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Fluorescent Anti-MUC5AC Brightly Targets Pancreatic Cancer in a Patient-derived Orthotopic Xenograft

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Abstract. *Background: Overexpression of mucin-5AC (MUC5AC) makes it a targetable biomarker in pancreatic cancer. The present study evaluated tumor targeting with a MUC5AC antibody conjugated to a near-infrared dye in a patient-derived orthotopic xenograft (PDOX) mouse model. Materials and Methods: MUC5AC monoclonal antibody was conjugated to the near-infrared dye IRDye800CW to synthesize MUC5AC-IR800. PDOX models were established by implanting a high-MUC5AC-expressing patient-derived pancreatic tumor on the pancreas of nude mice. After 4 weeks of PDOX tumor growth, mice were imaged after receiving MUC5AC-IR800 (75 μg) intravenously. Results: In the PDOX models, MUC5AC-IR800 selectively and brightly targeted the pancreatic tumor (tumor to background ratio: 2.46±0.465). Conclusion: MUC5AC-IR800 provides distinct visualization of pancreatic tumors. MUC5AC-IR800 may be used clinically in the future to improve pancreatic cancer resection. This novel fluorescent probe is also promising for targeting of pre-malignant pancreatic lesions with subsequent resection under fluorescence guidance.*

Pancreatic cancer is the fourth-leading cause of cancer death in the United States (1, 2). Over 60,000 people are estimated to be diagnosed with pancreatic cancer and >48,000 will die from it in the United States in 2021 (2). Of people diagnosed with pancreatic cancer, 90% of them will die from the disease (1).

The only current curative treatment for pancreatic cancer is surgical resection (1). However, most patients are diagnosed with advanced disease, with only 15-20% considered surgical candidates (3). Of those that undergo surgical resection, 50-80% have positive microscopic resection margins (R1), a poor-prognosis indicator. Over 60% of patients will develop recurrence of disease within 2 years of their operation (4, 5). Therefore, improved methods of pancreatic cancer therapeutics are needed.

Mucins, glycoproteins involved in cell signaling and barrier protection, are overexpressed in pancreatic cancer (6, 7); specifically, mucin-5AC (MUC5AC), which has minimal or no expression in healthy pancreatic tissue (8-10). Jonckheere *et al.* performed mucin staining in normal pancreatic tissue, pancreatic intraepithelial neoplasia (PanIN) and pancreatic ductal adenocarcinoma (PDAC). MUC5AC expression was found in 2-4% of normal ducts but its overexpression was found in 70% of PanIN IA cases and 85% of PDAC (9). In the present study, fluorescent-tagged antibodies to MUC5AC were tested with tumor lysates of pancreatic cancer and pancreatic-cancer patient-derived orthotopic xenograft (PDOX) models.

Materials and Methods

Mice. Athymic nude mice, aged 4-6 weeks, were purchased from The Jackson Laboratory (Bay Harbor, ME, USA). The mice were housed in a barrier facility and fed an autoclaved laboratory-approved diet. The mice were anesthetized with intraperitoneal injection of a solution of xylazine, ketamine and phosphate-buffered saline (PBS) prior to all surgical procedures. At the conclusion of the study, mice were euthanized with CO_2 inhalation or cervical dislocation. All studies were approved by the San Diego Veterans Administration Medical Center Institutional Animal Care and Use Committee (animal-use protocol A17-020).

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Key Words: Pancreatic cancer, mucin, MUC5AC, antibody, tumor-specific imaging, fluorescence guided surgery, PDOX.

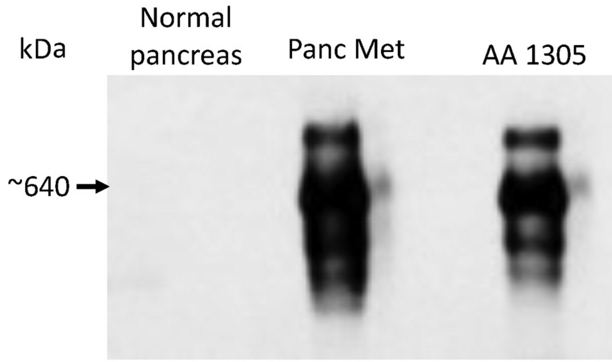

Figure 1. *Western blot showing a minimal level of expression of mucin-5AC on the normal pancreas but high levels of expression in a primary patient-derived pancreatic tumor, AA1305, and a patient-derived liver metastasis from a primary pancreatic cancer (PancMet).*

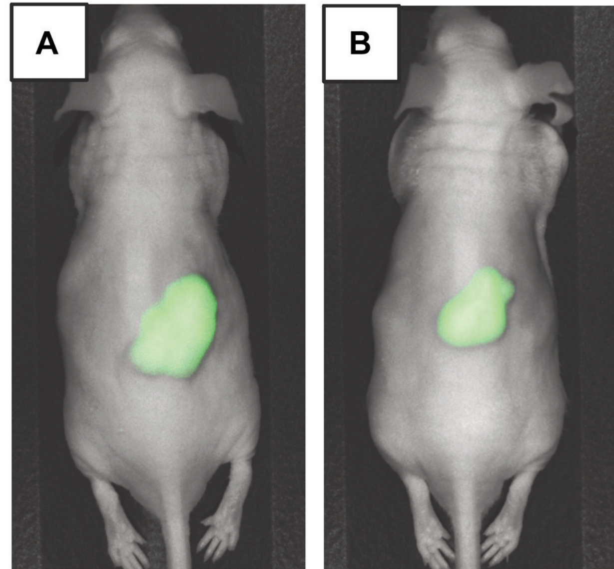

Figure 2. *Imaging of subcutaneous models 72 h after injection with 50 μg (A) and 75 μg (B) mucin-5AC monoclonal antibody conjugated to the near-infrared dye IRDye800CW.*

Antibody conjugation. Monoclonal MUC5AC antibody (Novus Biologicals, Littleton CO, USA) was conjugated to the near-infrared (NIR) dye IRDye800CW NHS ester (LI-COR Biosciences, Lincoln NE, USA) establishing MUC5AC-IR800. The dye was conjugated to the antibody per the manufacturer's protocol and incubated at room temperature for 2 h on a shaker plate. After incubation, the antibody–dye conjugate was added to a gel desalting column (Thermo Fisher Scientific, Waltham, MA, USA) to remove excess unbounded dye. The final product was stored at 4˚C.

Western blotting. Tumor lysates were made using a normal human pancreas, a patient-derived pancreatic tumor (AA1305) and a patient-derived liver metastasis (PancMet). Western blotting was performed as previously described (11). Total-protein lysates (80 μg) were electrophoretically separated in 2% sodium dodecyl sulfate-agarose gels prepared using 1.5 M Tris pH 8.8. Proteins were resolved by horizontal electrophoresis for 4 h at 100 V in Tris-glycine-sodium dodecyl sulfate buffer. The proteins were transferred onto polyvinylidene-difluoride membranes by overnight horizontal transfer and then blocked in 5% skim milk in PBS. After blocking, membranes were incubated with primary antibody (CLH2, cat. no. MAB2011; Millipore, Burlington MA, USA) at 4˚C overnight. Subsequently, membranes were washed with phosphate-buffered saline/tween solution three times and incubated with horseradish peroxidase-labelled anti-mouse secondary antibody for 1 hour at room temperature. After secondary-antibody incubation, membranes were washed three times with phosphate-buffered saline/tween solution. Protein bands were visualized using a chemiluminescence reagent (Luminol enhancer solution and peroxide solution; Thermo Fisher Scientific).

PDOX establishment. A patient-derived primary pancreatic cancer (AA1305) was used in the present study. To establish the cancer subcutaneously in nude mice, tumor fragments (~1 mm^3) made from a surgical specimen were implanted in the bilateral flanks and shoulders of the mice (n=6). Tumor fragments were allowed to grow for ~4 weeks. Next, subcutaneous-grown tumors were harvested, and tumor fragments (~1 mm^3) were implanted in the pancreas of the mice (n=6) to create a PDOX model. This was accomplished first by anesthetizing the mice then sterilizing their abdomen with a 70% ethanol solution. A small incision was made in the upper abdomen just left of midline. The mouse's spleen was carefully exposed and retracted cephalad, exposing the pancreas. The tumor fragment was sutured to the body of the pancreas using 8-0 nylon suture (Ethicon Inc., Somerville, NJ, USA) (12). The mouse organs were returned to the peritoneal cavity and the incision was closed with 6-0 nylon suture (Ethicon Inc.). Post-procedural pain was treated with subcutaneous buprenorphine (25 μl) reconstituted in PBS. The tumors were allowed to grow for 4 weeks. Male and female mice were used. Patient tumors and normal tissue were obtained with informed consent under UCSD Institutional Review Board approval.

Antibody-dye conjugate dosing and imaging. The antibody–dye conjugate was administered *via* tail vein injection. *In vivo* imaging with NIR wavelength (800 nm) was performed daily in subcutaneous models for 72 h using a Pearl Trilogy Small Animal Imaging System (LI-COR Biosciences). The Pearl Trilogy Small Animal Imaging System was used to quantify the strength of the NIR signal from the tumor and from the skin which was used as background. A pilot experiment used MUC5AC-IR800 doses of 25, 50 and 75 μg. After the subcutaneous tumors grew to 1 cm^3, mice were divided into three groups to receive 25, 50 or 75 μg of the antibody–dye conjugate. There were two mice in each group for a total of six subcutaneous models. The mice were imaged at 24, 48 and 72 h after receiving anti-MUC5AC. The tumor to background ratio (TBR) was calculated by dividing the peak intensity of the signal from the tumor by the peak intensity of the signal from the skin.

For the PDOX model, mice (n=6) received MUC5AC-IR800 *via* tail injection (75 μg). An incision was made in the left upper-abdominal quadrant of the PDOX models and the pancreas and

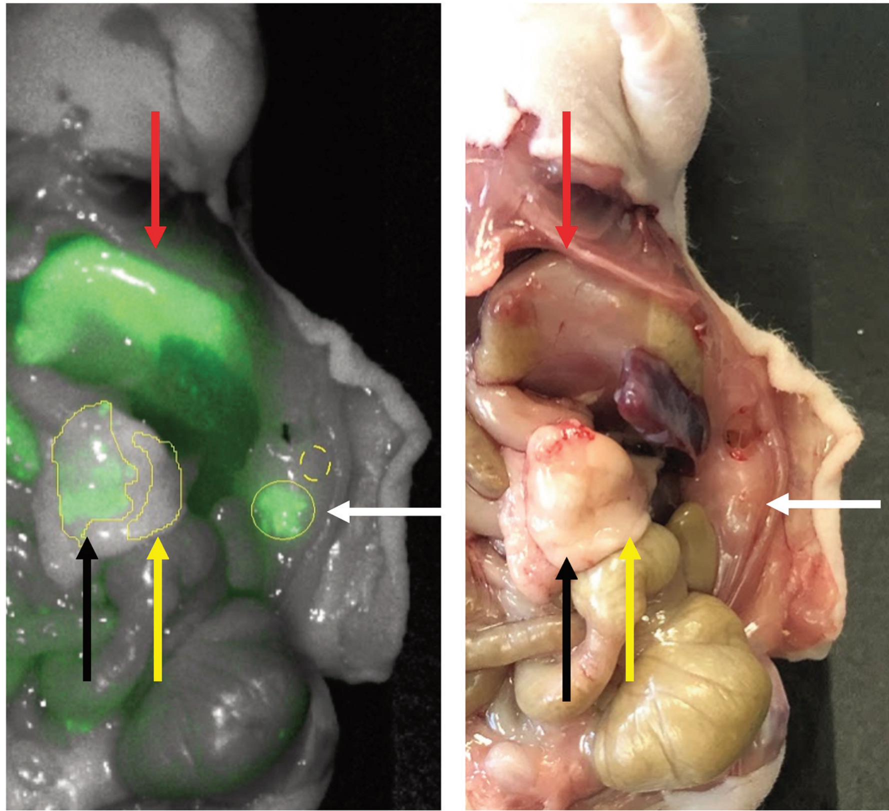

Figure 3. *Patient-derived orthotopic xenograft (PDOX) model of AA1305 72 h after injection of 75 μg of mucin 5AC (MUC5-AC) monoclonal antibody conjugated to the near-infrared dye IRDye800CW. The implanted pancreatic tumor (black arrow) brightly fluoresced compared to normal pancreas surrounding it (yellow arrow). There is also a fluorescence signal from local tumor invasion on the abdominal wall (white arrow) where the peritoneum overlaid the pancreas, as well as fluorescence on the mouse's stomach (red arrow). The fluorescence on the stomach does not represent metastatic spread but normal MUC5AC expression, possibly also fluorescence of the mouse chow.*

tumor were carefully exposed. In vivo imaging was performed at 48 hours with the Pearl Trilogy Small Animal Imaging System. At 72 hours, the mice were euthanized and a laparotomy incision was made, exposing the peritoneal cavity and abdominal contents. Imaging with the Pearl Trilogy Small Animal Imaging System was again performed. Normal pancreas was used to calculate the background tissue signal. The TBR was a calculated by dividing the peak tumor signal by the peak background signal.

Results

MUC5AC expression. Western blotting demonstrated minimal to no MUC5AC expression in normal pancreas obtained from the patient. However, there was a very high level of MUC5AC in the patient-derived pancreatic cancer, AA1305 (Figure 1).

TBR in subcutaneous models. Figure 2 shows imaging at 72 h of mice which received 50 μg and 75 μg of MUC5AC-IR800, respectively. The TBRs (average±SD) at 24, 48 and 72 h for the 25 μg group were 1.25±0.339, 1.67±0.679 and 1.84±0.700, respectively. For the 50 μg group, they were 2.44±1.209, 3.42±1.739 and 3.83±1.874, respectively. For the 75 μg group, the TBRs were 2.19±0.085, 2.82±0.177 and 4.35±0.177, respective. The highest TBR was measured at 72 h in the 75 μg-treated group and these conditions were used for the PDOX models.

TBR in the PDOX models. The average TBR at 48 h was 2.46±0.465 and that at 72 h was 2.183±0.619 (Figure 3).

No toxicity was observed in the subcutaneous or orthotopic models.

Discussion

The need for R0 resection is paramount as surgical resection is the only current curative treatment for pancreatic cancer (4, 13). Intraoperatively, the surgeon is dependent on visual and tactile cues of the unlabeled tumor to determine appropriate extent of

resection (13). The need for improvement is shown by the high rates of R1 resection, reportedly as high as 50-80% (4, 13). There is controversy regarding the wide range of R1 rates (<20% to >80%) reported in the literature (4). This is due to the lack of consensus in defining R1 (although it is accepted that the true R1 rate is under-reported given the high rate of recurrence in R0 resections) (4, 5, 14). In the UK and Germany, the superior-mesenteric-vein groove is routinely evaluated and considered a margin while in the United States, the superior-mesenteric-vein groove may be evaluated but only the portion facing the superior mesenteric-artery requires mandatory evaluation (14). Moreover, the International Union Against Cancer classification defines R1 as microscopic tumor at specimen edge (0 mm margin), whereas more recent protocols such as guidelines from the British Royal College of Pathologists define R1 as tumor within 1 mm of the specimen edge (15, 16).

Specific fluorescence tumor labeling should improve visualization of primary and metastatic disease wherever it is located (including the uncinate or the superior-mesenteric-artery groove), enabling more aggressive resection if indicated and safe. Improved tumor margin visualization could lead to an improved rate of R0 resection and improved disease-free and overall survival. This is the aim of fluorescence-guided surgery (FGS) in oncology.

In the present study, we demonstrated the ability of fluorescent MUC5AC antibodies to selectively target pancreatic cancer tissue compared to normal pancreatic tissue. Western-blot analysis of patient-derived tumor samples demonstrated high expression of MUC5AC, consistent with previously-published data regarding mucins and pancreatic cancer (6, 8, 10). Kim *et al.* reviewed 68 cases of PDAC. They found MUC5AC staining in the majority of PanIN 1A (71%), PanIN 1B (89%), PanIN 2 (88%), and PanIN 3 (90%) cases (17). MUC5AC staining was also demonstrated in well-differentiated (100%), moderately-differentiated (96%) and poorly-differentiated (59%) PDAC specimens (17). This was confirmed by high TBRs in both our subcutaneous and orthotopic models. The different TBRs between the models is expected given the difference between skin and pancreatic tissue. The subcutaneous models are useful for dose-ranging and timing of imaging; however, the orthotopic models are considered more clinically relevant (18). In the orthotopic model, the TBR decrease between 48 and 72 h is due to the signal of the tumor being stable from 48 to 72 h but the background tissue (normal pancreas) having a slight increase in signal strength.

A concern with using an antibody to MUC5AC is its binding to other locations expressing MUC5AC (‘off-target binding’). MUC5AC is a secretory mucin mainly found on the apical surface of epithelial cells in the respiratory and gastrointestinal. tract (6). MUC5AC expression has been reported in the tracheobronchial lining, gallbladder and the lumen of the lacrimal ducts (6). These sites may have off-target binding and their potential interference with tumor imaging using MUC5AC fluorescence antibody must be considered. However, in the present study, there was no off-target labeling which interfered with the pancreatic-cancer fluorescence signal.

Since the early 1990s, fluorescence-tagged antibodies have been used to image tumors (13). Our laboratory pioneered FGS of pancreatic cancer in PDOX models using fluorescent antibodies to selectively visualize the tumor, resulting in improved R0 resections, reduced recurrence and increased survival of the mouse models compared to bright light surgery (BLS) (19-21). McElroy *et al.* demonstrated strong pancreatic-cancer-cell binding by a fluorescent antibody probe with minimal background peritumoral-stroma staining (22). Maawy *et al.* compared carcinoembryonic antigen antibodies conjugated to different dyes of varying wavelengths, showing longer wavelengths were associated with higher TBRs and greater tissue penetration (23). Kaushal *et al.* demonstrated improved visualization of pancreatic-cancer cells in vitro and in subcutaneous pancreatic cancer models in vivo with fluorescent antibodies to carcinoembryonic antigen (24). Metildi *et al.* compared FGS to BLS in orthotopic mouse models of pancreatic cancer and showed improved resection rates, disease-free and overall survival in the FGS arm (19, 20). Hiroshima *et al.* randomized pancreatic PDOX models to neoadjuvant chemotherapy (NAC) with gemcitabine with FGS, FGS alone, BLS with NAC or BLS alone, to determine if FGS in combination with NAC would lead to better outcomes. Their results showed the FGS-plus-NAC arm had reduced rates of metastatic recurrence (25). Furthermore, our laboratory is the pioneer of using fluorescent proteins for in vivo imaging (18, 26, 27). We previously showed fluorescent protein-labeled tumors are powerful tools for FGS (28, 29) and FGS can be used for resection of recurrent tumors expressing the fluorescent protein, which serves as a genetic reporter (30).

Limitations of the present study include the use of immunocompromised mice in the establishment of the PDOX models and the lack of murine pancreas evaluation in the western blot. Another limitation of the PDOX model is the pancreatic tumor is sutured onto the pancreas rather than growing from within it. Another model which may represent a pancreatic tumor growing in the pancreas is the use of patient-derived explants/spheroids/organoids *in vitro*. Our laboratory has pioneered 3-D sponge-gel histoculture of patient tumors, including pancreatic cancer (31). In future experiments, pancreatic organoids will be labeled with MUC5AC fluorescent antibodies (32). We also did not perform immunohistochemistry with the AA1305 tumors. We have previously studied immunohistochemical staining of individual cells with a labeled antibody to cancer-antigen 19-9 in an orthotopic model of pancreatic cancer (22). We will perform a similar experiment with the MUC5AC antibody and pancreatic cancer in future studies. Another limitation is the lack of control animals. McElroy *et al.* used control mice with unlabeled tumors with no fluorescent-tagged antibody

which demonstrated no fluorescence signal, indicating the signal from the fluorescent antibody-labeled tumor was not from background tissue (22). Hollandsworth *et al*. used control animals receiving NIR dye alone (not conjugated to an antibody) in a colon-cancer orthotopic model experiment. The control mice demonstrated non-specific fluorescence without localization to the tumor, indicating the fluorescent-tagged protein signal was specific (33, 34).

The verification of a new antibody to target pancreatic cancer is an important development of the present study. Next steps in developing fluorescent MUC5AC antibodies targeting pancreatic cancer in the PDOX model include determination of the minimal TBR associated with an increased rate of R0 resection with FGS.

Conclusion

The anti-MUC5AC fluorescent-tagged antibody successfully targeted pancreatic tumors in subcutaneous and PDOX mouse models. The fluorescent anti-MUC5AC antibody will be used to confirm the principle that FGS can aid in intraoperative decisions for complete resection of pancreatic cancer with minimal disruption of normal tissue.

Conflicts of Interest

AntiCancer Inc uses PDOX models for contract research. Robert M. Hoffman is a non-salaried associate of AntiCancer Inc. All other Authors have no disclosures.

Authors' Contributions

Michael A. Turner wrote the manuscript and was actively involved in each step of the experimental process. Hannah Hollandsworth, Siamak Amirfakhri, and Thinzar Lwin were actively involved in the experimental process and contributed to the Materials and Methods section of the article. Surinder K. Batra, Sukhwinder Kaur, Gopalakrishnan Natarajan, and Kavita Mallya contributed antibodies for the experiments and performed western blotting. Robert M. Hoffman revised the manuscript. Michael Bouvet was involved in oversight of the research design, implementation and editing of the article.

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