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Ratiometric Activatable Cell-Penetrating Peptides Label Pancreatic Cancer, Enabling Fluorescence-Guided Surgery, Which Reduces Metastases and Recurrence in Orthotopic Mouse Models

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Abstract

Background—The aim of this study was to evaluate the efficacy of using matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9)-cleavable ratiometric activatable cell-penetrating peptides (RACPPs) conjugated to Cy5 and Cy7 fluorophores to accurately label pancreatic cancer for fluorescence-guided surgery (FGS) in an orthotopic mouse model.

Methods—Orthotopic mouse models were established using MiaPaCa-2-GFP human pancreatic cancer cells. Two weeks after implantation, tumor-bearing mice were randomized to conventional white light reflectance (WLR) surgery or FGS. FGS was performed at far-red and infrared wavelengths with a customized fluorescence-dissecting microscope 2 h after injection of MMP-2 and MMP-9-cleavable RACPPs. Green fluorescence imaging of the GFP-labeled cancer cells was used to assess the effectiveness of surgical resection and monitor recurrence. At 8 weeks, mice were sacrificed to evaluate tumor burden and metastases.

Results—Mice in the WLR group had larger primary tumors than mice in the FGS group at termination [1.72 g ± standard error (SE) 0.58 vs. 0.25 g ± SE 0.14; respectively, $p = 0.026$). Mean disease-free survival was significantly lengthened from 5.33 weeks in the WLR group to 7.38 weeks in the FGS group ($p = 0.02$). Recurrence rates were lower in the FGS group than in the WLR group (38 vs. 73 %; $p = 0.049$). This translated into lower local and distant recurrence rates for FGS compared to WLR (31 vs. 67 for local recurrence, respectively, and 25 vs. 60 % for

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distant recurrence, respectively). Metastatic tumor burden was significantly greater in the WLR group than in the FGS group (96.92 mm^2 ± SE 52.03 vs. 2.20 mm^2 ± SE 1.43; respectively, χ^2 = 5.455; p = 0.02).

Conclusions—RACPPs can accurately and effectively label pancreatic cancer for effective FGS, resulting in better postresection outcomes than for WLR surgery.

A number of different methods have been used to label tumors for fluorescence-guided surgery (FGS).[1,2] Several of these methods include fluorophore-conjugated antibodies to unique surface markers expressed by individual tumor types[3–9] and replication-competent viruses engineered to express the green fluorescent protein (GFP) in the presence of activated telomerase[10,11] to selectively label tumors for effective FGS.

Another technique for in vivo labeling of cancer cells involves the use of activatable cell-penetrating peptides (ACPPs).[12–16] Polycationic cell-penetrating peptides (CPPs) are connected via a cleavable linker to a neutralizing polyanion whose adsorption and uptake into cells are inhibited until the linker is proteolyzed. With the upregulation of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in most solid tumors, exposure to these proteases results in cleavage and dissociation of the linked peptide, allowing it to bind to and enter tumor cells. Conjugating CPPs to a fluorophore(s) enables the tumor to acquire a fluorescent label.

The aim of this study was to develop a method to accurately visualize primary pancreatic tumors labeled with ratiometric activatable cell-penetrating peptides (RACPPs), conjugated to Cy5 and Cy7 fluorophores, in orthotopic murine models of human pancreatic cancer for effective FGS. The advantages of the use of RACPPs over single fluorophore ACPPs for tumor imaging include imaging and quantification that do not depend on the total probe uptake, varying washout of the ACPPs, or on thresholding. Also, RACPPs have a rapid change that can be detected within 1–2 h (vs. 6 h for single fluorophore versions[12,15] and 24 h for dequenching probes[17]), which is more applicable to rapid, intraoperative use. Finally, the RACPP dequenching mechanism improves the contrast of Cy5 alone compared with a single fluorophore ACPP,[16] enhancing the detection specificity/sensitivity and making ACPPs immediately adaptable to the equipment already available in FGS clinical operating rooms.

MATERIALS AND METHODS

Cell Culture

MiaPaCa-2–green fluorescent protein (GFP)-expressing pancreatic cancer cells were maintained as previously described in RPMI 1640 medium with 10 % fetal bovine serum.[18]

Peptide Synthesis and Fluorophore Labeling

Cy5 and Cy7 ratiometric activatable cell-penetrating peptides (RACPPs) were synthesized on an automatic peptide synthesizer using standard protocols for fluorenylmethoxycarbonyl solid-phase synthesis as previously described.[16] All reactions were monitored with analytic

HPLC and mass spectrometry (MS). According to characterization with HPLC-MS, all products were >95 % pure.

Animal Care

Female athymic *nu/nu* nude mice were maintained in a barrier facility on high-efficiency particulate air-filtered racks. 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. A total of 32 mice were used for these experiments. All animal studies were approved by the University of California, San Diego Institutional Animal Care and Use Committee and were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals.

Subcutaneous Tumor Cell Implantation

Human MiaPaCa-2–GFP pancreatic cancer cells were harvested by trypsinization and prepared for injection. Cells (2×10^6 in 100 μL 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 tumors for orthotopic implantation.

Orthotopic Implantation of Tumor Tissue Fragments

Surgical orthotopic implantation of single 1 mm^3 tumor fragments from the fluorescent MiaPaCa-2–GFP subcutaneous tumors were used to establish orthotopic models in nude mice. The tail of the pancreas was delivered through a small 6–10 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 nonabsorbable sutures (Ethicon Inc., Somerville, NJ).[19–21]

Imaging and Resection of Tumors

Thirty-two tumor-bearing mice were randomized to undergo FGS or WLR. For FGS, resection of the primary pancreatic tumor using fluorescence-guided molecular navigation was performed 2 h after mice were injected with RACPPs (10 nmol, i.v.). A customized fluorescence dissecting microscope (MVX10, Olympus Corporation, Tokyo, Japan) with an exposure time of 0.5–1 s, excitation of 620/60 nm, and emission of 700/75 nm (Cy5) or 785/62 nm (Cy7) was used for intraoperative optical imaging. The ratio of Cy5 to Cy7 signal (representing cleaved, activated peptide over uncleaved, intact peptide) was imaged with custom-designed software displaying a blue (low ratio, mostly uncleaved) to red (high ratio, mostly cleaved) color scale for surgical guidance. Tissue was considered tumor and resected if it had a >20 % ratio increase over background pancreas tissue. Tumor-bearing mice randomized to the WLR arm underwent resection under standard bright light. Whole body preoperative and postoperative images (GFP, excitation of 466/44 nm, emission tuned to 520 nm) were performed on all mice using a Maestro CRI Small Animal Spectral Separation Imaging System (Cambridge Research & Instrumentation, Inc. Woburn, MA) to confirm the presence of the tumor and completeness of the resection. Postoperatively, the

mice underwent weekly whole body imaging of GFP to assess for recurrence using the Maestro. Eight weeks postoperatively, the surviving mice (n = 31) were terminated for intravital and ex vivo imaging, using the OV-100 Small Animal Imaging System (Olympus Corporation) with an exposure time of 1.5–3 s, excitation of 460–490 nm, and emission of 510 nm long-pass to assess for local and distant recurrence, and overall pancreatic and metastatic tumor burden. All images were analyzed with Image J version 1.440 (National Institutes of Health, Bethesda, MD). The intensity of the fluorescence signal per pixel can be measured with this software, and the area of fluorescence can be calculated with a set scale.

Tissue Histology

Samples were collected during the experiments and at necropsy for histologic preparation with hematoxylin and eosin (H&E) staining. Fresh tissue samples were fixed in Bouin's solution and regions of interest were embedded in paraffin prior to sectioning and staining with H&E for standard light microscopy.

Data Processing and Statistical Analysis

SAS, version 9.2 (The SAS Institute, Inc.) was used for statistical analyses. Continuous variables (tumor weight 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. Categorical variables (complete resection, local and distant recurrence, and presence of tumor at termination) were expressed as counts and percentages, and tests of significance used Fisher's exact test. We compared the disease-free survival (DFS) between treatment groups using a log rank test. A two-sided p value ≤0.05 was considered statistically significant for all comparisons.

RESULTS

Our primary aim was to evaluate the efficacy of using Cy5 and Cy7-conjugated RACPPs to guide resection of the fluorescently labeled tumor. Orthotopic mouse models of the human pancreatic cancer MiaPaCa-2–GFP were established in 32 mice. Tumors were permitted to grow for 2 weeks after which mice were randomized to FGS or WLR. FGS was performed with the MVX10 fluorescence-dissecting microscope 2 h after injection of ACPPs (Fig. 1).

With fluorescence guidance, all 16 mice underwent a complete resection as evident by lack of GFP fluorescence in postoperative images. However, 1 of the 16 mice in the WLR group had evidence of residual tumor postoperatively and was not included in later analysis. The WLR-treated mice had early recurrence of tumor and significant tumor growth over the postoperative period that led to premature morbidity, whereas a larger percentage of the FGS-treated mice remained tumor free and healthy (Fig. 2).

All mice were followed postoperatively for 8 weeks with whole body imaging of the GFP-expressing pancreatic cancer to assess for recurrence. FGS significantly lengthened the DFS from 5.33 weeks in the WLR group to 7.38 weeks (p = 0.02) (Fig. 3a). Furthermore, with improved visualization of tumor margins and thus enhanced resection, recurrence rates were significantly decreased with FGS from 73 38 % (p = 0.049) (Fig. 3b). At 8 weeks

postoperatively, all mice were terminated and their abdomens were exposed to assess primary and metastatic tumor burden and local and distant recurrence rates between the two groups. Mice in the WLR group demonstrated significantly larger primary pancreatic tumors compared to mice in the FGS group (1.72 g ± SE 0.58 vs. 0.25 g ± SE 0.14; $p = 0.026$) (Fig. 3c). The metastatic tumor burden was also significantly greater in the WLR group (96.92 mm^2 ± SE 52.03 vs. 2.20 mm^2 ± SE 1.43; $\chi^2 = 5.455$; $p = 0.02$) (Fig. 3d). Furthermore, mice in the FGS group experienced lower local and distant recurrence rates (31 vs. 67 and 25 vs. 60 %, respectively) compared to mice in the WLR group.

DISCUSSION

In this study, we demonstrated that FGS with RACPPs can enhance pancreatic tumor resection. RACPPs, once exposed to proteases that are characteristic of pancreatic tumors, were cleaved and absorbed by cancer cells, thereby highlighting the delineation of invasive tumor from surrounding normal tissue. RACPPs enabled high absolute tumor fluorescence and, with minimal uptake by surrounding tissues such as the skin, provided a strong tumor-to-background fluorescence contrast that allowed the surgeon to better distinguish the tumor from normal tissue.

FGS can enhance detection of tumor margins and thus provide a more objective means of identifying and ultimately resecting solid tumors. Initial studies explored the use of a near-infrared fluorescent dye, indocyanine green (ICG), to label sentinel lymph nodes in breast cancer patients.[22] Other surgeons have used ICG as an aid in liver resection.[23] However, indocyanine green does not specifically label cancer cells. Another method was described by Stummer et al.[24] in which the metabolite 5-aminolevulinic acid, a precursor of hemoglobin, resulted in accumulation of porphyrins within malignant glioma. In a clinical trial, this method of FGS led to more complete resections and improved 6-month progression-free survival.

Numerous preclinical methods of more specifically labeling tumor cells have been described and tested in mouse models. For example, genetic labeling of tumors with green fluorescent protein in situ has been achieved. Stiles et al.[25] were able to label metastatic lung tumor foci of >1 mm in a mouse model using a herpes simplex virus (NV1066) that carried the gfp gene. Kishimoto et al.[10] used a telomerase-dependent adenovirus (OBP-401) also containing the gfp gene to accurately label tumors. Once labeled, these tumors could be resected under fluorescence guidance.[11] Furthermore, tumors that recurred also maintained GFP expression, making detection of recurrence and future metastasis possible.[26]

Our laboratory has demonstrated the efficacy of using fluorescently labeled antibodies directed toward specific tumor antigens, a more clinically translatable method, to improve resection rates and thus surgical outcomes in mouse models of pancreatic and colon cancer.[3,4,6–9] This method was tested in a clinical trial for patients with ovarian cancer by van Dam et al.[27] in which they targeted their fluorescently labeled antibodies toward the folate receptor-α. In this study, the number of tumor deposits detected by surgeons when guided by tumor-specific fluorescence was significantly higher than with visual observation alone. However, two of five patients in this study showed weak or no fluorescence.

Although antibody approaches may be successful for individual tumor types that express unique surface markers, they are often not applicable to all tumor types.[3,4] RACPPs are molecular probes with wide applicability in a variety of tumor types. In addition, RACPPs bind tightly to tumors, providing ample signal with low background illumination, as previously demonstrated.[12–16] RACPPs can also be used at multiple stages in the evaluation of pancreatic cancer, such as preoperative and postoperative imaging in addition to surgical guidance. Although the RACPP labeling method is useful only in those tumors that express an appropriate protease that can cleave and activate the RACPP, MMP-2 and MMP-9 activity is reported to be upregulated in human pancreatic cancer.[28–30] Also, all of more than 20 human xenograft and murine syngeneic tumor lines have sufficient MMP-2 and MMP-9 activity to be detectable with RACPPs, in our experience.

CONCLUSIONS

In this study, we demonstrated that accurately labeling pancreatic tumors with RACPPs in orthotopic mouse models of human pancreatic cancer improved surgical resection under fluorescence guidance, decreased recurrence, and increased tumor-free survival compared to bright light surgery. Future studies will determine whether RACPPs are superior to antibodies for imaging pancreatic cancer.

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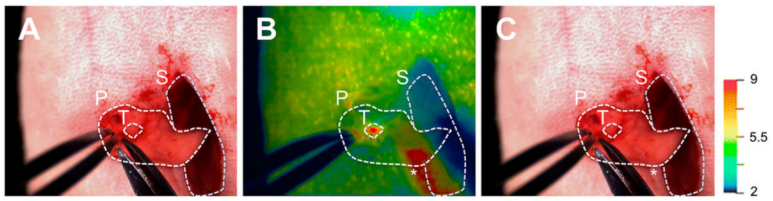

FIG. 1.

Comparison of FGS and WLR imaging. Intraoperative images during FGS of a mouse showing a white light reflectance view (**a**), ratiometric fluorescence view (**b**), and the overlay of the two (**c**). A tumor that is difficult to detect with bright field imaging has clear margins with ratiometric imaging. *White dashed lines* indicates the pancreas, spleen, and tumor outlines. The displayed ratio scale ranges from 2 to 9. *P* pancreas, *S* spleen, *T* tumor *Region with motion artifact from movement of the surgical scissors; also note that the scissors were in different positions in **a** and **b**

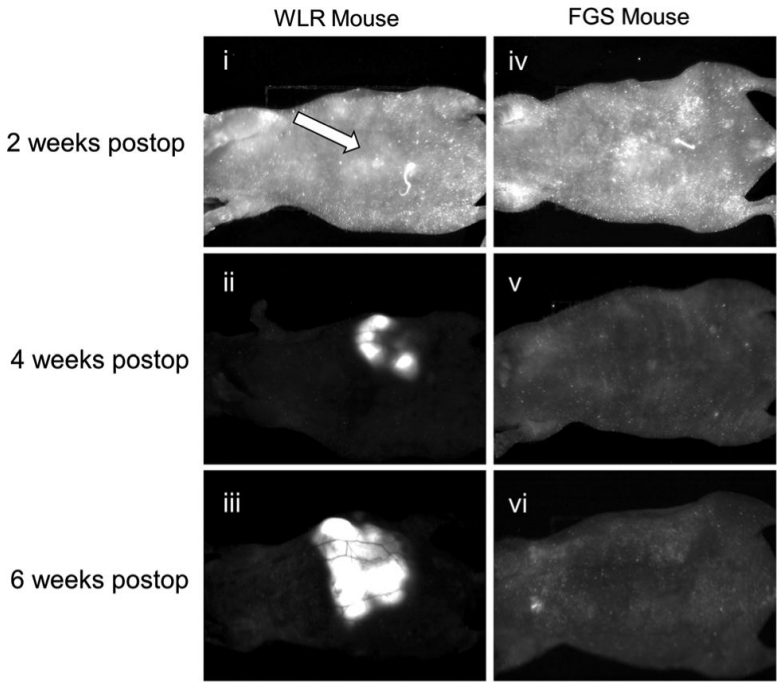

FIG. 2.

Comparison of resection with FGS or WLR. Postoperative whole body noninvasive images of the white light reflectance (WLR) and fluorescence guided surgery (*FGS*) of the mice. The WLR treated mouse (*i–iii*) had an incomplete resection that resulted in early recurrence of the tumor by 2 weeks (*white arrow*) and a significant tumor growth over the next 6 weeks that led to early morbidity. In contrast, the FGS mouse (*iv–vi*) that underwent a complete resection remained tumor free in the postoperative period. The Maestro imaging settings were as follows: excitation 466/44 nm, emission 515 nm long pass (limited to 520–530 for *ii–vi*), and exposure time of 1 s

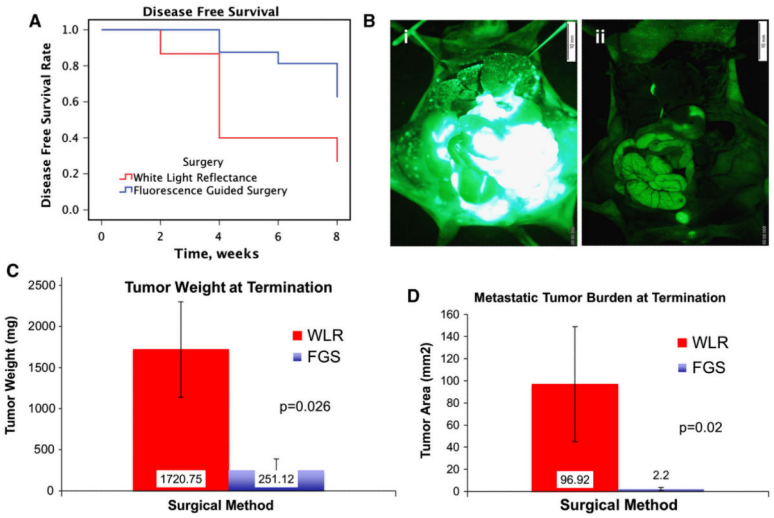

FIG. 3.

Disease free survival, tumor weight at termination, and meta static tumor burden. **a** Recurrence in the postoperative period was defined as the presence of a green fluorescent protein (GFP) signal on whole body noninvasive imaging. Mice from the white light reflectance (WLR) group demonstrated shorter time to recurrence and thus a significantly shortened disease free survival (DFS) ($p = 0.02$). Median DFS for the WLR was 4 weeks. Fewer than 50 % of mice in the fluorescence guided surgery (FGS) group had evidence of recurrence during the follow up period. **b** Mice were terminated at 8 weeks postoperatively. The WLR group (*i*) demonstrated greater metastatic burden and higher local and distant recurrence rates compared to the FGS group (*ii*) (31 vs. 67 % for local recurrence, respectively; and 25 vs. 60 % for distant recurrence; respectively). **c** Improved resection rates in the FGS group resulted in a significantly smaller primary pancreatic tumor burden than in the WLR group at 8 weeks (1.72 g ± SE 0.58 vs. 1.25 g ± SE 0.14; respectively; p 0.026). **d** Metastatic tumor burden was also significantly less in mice from FGS compared to those in the WLR group at 8 weeks ($\chi^2 = 5.455$; $p = 0.02$)