.

Statistical analysis

PASWStatistics 18.0 (SPSS, Inc) was used for statistical analyses. Tumor weight is expressed as mean ± SD. The two-tailed Student's *t*-test was used to compare continuous variables between 2 groups. Comparisons between categorical variables were analyzed with Fisher's exact test. A p value < 0.05 was considered statistically significant for all comparisons.

RESULTS

Antibody labeling

The pancreatic PDOX tumor was diagnosed as moderately differentiated adenocarcinoma with H&E staining (Figure 1A). Based on immunohistochemistry, the PDOX tumor was found to be CEA-positive (Figure 1B). The PDOX was clearly labeled with anti-CEA-650 (Figures 1C and 1D). These results were consistent with the immunohistochemical results, and based on them, it was decided to use anti-CEA-650 to label the PDOX for FGS. Anti-CEA-650 was injected in the tail vein of the mice with PDOX tumors 24 hours before FGS.

Resistance of the PDOX to NAC

The PDOX mice were randomized to 4 groups; BLS only; BLS + NAC; FGS only; FGS + NAC. Each treatment arm involved 8 tumor-bearing mice. The mice randomized to NAC group were treated with GEM on days 8, 15 and 22. All animals underwent surgery on day 29 (Figures 2 and 3). The average excised tumor weight was 168.3 ± 88.1 mg in BLS only mice, 153.4 ± 54.8 mg in the BLS + NAC, 284.0 ± 80.9 mg in the FGS only mice, and 231.2 ± 77.8 mg in the FGS + NAC group mice. There was no difference between BLS only and BLS + NAC, and between FGS only and FGS + NAC ($p = 0.690$ and $p = 0.205$, respectively).

Upon histological examination, only few (20–30%) cancer cells were replaced by stromal cells in the tumor with NAC treatment (FGS + NAC), and the glandular formation was preserved as well as in the tumor without GEM treatment (FGS only) (Figure 4). The treatment effect of GEM was judged as grade IIa (Figure 4D). These results suggest that the PDOX is resistant to NAC treatment with GEM. Fluorescence was clearly detected in the PDOX with GEM treatment (Figure 3B and 4B). Fluorescence decreased in some areas of the tumor treated with GEM, however not significantly enough to affect FGS (Figure 4B).

FGS enabled resection of residual tumors that could not be detected with BLS

FGS on the PDOX succeeded in resecting residual tumor that could not be detected with BLS. FGS + NAC resected significantly more tumor weight than BLS + NAC ($p = 0.036$) (Figures 5 and 6). The average excised tumor weight in FGS-treated mice was significantly more than BLS-treated mice ($p = 0.001$). These results suggest that FGS may allow for resection of residual tumor undetectable with BLS, resulting in a more complete resection with FGS.

FGS decreases tumor recurrence

FGS-only significantly reduced the total recurrence rate of the PDOX compared to BLS only ($p = 0.041$), and FGS + NAC also significantly reduced the total recurrence rate compared to BLS + NAC ($p = 0.007$) (Table 1). The average local recurrent tumor weight was 308.8 ± 382.2 mg for BLS only; 386.5 ± 351.4 mg for BLS + NAC; 8.5 ± 24.0 mg for FGS; and 0 ± 0 mg for FGS + NAC (Figure 7). The average local recurrent tumor weight in FGS + NAC was significantly less than BLS + NAC ($p = 0.017$). These results suggest that FGS reduces local recurrence by eradicating the residual tumor not detectable with BLS.

DISCUSSION

In a previous study, we investigated the use of fluorophore-labeled anti-carcinoembryonic antigen (CEA) monoclonal antibody to aid in cancer visualization in nude mouse models of human colorectal and pancreatic cancer.[4] The results indicated that fluorophore-labeled anti-CEA offers a novel intraoperative imaging technique for FGS. In another study, we evaluated whether FGS with a fluorophore-conjugated antibody to CEA, to highlight the tumor, can improve surgical resection and increase disease-free survival (DFS) and overall survival (OS) in orthotopic mouse models of human pancreatic cancer.[22]

There are several novel aspects to the present study that should be emphasized. The present study took advantage of a longer wavelength dye, DyLight 650, which we have previous shown has better tissue penetration compared to AlexaFlour 488.[19] In addition, the PDOX model developed in our laboratory, and used in the present study, allows for individualized therapy that is not available with pancreatic cancer cell line models.[7, 8, 18, 23] The imaging system utilized for FGS was a hand held portable system that could easily be translated into the operating room.[7, 23]

Local and distant recurrence of pancreatic cancer is high and many of these patients usually only receive palliative chemotherapy and have a short survival. The present study was a proof-of-principle study using a chemoresistant tumor rather than a broad survey of many tumors. Despite the chemoresistance of the tumor, we lowered the recurrence rate with FGS, thereby establishing the principle that FGS can lead to an R0 resection. Future studies by our group will use on additional chemoresistant pancreatic cancer PDOXs for testing of FGS strategies in these models.

In summary, the results from the present study demonstrate that FGS can significantly reduce local recurrence compared to BLS in pancreatic cancer resistant to NAC.

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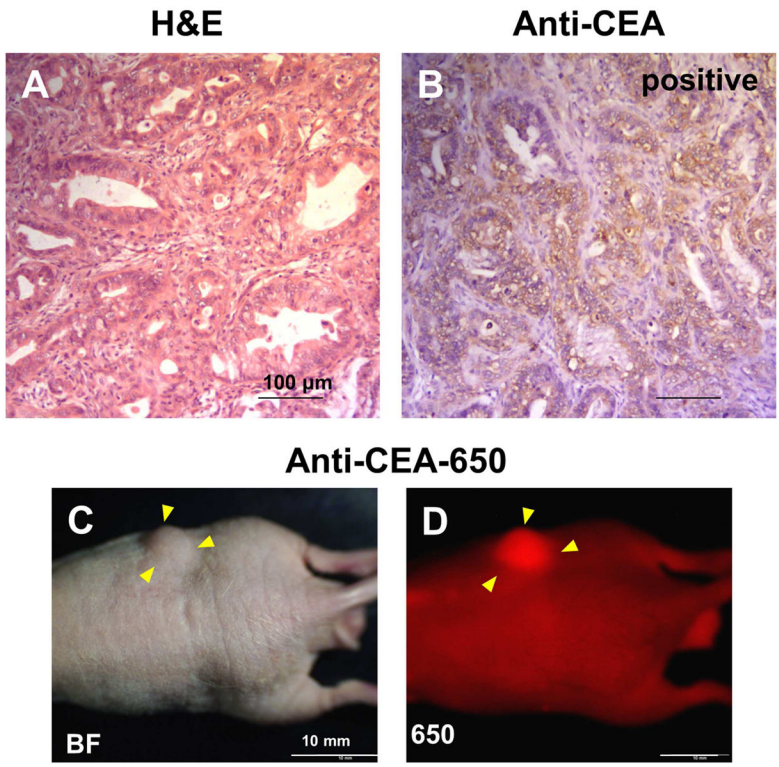

Figure 1.

Characterization of the pancreatic tumor. The pancreatic cancer was diagnosed as moderately differentiated adenocarcinoma with H&E staining (A). The tumor strongly stained with anti-CEA antibody (B). Scale bars: 100 μm. (C) Whole body images of subcutaneous tumor with anti-CEA-650. anti-CEA-650 (50 μg) was injected in the tail vain of the mice with subcutaneous tumors. Twenty-four hours later, whole body images were taken with the OV100 (Olympus). Yellow arrowheads indicate subcutaneous tumors. The subcutaneous tumors were clearly labeled with anti-CEA-DyLight 650. Scale bars: 10 mm.

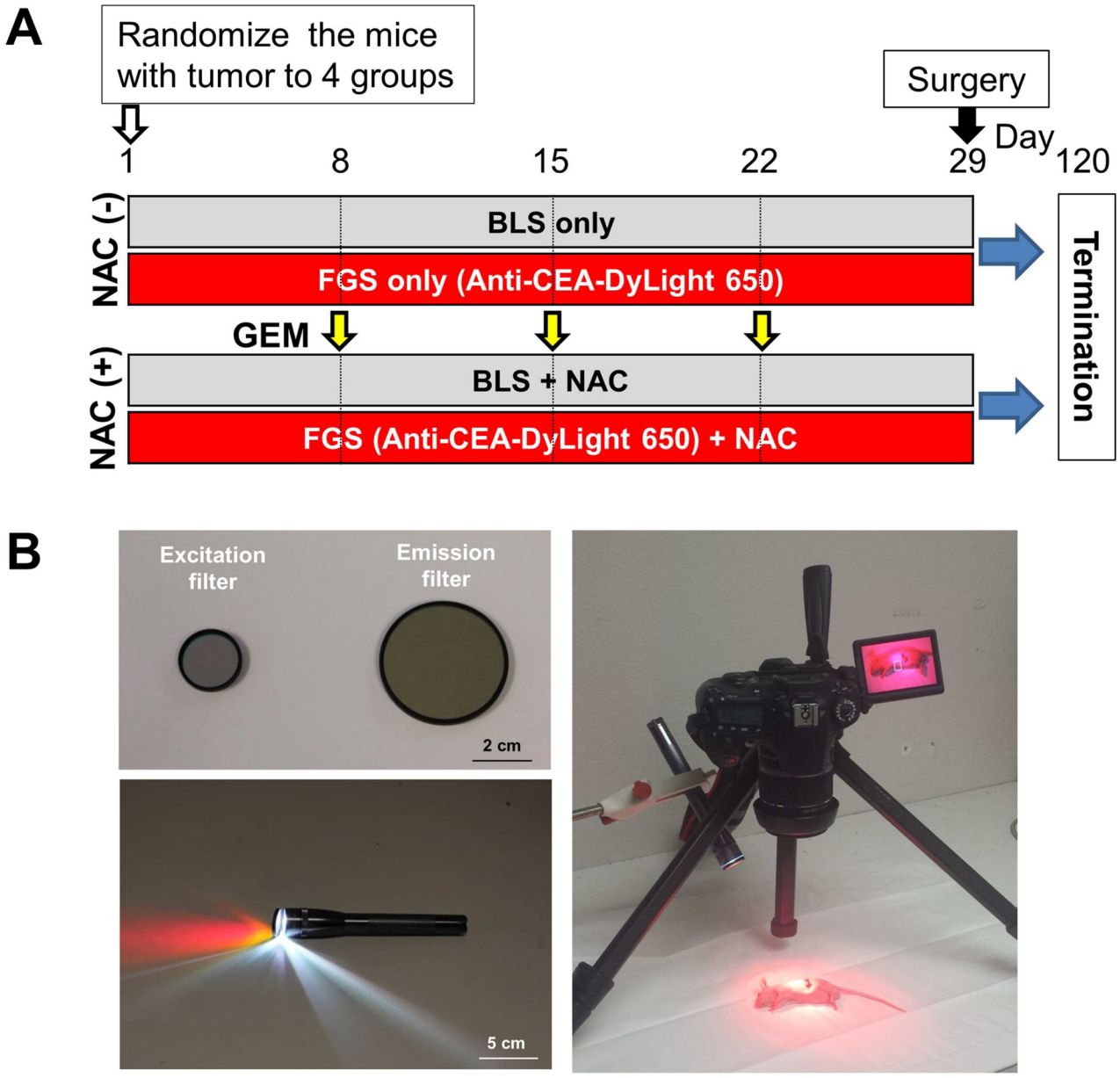

Figure 2.

(A) Schema of the experimental design. After confirmation of tumor growth, the PDOX nude mouse models were randomized to 4 groups: BLS only; BLS + NAC; FGS only; and FGS + NAC. Each treatment arm contained 8 PDOX nude mice. The mice randomized to NAC (+) groups were treated with GEM on day 8, 15 and 22. All animals underwent surgery on day 29. BLS was performed under standard bright-field using the MVX10 microscope. Anti-CEA antibody conjugated with DyLight 650 (50 μg) was injected in the tail vain of the mice in the FGS groups 24 hours before surgery. FGS was performed using the MINI MAGLITE® LED PRO flash light (MAG INSTRUMENT, Ontario, CA, USA) with an excitation filter (ET640/30X, Chroma Technology Corporation, Bellows Falls, VT, USA) and the Canon EOS 60D digital camera with an EF–S18–55 IS lens (Canon, Tokyo,

Japan) and an emission filter (HQ700/75M-HCAR, Chroma Technology Corporation) (B). Twelve weeks after surgery, animals underwent laparotomy, and the tumors were imaged and weighed and harvested for analysis. Scale bars: 2cm (filters) and 5 cm (flash light).

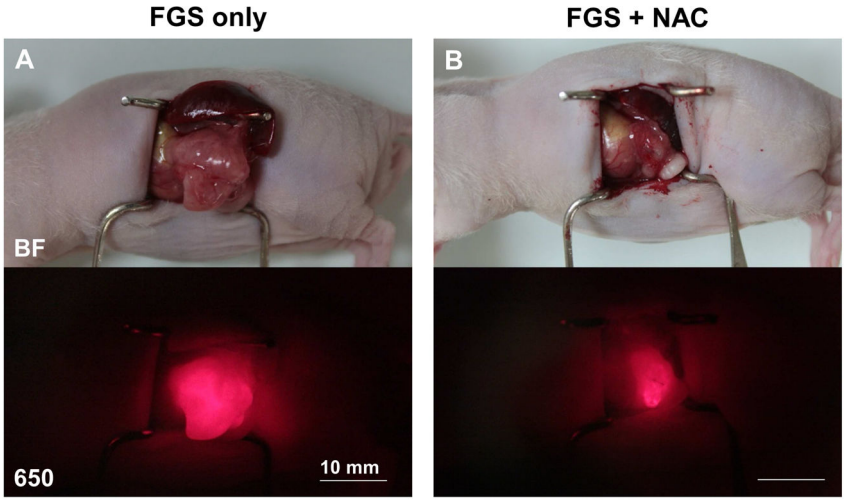

Figure 3.

Representative images of FGS on each treatment group. Upper panels indicate bright field (BF) images and lower panels indicate fluorescence images for DyLight 650 (650). A) FGS B) FGS + NAC. Scale bars: 10 mm.

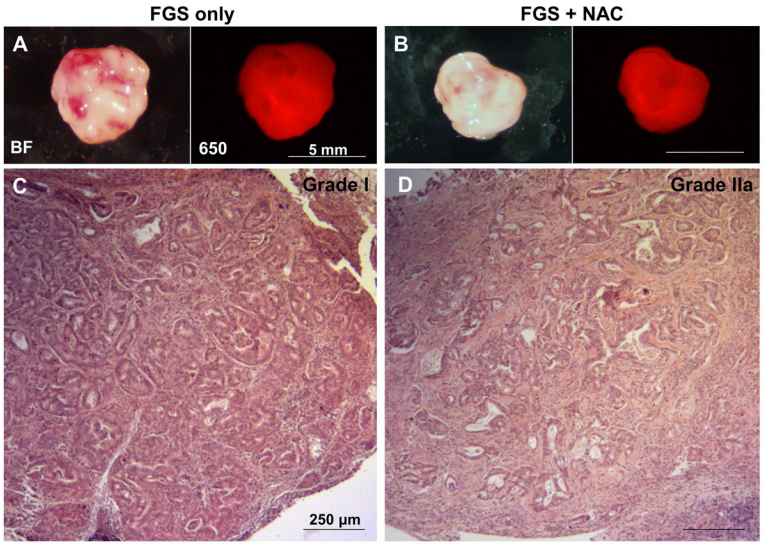

Figure 4.

Representative gross and histological images of excised tumors in each treatment group. Left panels of (A) and (B) indicate bright field (BF) images and right panels indicate fluorescence images for Anti-CEA DyLight 650 (650). Histopathological response to GEM treatment was defined according to Evans's grading scheme. The tumors without GEM treatment (FGS only) were occupied by viable cancer cells which formed glandular structure and were judged as Grade I (C). In the tumors with GEM treatment (FGS + NAC), there were 20–30% fewer cancer cells than FGS-only, but the glandular formation was still preserved as well as the tumor without GEM treatment (D). Treatment effect of GEM on the pancreatic tumor was judged as grade IIa (D). Scale bars: 5 mm (A and B), 250 μm (C and D).

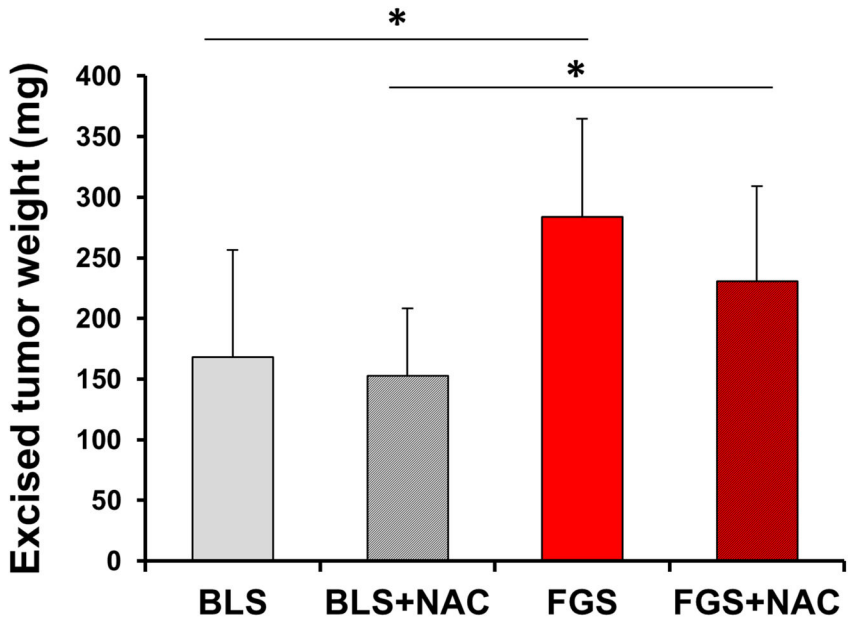

Figure 5.

Excised tumor weight. Excised tumor weight of BLS-only; BLS + NAC; FGS-only; and FGS + NAC. The average excised tumor weight in FGS-only was significantly more than BLS-only ($p = 0.016$). The average excised tumor weight in FGS + NAC was significantly more than BLS + NAC ($p = 0.036$). Bar graphs of the excised tumor weight in BLS and FGS groups (B), and in NAC (−) and NAC (+) groups (C). The average excised tumor weight of FGS group was significantly more than BLS group ($p = 0.001$). There was no difference in the excised tumor weight between NAC (−) and NAC (+) group. ($p = 0.294$). * $p < 0.05$, ** $p < 0.01$.

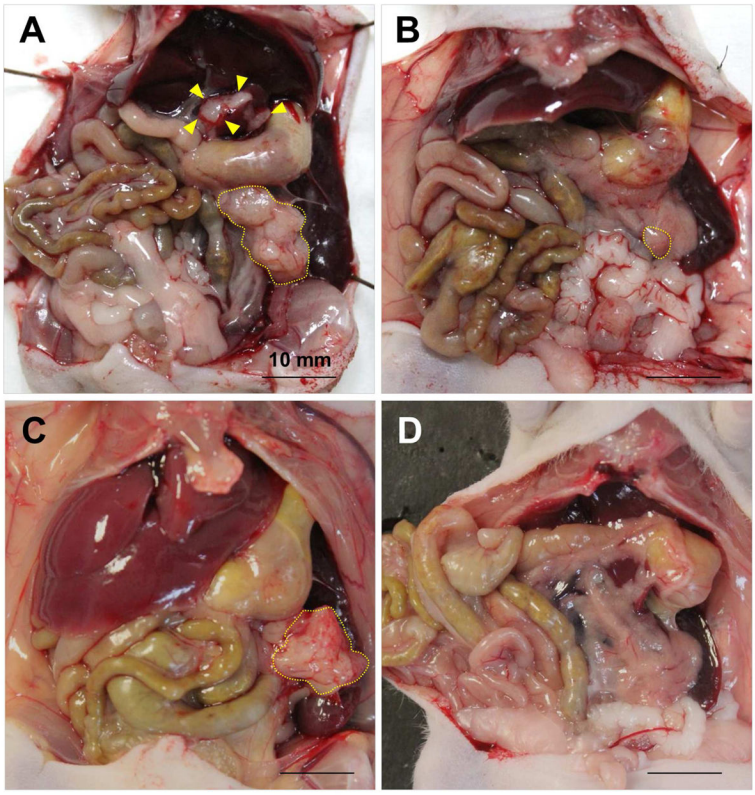

Figure 6.

Representative images of recurrence. (A) A large local recurrent tumor (surrounded by a yellow broken line) and portal lymph-node metastases (yellow arrowheads) in a BLS-only treated mouse. (B) A local recurrent tumor (surrounded by a yellow broken line) in the BLS + NAC-treated mice. (C) A small local recurrent tumor (surrounded by a yellow broken line) in an FGS-only treated mouse. (D) No recurrent tumor in FGS + NAC mice was detected. Scale bars: 10 mm.

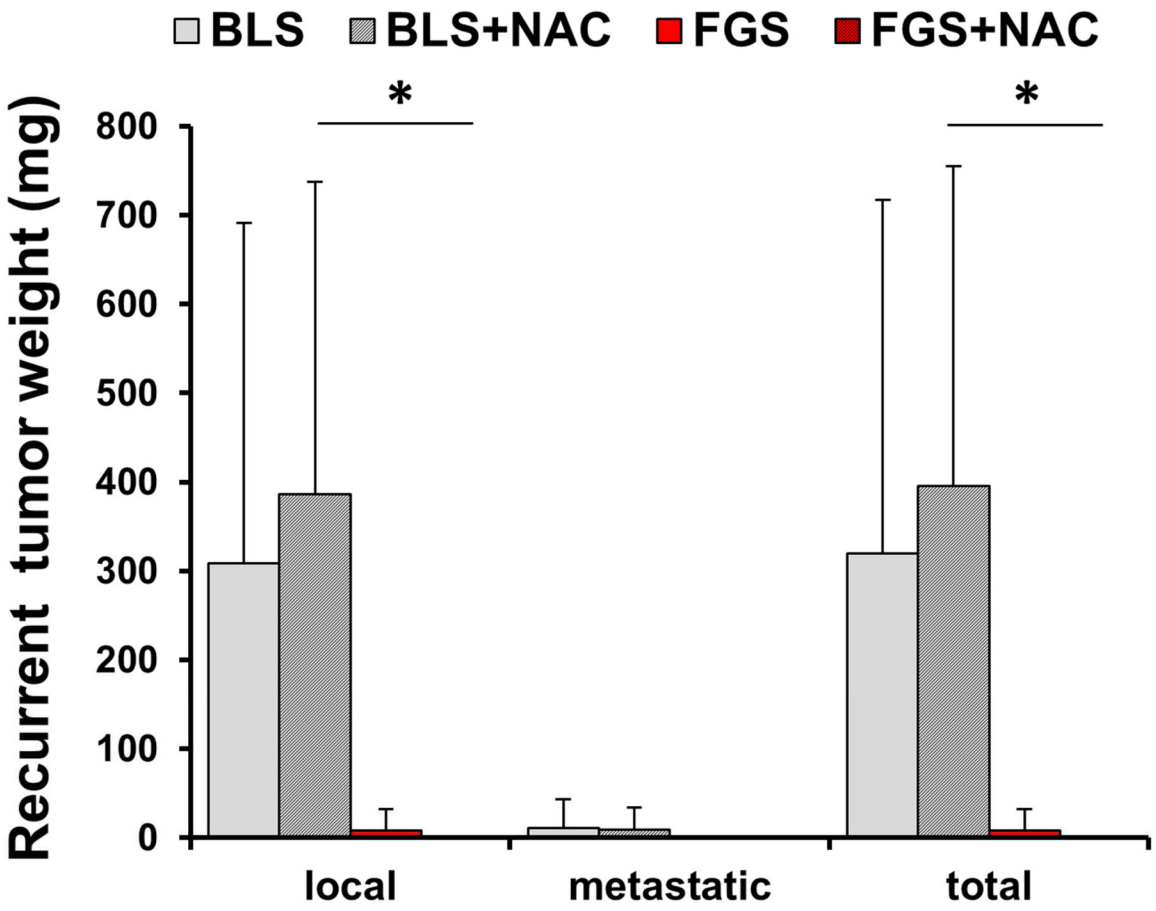

Figure 7.

Recurrent tumor weight. The average local recurrent tumor weight in FGS or FGS + NAC mice was significantly less than BLS or BLS + NAC mice ($p = 0.017$).

TABLE 1

Recurrence rate of PDOX in each treatment group

	local	metastatic	total
BLS only	6 / 8 (75%)	1 / 8 (12.5%)	6 / 8 (75%)
BLS + NAC	6 / 8 (75%)	1 / 8 (12.5%)	6 / 8 (75%)
FGS only	1 / 8 (12.5%)*	0 / 8 (0%)	1 / 8 (12.5%)*
FGS + NAC	0 / 8 (0%)**	0 / 8 (0%)	0 / 8 (0%)**

* p = 0.041, compared to BLS only

** p = 0.007, compared to BLS + NAC