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Perfusion CT estimates photosensitizer uptake and biodistribution in a rabbit orthotopic pancreas cancer model: a pilot study

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Abstract

Objectives—It was hypothesized that perfusion computed tomography (CT) blood flow (BF), blood volume (BV) and vascular permeability surface-area (PS) product parameters would be predictive of therapeutic anti-cancer agent uptake in pancreatic cancer, facilitating image-guided interpretation of human treatments. The hypothesis was tested in an orthotopic rabbit model of pancreatic cancer, by establishing the model, imaging with endoscopic ultrasound and contrast CT, and spatially comparing the perfusion maps to the *ex vivo* uptake values of injected photosensitizer, verteporfin.

Materials and Methods—Nine New Zealand White rabbits underwent direct pancreas implantation of VX2 tumors and CT perfusion or endoscopic ultrasound was performed 10 days post-implantation. Verteporfin was injected during CT imaging and tissue was removed 1 h post-injection for frozen tissue fluorescence scanning. Region-of-interest comparisons of CT data with *ex vivo* fluorescence and histopathological staining were performed.

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Results—DCE-CT showed enhanced BF, BV, and PS in the tumor rim, and decreased BF, BV and PS in the tumor core. Significant correlations were found between *ex vivo* verteporfin concentration and each of BF, BV, and PS.

Conclusions—The efficacy of verteporfin delivery in tumors is estimated by perfusion CT, providing a non-invasive method of mapping photosensitizer dose.

Keywords

Pancreatic cancer; CT perfusion; drug delivery; photodynamic therapy; dosimetry; pharmacokinetics

Introduction

The five-year survival of pancreatic adenocarcinoma is approximately 3%, amongst the most dismal prognoses in all of oncology (1). Radical resection with or without adjuvant or neoadjuvant treatment is the only means of improving long-term survival, but is only an option in approximately 25% of patients (2). For locally advanced and unresectable disease, a main focus of patient management is the preservation of an acceptable quality of life for as long as possible through palliative therapy (3). Treatments with minimal side-effects that are able to provide some degree of tumor control are preferred in these cases but options are currently limited. New therapeutic options—including photodynamic therapy (PDT) (4, 5), radiofrequency ablation (6), tumor vaccines (7) and targeted nanoparticles (8)—may be treatment options for unresectable pancreatic cancer and the advance of suitable pre-clinical animal models will be critical to the development and implementation of each to accurately assess their efficacy prior to widespread clinical translation. For example, in a recent phase I/II study, verteporfin-based PDT was delivered to locally advanced pancreatic cancer (9). While PDT was clearly able to induce dose-dependent damage to the cancers, it was also noted that the treatment induced a necrotic volume that was inversely related to the observed contrast CT enhancement (10). Beyond this, it is well known that drug permeability in cancer is directly related to perfusion in a number of cancer and drug combinations (11, 12), and so perfusion imaging is a reasonable way to diagnostically assess the penetration of therapeutics into the lesion. The goal of this paper was to utilize a rabbit orthotopic model of cancer in the pancreas, and use it with conventional ultrasound and CT imaging to characterize uptake and dose delivery of verteporfin, in order to better understand the role of this type of imaging in human PDT treatments.

Photodynamic therapy is an alternative treatment for cancer that uses the interaction of light and a photosensitizer, in the presence of oxygen, to produce singlet oxygen (13). The singlet oxygen causes direct cell death through necrosis (14), as well as indirect cell death by vascular damage induced hypoxia. A major benefit of PDT is that it exerts its effect photochemically, rather than thermally, so connective tissues such as collagen and elastin remain intact, preserving the mechanical integrity of tissue (15). The photosensitizer drug, such as verteporfin used in this study, is most often delivered intravenously. Localized tissue necrosis can be produced by delivering light from a low-power laser directly to the tumor through a small diameter fiber (9). Since both drug delivery and light delivery affect the

volume of tumor necrosis, dynamic CT imaging is proposed for planning and evaluating PDT treatment in pancreatic cancer.

Preclinical studies are necessary to further the understanding of drug delivery, and to establish patient-specific dosage and treatment planning protocols, similar to the current standard-of-practice in radiation therapy (16). In particular, clinical imaging is an ideal candidate to estimate the uptake of a photosensitizer, correct for light-absorption due to blood volume (which results in underdosing) and optimize light probe placement. Unfortunately, clinical CT imaging is not compatible with the small animal models typically used in preclinical cancer research, as the spatial resolution of CT and ultrasound devices becomes a limiting factor when animal size is scaled down. Recently, a rabbit model of pancreatic cancer was described (17), and used to investigate MRI (18). We utilize this rabbit model of pancreatic cancer to complete comparative imaging and drug delivery studies. Clinical CT and endoscopic ultrasound (EUS) imaging was carried out on these animals, including contrast-enhanced CT, parametric maps of blood flow (BF), blood volume (BV), and vascular permeability surface-area (PS) product. As a corollary, the relationship between CT parameters measured in healthy and tumor regions of the pancreas and the uptake of the photosensitizer verteporfin were investigated to highlight applications in individualized dosimetry.

Materials and Methods

Animal experiment

All experiments were carried out according to an animal use protocol approved by the Institutional Animal Care and Use Committee (IACUC) at [institution removed]. Nine female New Zealand White rabbits (approximately 3 kgs) were obtained from a local supplier (Millbrook Labs, Amherst, MA) and housed in the Center for Comparative Medicine and Research. The tumor propagation procedure followed standards developed for this tumor model over the past few decades (17–19). In brief, the VX2 carcinoma cells were proliferated in live animals to establish tumorigenic potential (three rabbits were prepared for subcutaneous thigh tumor implantation and not used for subsequent imaging studies). The right flank was sterilized with chlorhexiderm and locally anesthetized with bupivacaine (< 8 mg/kg). A 1-cm incision was made in the flank, and small pieces of solid VX2 tumor were placed into a pocket formed under the skin. The incision was closed with nylon dextron suture. After about 2 weeks, re-excision surgery was performed using the same sterilization and anesthetization protocol as above. The tumor mass was resected from the leg, and the animal was euthanized. The tumor resection was cut up into 1 mm³ sections for implantation into other rabbits.

Pancreatic tumor implantation was performed on the remaining six animals. An incision was made through the upper median line of the abdomen. The pancreas was located just below the incision as attached to the spleen, and a small stainless steel 28-Ga suture was placed in the head of the pancreas to allow localization on CT images during imaging. Approximately 5–10 mm from the suture, 2 small pockets were made in the pancreas, and a small piece of VX2 tumor was implanted into each site. Absorbable hemostats (Surgicel® Original, Ethicon, Inc., Somerville, NJ) were put over the incision site, and the abdomen wall and skin

were closed with 6-0 and 4-0 Dexon nylon suture, respectively. Additional details about these experiments can be found in Ref. (19). Animals were used for imaging studies at approximately 10 days after implantation.

Image Acquisition

Five animals were scanned in a clinical CT scanner (LightSpeed RT16, GE Healthcare, Waukesha, WI). Dynamic contrast-enhanced CT images were acquired following a 1 ml/sec injection of iohexol, an iodine-based contrast agent (Omnipaque 300, 1 ml/kg, GE Healthcare). After acquiring a scout axial scan for slice selection, two-phase cine helical scans were acquired with a beam energy and current of 120 kVp and 200 mA. Eight 2.5-mm thick axial slices were acquired every 0.5 seconds for 30.5 seconds, then every 10 seconds out to about 2 minutes. To minimize breathing motion artifacts, a breath hold was executed during the first phase of imaging by temporarily turning off the ventilator. During the second phase, the ventilator was synchronized to the image acquisition. Image acquisition parameters were based on a previous validation study in the same animal species (20).

Endoscopic ultrasound images were obtained from the animal that did not undergo CT scanning or verteporfin injection, post-mortem using a curved linear array EUS device (GF-UC140P, Olympus America Inc., Center Valley, PA). Laparotomy was performed by making a midline incision above the stomach, which was exposed and opened. The EUS probe was placed under direct visualization into the stomach, and scanned images were digitally saved using the ALOKA 5.0 processing system.

Computed tomography analysis

Dynamic-contrast enhanced CT data was analyzed with the adiabatic approximation to the tissue homogeneity (AATH) (21) model using CT PERFUSION 4 (GE Healthcare). In contrast to the extended Kety model, the AATH model is used in DCE-CT and DCE-MRI kinetic analysis of tumors because it accounts for differences in vascularization kinetics, in addition to compartment exchange (22). Parametric maps of BF, BV, MTT and PS were determined by deconvolving the time-enhancement curves for each 2×2 pixels by the arterial input function obtained from pixels corresponding to the abdominal aorta. Partial volume correction was performed by a previously described method (23), using venous concentration obtained from the inferior vena cava. Regions-of-interest were hand-drawn by two of the authors based on localization of a small metallic suture placed rostral to the tumor mass during the time of implantation. The core was defined as the hypointense contiguous region evident on the enhanced CT image post-contrast. The tumor rim was defined as the hyperintense region in a 4 pixel thick region surrounding the core contour. A circular region in the normal pancreas of approximately the same size as the tumor was chosen so that it was not directly adjacent to the rim or core. All parametric maps were analyzed with in-house developed software (IDL v6.0, Exelis VIS, Boulder, CO).

Ex-vivo fluorescence imaging and histopathology

Verteporfin was administered intravenously in four animals at 1 mg/kg (active benzoporphyrin derivative sensitizer dose) immediately after imaging and animals were sacrificed 60 minutes post-injection. Following euthanasia, the pancreas and surrounding

tissue were removed and frozen. *Ex vivo*, 2.5-mm thick tissue slices were obtained using an in-house built acrylic tissue matrix, starting approximately 5 mm from the rostral aspect of the tumor and moving in the caudal direction. Slices were placed directly on a fluorescence scanning system (Typhoon 9410, GE Healthcare) and fluorescence was measured using 633 nm excitation and a 685 nm long-pass filter for emission collection. After imaging, the tissue slices were fixed in 10% buffered formalin, processed using standard procedure, and then embedded in paraffin. After embedding, 4-micron-thick tissue slices were obtained, stained with hematoxylin and eosin (H&E), and mounted on slides. Slides were examined using a light microscope (BX50, Olympus America Inc., Center Valley, PA) and images of the microscope field of view were acquired with a CCD camera (Insight 2.0 color, SPOT Imaging Solutions, Sterling Heights, MI). One set of images was discarded due to poor quality, and the remaining three sets of micrographs were analyzed. Fluorescence imaging data is presented in relative fluorescence units (RFU).

Statistical analysis

All statistical tests were performed using SPSS software (version 15.0, IBM, Armonk, NY). Group data were reported as mean $\pm$ standard error unless otherwise stated, and significance was defined as $p < 0.05$. Differences in group averages were determined by t-test, and a modified regression analysis was performed to account for multiple measurements in the same rabbit (24). To determine region-of-interest reproducibility, both the interclass correlation coefficient and the coefficient of variation (CV) between two ROI sets drawn independently were calculated.

Results

Computed tomography was performed on five rabbits. Representative examples of six CT images in two rabbits with pancreatic tumors are shown in Fig. 1. Pre-contrast images (Fig. 1A,D), as well as images obtained at peak arterial contrast 10 s following contrast injection (Fig. 1B,E), and images acquired 70 s after injection showing ring enhancement of the tumor (Fig. 1C,F) are shown.

In addition to these contrast-enhanced CT images, the AATH model was used to calculate parametric maps of BF, BV, and PS product in a subset of five rabbits, a representative example of which is shown in Fig. 2. Region-of-interest analysis was performed on a total of 15 slices from 5 rabbits, for normal pancreas, tumor rim, and tumor core. The average BF was 59.8 ± 3.1 , 70.5 ± 4.1 , and 27.1 ± 3.4 ml/min/100g, for the normal pancreas, tumor rim and tumor core, respectively. The average blood volume was 15.4 ± 1.8 , 15.4 ± 1.4 , and 4.8 ± 1.0 ml/100g, and the average PS product was 20.4 ± 2.5 , 29.0 ± 2.5 , and 10.2 ± 1.1 ml/min/100g, respectively, for the same three regions. For all three parameters, the average values of the tumor core were significantly different from the normal pancreas ($p < 0.001$) and tumor rim ($p < 0.001$). The PS product measured in the tumor rim was also significantly different from the PS product measured in the normal pancreas ($p < 0.05$). Coefficients of variation were calculated to determine the reproducibility between animals; these were 8.1, 12.9, and 12.0% for BF, BV, and PS in the tumor rim; 25.5, 33.2, and 22.3% for BF, BV, and PS in the tumor core; and 5.11, 11.0, and 12.4% in the normal pancreas. To test

reliability in the ROI analysis, BF, BV, and PS calculated with two independently drawn sets of ROIs had an interclass correlation of 0.992 and a CV of 6.77%

An EUS image was acquired in one rabbit and is summarized in Fig. 3. The image demonstrates a 13 mm hyperechoic mass in the pancreatic tail. The mass is well demarcated, does not involve the main pancreatic duct, and is seen easily under EUS guidance.

Fluorescence images were acquired on ex-vivo tissue sections from 3 rabbits, 1 hour post-injection of verteporfin. On average, the fluorescence intensity measured in the rim of the tumor was 18% higher than in the normal pancreas, whereas the fluorescence intensity of the tumor core was 14% lower than in the normal pancreas. Region-of-interest analysis was performed on fluorescence images, and a linear regression analysis was used to determine the presence of a correlation between relative fluorescence and CT parameteric data across regions and across animals. The results of the regression analysis are presented in Fig. 4. A very strong correlation was detected between *ex vivo* verteporfin fluorescence and BF (slope = $203.5 \pm 28.9 \text{ RFU} \cdot \text{min} \cdot 100\text{g} \cdot \text{ml}^{-1}$, y-int = $24304 \pm 966 \text{ RFU}$, $r = 0.84 \pm 0.04$, $p < 0.001$) and between *ex vivo* verteporfin fluorescence and PS product (slope = $420 \pm 75 \text{ RFU} \cdot \text{min} \cdot 100\text{g} \cdot \text{ml}^{-1}$, y-int = $25744 \pm 445 \text{ RFU}$, $r = 0.81 \pm 0.03$, $p < 0.001$). A strong correlation was also detected between *ex vivo* verteporfin fluorescence and BV (slope = $682 \pm 40 \text{ RFU} \cdot 100\text{g} \cdot \text{ml}^{-1}$, y-int = $26937 \pm 2299 \text{ RFU}$, $r = 0.76 \pm 0.08$, $p < 0.05$). Finally, a correlation between BF and BV was detected (slope = $0.24 \pm 0.03 \text{ min}^{-1}$, y-int = $-1.10 \pm 0.83 \text{ ml/min/100g}$, $r = 0.90 \pm 0.03$, $p < 0.001$), but no correlations between PS and BF or PS and BV were detected. The average CT perfusion parameters measured in the subset of three rabbits used in the comparison with verteporfin fluorescence did not differ significantly from the group averages presented in the above paragraph.

Figure 5 presents representative high magnification micrographs of H&E stained tissue sections that have been annotated to highlight certain morphologies. Both normal pancreas and carcinoma were visualized, and invasion of cancer into normal pancreas tissue was readily observed in panel A and D. The tumor regions are characterized by high amounts of tumor-related stroma (panel B), including desmoplastic fibrosis. Finally, mild immune responses, such as the invasion of neutrophils and mononuclear agranulocytes, were observed in some locations.

Discussion

The management of advanced unresectable cancer is challenging and novel therapeutic treatments are needed to provide palliative care and improve quality of life. However, while small single-center clinical trials can give some idea on the tolerance and feasibility of a proposed treatment, appropriate animal models must be used to refine therapeutic approaches before translation into a clinical setting will be successful. For example, the use of verteporfin was recently investigated in a clinical trial involving patients with unresectable locally advanced pancreatic cancer (10). In this study, a large variability of tumor control was observed, and it was hypothesized that this variability was attributable to differences in tumor BV, which diminished effective dose delivered because of light absorption by blood. However, in humans the delivery of verteporfin into pancreas tumors is

difficult to assess because biopsy for assessment is infeasible. Additionally, fiber-based fluorescence assay of the signal is logistically challenging (25, 26), leading to a nearly complete lack of information about the success of local delivery of the drug to the tumor and the normal pancreas. It is well recognized that perfusion CT can provide a surrogate for drug assay *in vivo*, and areas of high perfusion, BV, and vascular permeability in tumors are likely to correlate with delivery distributions of most small to medium sized molecules. Thus, the overall goal of this work was to use a large animal model to test if imaging could be used to better understand the delivery of verteporfin in a pancreas tumor; for example, to determine whether the potential decrease in treatment efficacy caused by poor drug delivery in hypovascular tumors is offset by the potential increase in light delivery caused by reduced blood absorption.

Since CT imaging is the most widely used modality to detect, stage and monitor the progression of pancreatic cancer, it is necessary that the proposed animal model be compatible with standard clinical CT imaging protocols. A two-phase CT perfusion protocol was employed to acquire hemodynamic parameters. Contrast-enhancement of the tumor rim was observed in all animals that underwent CT imaging. Region-of-interest analysis on tumor rim, tumor core, and normal pancreas showed good reproducibility of values between subjects based on the CV analysis. According to an F-test of McKay's approximation for CV (27), BF measurements were more reproducible than BV and PS, agreeing with recent DCE-CT studies of reproducibility in nasopharyngeal carcinoma (28). Additionally, by the same statistical test, reproducibility was significantly poorer in the tumor core, compared with tumor rim or normal pancreas, most likely due to the lack of enhancement attributed to low perfusion. Significant differences between tumor rim and the other two regions were detected for BF and PS. There was a significant difference between BV in the rim compared with the core, but not between the rim and the normal pancreas. These findings are mostly in agreement with clinical studies of pancreatic cancer using CT perfusion imaging (29–31), where the tumor core is typically hypoperfused, highlighting the suitability of this model in evaluating drug delivery. However, typical clinical presentation does not include a tumor rim that is hyperperfused compared with the normal pancreas; this is likely a feature unique to the VX2 tumor line, and is reflected in both the fluorescence and CT perfusion parameters. To provide further clinical context, we also performed imaging using an endoscopic ultrasound, to demonstrate the feasibility of this type of imaging in the rabbit model. Although *in vivo* endoscopic ultrasound was not possible due to the diameter of the scope that was available during the study, the *ex vivo* procedure very closely approximated what would be done *in vivo*, the only difference being that the probe was inserted into the stomach via an incision rather than by esophageal insertion. The tumor mass was well demarcated on the ultrasound image. This animal model will be useful to investigate the use of EUS, including contrast-enhanced EUS, for treatment planning and PDT fiber guidance in future studies.

As a corollary to the description and evaluation of the rabbit pancreatic cancer model, the ability for CT perfusion to predict the uptake of verteporfin, a photosensitizer used in photodynamic therapy, was evaluated. The results of the regression suggested that the uptake of the drug at one h is strongly related to the kinetic parameters of the tissue regions-

of-interest. In particular, all of BF, BV, and PS showed strong correlations with verteporfin fluorescence. This extends the previous work by our group that showed a correlation between uptake of gadolinium contrast and verteporfin in a mouse model of orthotopic pancreatic cancer (32). In this study, we show that the kinetic parameters obtained from CT perfusion provide a surrogate measure of local verteporfin kinetics, and provide a framework using the rabbit pancreas tumor model to evaluate image guided and image planned therapeutic interventions for direct translation to human end-stage pancreatic cancer.

While the VX2 tumor model has been used as a model of invasive pancreatic cancer before (17, 18), this complimentary study is the first to investigate clinical imaging in rabbit pancreatic cancer with the goal of PDT treatment planning. The degree to which VX2 tumors mimic the morphology and behaviour of pancreatic tumors found in humans is unknown and since the VX2 cell line is a naturally occurring rabbit squamous cell carcinoma derived from virus-induced papilloma (33), it cannot be assumed to have any structural or molecular similarity to human pancreatic cancer. In fact, previous work by our team has found VX2 tumors implanted into the liver and thigh to have a soft, diffuse consistency, which is in contrast to the classical description of human ductal adenocarcinomas of the pancreas as firm, fibrous and marble-like in nature (34–36). In this study we found that, while the consistency of these tumors was not rock-hard, it was much more firm than that observed in other implantation sites, suggesting that the environment plays a part in the morphology of the growing mass. In rodent studies, the use of athymic mice and rats enables the implantation of human pancreatic cells, which may more closely mimic the morphology of the human disease. While the use of a non-pancreas origin tumor is a limitation of this study the VX2 tumor is used out of necessity due to the intact immune system of rabbits. Nevertheless, as a model for evaluating clinical imaging techniques, especially pertaining to treatment planning in pancreatic cancer, the results of this study suggest that this VX2 tumor model is a suitable choice.

Conclusion

The animal model described in this paper enabled the investigation of clinical CT imaging and endoscopic ultrasound imaging for better understanding photosensitizer delivery and eventual treatment planning in advanced pancreatic cancers. The development of alternate treatment strategies is especially important to improving the quality of life for patients with advanced unresectable pancreatic cancer. To date, no suitable animal model exists to plan, evaluate and determine the mechanistic underpinnings of treatments such as photodynamic therapy in this clinical context, and the resulting strong correlations between perfusion parameters and drug delivery appears to be consistent with the idea that perfusion CT could provide eventually be used as individualized diagnostic data about drug delivery in patients being planned for therapy.

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Fig. 1. Iohexol contrast-enhanced CT images acquired in two rabbits showing (A,D) before enhancement, (B,E) at the peak arterial enhancement, and (C,F) during contrast washout phase. The arrow indicates the location of tumor with ring enhancement pattern apparent in both animals.

Fig. 2.

Dynamic contrast-enhanced CT parametric maps of (A) blood flow, (B) blood volume and (C) permeability surface-area product, and (D) the post-enhancement image of the same region. In all cases, the tumor core is visible as very low values (white arrowhead). The arterial input function and time-density curves (E) show the dynamic behaviour of contrast associated with the regions-of-interest. (F) The group averages for the three regions-of-interest are presented. Error bars denote SEM and significant differences between normal pancreas and either tumor rim or tumor core ($P < 0.001$) are indicated by *a* or *b*, respectively.

Fig. 3.
Endoscopic ultrasound image acquired in a rabbit, showing the hyperechoic tumor mass (white arrowhead).

Fig. 4.

Linear regression plots showing the relationship between *ex vivo* verteporfin fluorescence and (A) blood flow, (B) blood volume, (C) and permeability surface-area product. Symbols indicate results from the three regions: normal pancreas (squares), tumor rim (triangles) and tumor core (circles). Symbols of the same color represent the same animal. Lines-of-best-fit for each animal (dashed lines) as well as the average line-of-best-fit (solid line) are shown. (D) A representative fluorescent tissue section showing accumulation of verteporfin in the rim of the tumor (arrows).

Fig. 5.

Hematoxylin and Eosin stain of excised tissue samples containing normal pancreas tissue and cancerous tissue. (A) The carcinoma cells (*Ca*) are seen invading into the healthy pancreatic exocrine acinar cells (*Ac*). (B) Highly fibrous regions (*F*) of tumor are observed, surrounded by cancer cells. (C) A mild immune response is suggested by the appearance of leukocytes (*Le*) adjacent to the arteriole vessel (*V*) and (D) Both normal acinar cells and cancer cells are observed, with large areas of desmoplastic fibrosis (40× magnification).