

Molecular imaging-based pancreatic cancer characterization

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Introduction: Pancreatic cancer is the fourth leading cause of cancer death in the USA, and there are approximately 227,000 deaths per year worldwide (1). Early-stage pancreatic cancer is usually clinically silent, and most patients presenting symptoms attributable to pancreatic cancer have already advanced disease. Pancreatic cancer only becomes apparent after the tumor invades surrounding tissues or metastasizes to distant organs. Typical symptoms include abdominal or mid-back pain, obstructive jaundice, and weight loss, that can arise from anorexia, maldigestion from pancreatic ductal obstruction, and from cachexia (1). Better understanding of the disease, effective early detection methods, and new therapeutic targets are urgently needed to improve the outcome of the disease. Here, our aims are to characterize the metabolism of multiple pancreatic ductal adenocarcinoma tumors with ¹H MRS, and to assess the effect of tumors on body weight. We have also characterized the expression of choline kinase (Chk) and cyclooxygenase 2 (COX-2). Chk, the enzyme that converts free choline (Cho) into phosphocholine (PC), is known to be overexpressed in aggressive cancer, and COX-2 a critically important inflammation mediator, significantly influences cancer angiogenesis, invasion and metastasis. To further understand the interaction between the tumor and host, we are developing optical reporter systems that will switch on in muscle in response to cachectic signaling. We have performed initial studies to demonstrate the effect of conditioned medium from one of the cachexia-inducing cancer cell lines on a cell-based optical biosensor using genetically engineered myoblasts.

Methods: A6L, JD13D, Panc1, and BxPC3 human pancreatic adenocarcinoma cell lines, obtained from Dr. Maitra at the Johns Hopkins University, were used in the study. Panc1 and BxPC3 were obtained originally from the American Type Culture Collection. JD13D and A6L originated from pancreatic ductal adenocarcinoma. 2 x 10⁶ cells were inoculated subcutaneously in severe combined immunodeficient (SCID) male mice. Mouse-weight was followed for approximately 6 weeks. Once tumors reached ~ 500 to 600 mm³, they were excised, freeze-clamped, and stored at -80° C. For immunoblot analyses, 50 mg of each tumor was used, and the rest was analyzed with high-resolution ¹H MRS. Proteins were extracted from freeze-clamped tumors using RIPA buffer fortified with protease inhibitor cocktail, dithiothreitol, phenylmethylsulfonyl fluoride, sodium orthovanadate, and sodium fluoride. About 100 µg of protein was resolved on 10% SDS-PAGE, transferred onto nitrocellulose membranes, and probed with antibodies directed against Chk, and COX-2, with actin used as a loading control. Immunoblots were developed using SuperSignal West Pico Chemiluminescent substrate kit. A dual-phase extraction method based on methanol/chloroform/water was used to obtain lipid and water-soluble fractions from the tumors (2). Fully relaxed ¹H MR spectra of tumor extracts were acquired on an 11.7T spectrometer using a 5-mm HX inverse probe and the following acquisition parameters: 30° flip angle, 6000 Hz sweep width, 12.7 s repetition time, time-domain data points of 32K, and 128 transients. Spectra were analyzed using Bruker XWIN-NMR 3.5 software (Bruker BioSpin). Integrals of the metabolites of interest were determined and normalized to the tumor weight. Metabolite concentrations were obtained from ¹H spectra using an internal standard. To determine the effect of pancreatic cancer cells on muscle, we transiently co-transfected primary human myoblasts with EF-1α driven eGFP (constitutive expression) and with either a control vector, triple-tandem repeat of a NFκB *cis*-element from the mouse YY-1 gene lacking a minimal promoter (mp) sequence (Basic), or with the same tandem repeat fused to a minimal promoter sequence (3xNFκB) driving tdTomato expression. The transfected cells were differentiated and then treated for 24 h with conditioned medium from pancreatic tumor cells.

Results and Discussion: Amongst the four cell lines tested, we observed a dramatic loss of weight with growth of JD13D and Panc1 tumors (**Figure 1**). Pancreatic cancer patients often present with cachexia-induced weight loss, and tumors from these two cancer cell lines replicated this syndrome in mice. Immunoblots showed that Panc1 tumors exhibited high expression levels of COX-2 and the highest levels of Chk (**Figure 2**), and induced a loss of weight. However, JD13D tumors showed low levels of those two proteins (**Figure 2**), but also induced loss of weight. Thus, other protein markers associated with a cachexia syndrome await identification.

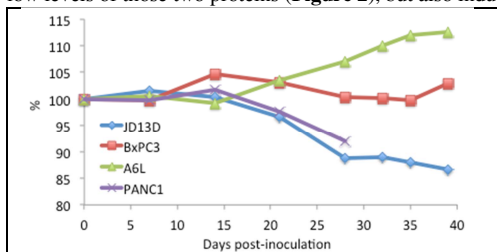


Figure 1: Normalized weight loss in SCID mice inoculated with JD13D, BxPC3, A6L, and Panc1 cells (n = 3).

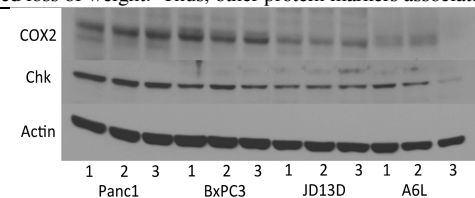


Figure 2: Western blot analysis of COX-2, Chk and Actin expression in Panc1, BxPC3, JD13D, and A6L tumor extracts (n = 3).

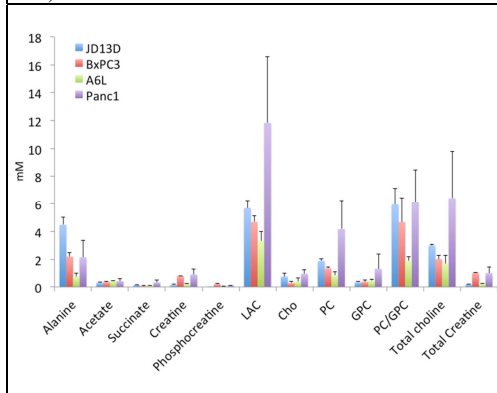


Figure 3: Metabolite quantification in tumor extracts with high-resolution ¹H MRS (Cho: free choline, PC: phosphocholine, GPC: glycerophosphocholine, LAC lactate) (n = 3; mean +/- sem).

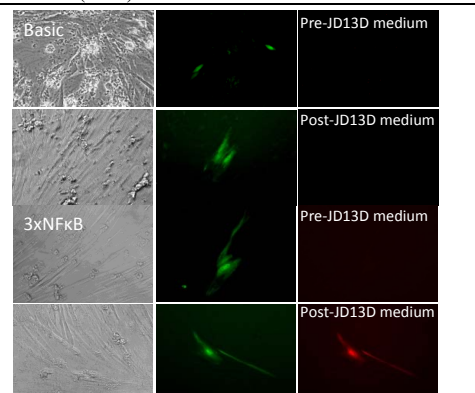


Figure 4: Basic and 3xNFκB transfected myoblasts showing constitutive expression of GFP, and inducible expression of tdTomato red protein visible in presence of JD13D conditioned medium.

¹H MRS analysis revealed that Panc1 tumors contained the highest level of total choline, mainly due to a high level of PC (**Figure 3**), correlating with the high level of Chk observed in the immunoblots. Panc1 tumors were also characterized by a high level of lactate compared to the three other cell lines. JD13D tumors that also induced weight loss presented with the second highest levels of total choline and lactate. No major differences in the lipid patterns were observed amongst the four tumor types. In the transfected myoblasts that were differentiated into myotubes, we observed that only the mp containing 3xNFκB promoter was inducible in the presence of conditioned medium obtained from JD13D cells (**Figure 4**), that induced mouse weight loss *in vivo* (**Figure 1**). No red fluorescence was induced in undifferentiated myoblasts or in confluent myoblasts (data not shown). The acquisition of *in vivo* ¹H MRS is on going, and should further improve the characterization of those pancreatic ductal adenocarcinoma cell lines, and the correlation between the metabolites measured *in vivo* with the loss of weight observed in the mice. Our data identify increased total choline and lactate as potential imaging biomarkers to detect pancreatic cancer, and Chk and COX-2 as potential therapeutic targets of this devastating disease.

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