Intra-tumoral heterogeneity of gemcitabine delivery and mass transport in human pancreatic cancer

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Abstract

There is substantial heterogeneity in the clinical behavior of pancreatic cancer and in its response to therapy. Some of this variation may be due to differences in delivery of cytotoxic therapies between patients and within individual tumors. Indeed, in 12 patients with resectable pancreatic cancer, we previously demonstrated wide inter-patient variability in the delivery of gemcitabine as well as in the mass transport properties of tumors as measured by computed tomography (CT) scans. However, the variability of drug delivery and transport properties within pancreatic tumors is currently unknown. Here, we analyzed regional measurements of gemcitabine DNA incorporation in the tumors of the same 12 patients to understand the degree of intra-tumoral
Introduction

There is increasing awareness that the heterogeneity of cancer is a substantial challenge to achieving a cure. This is well illustrated in the case of pancreatic cancer [1, 2]. Although this disease generally has a poor prognosis, there is substantial variability in the response to therapy between patients and within tumors [3]. This indicates that the reasons for the generally poor outcomes of pancreatic cancer are not uniform [4–6]. Indeed, while the majority of patients die of distant metastasis, about 30% of patients die of only locally progressive disease [7]. Differences between patients may contribute to this observation (i.e., inter-patient heterogeneity), but it is also important to consider the differences within their tumors (i.e., intra-tumoral heterogeneity) [6]. While there is evidence that there is intra-tumoral heterogeneity in the genomics of pancreatic cancer [8, 9], it is unclear what degree of variability exists in terms of the physical properties within a pancreatic tumor. Furthermore, the translational importance of intra-tumoral heterogeneity in pancreatic cancer is unclear, and connecting this phenomenon to clinical outcome measurements remains a challenge for the field.

Toward addressing the basic, translational, and clinical questions related to the inter-patient and intra-tumor heterogeneity of pancreatic cancer, we have conducted studies to understand the clinical and pathological factors related to the variable response of patients. For example, we have previously documented long-term survival in patients who achieve a pathological complete response after neoadjuvant therapy and resection, but these patients represent a very small fraction of all patients (2.7%) [3]. To understand this phenomenon in greater depth, we performed a clinical trial of intraoperative gemcitabine infusion during curative-intent surgery for 12 patients with resectable pancreatic cancer. In this trial, we observed significant inter-patient variability in gemcitabine incorporation within their primary pancreatic tumors [10]. We discovered that this variability could be accounted for using quantitative mass transport properties, derived from routine pancreatic protocol computed tomography (CT) scans. However, the degree of intra-tumoral heterogeneity in the delivery of gemcitabine and in the mass transport properties of the tumors remained unknown.

Here, we carefully cataloged the sites of sampling in each primary pancreatic tumor for gemcitabine incorporation in our original trial of 12 patients to gauge intra-tumoral heterogeneity in gemcitabine delivery. Furthermore, we developed a volumetric segmentation methodology to derive mass transport properties from CT scans, with the goal of achieving high inter-observer agreement. Finally, we assessed the ability of the pre-therapy CT-derived transport properties from each observer to describe the variable gemcitabine incorporation in this cohort.

Methods

Intraoperative gemcitabine infusion clinical trial

The details of our original trial design were described previously, including patient eligibility, intraoperative gemcitabine infusion procedure, sampling and measurement technique for gemcitabine incorporation, and histological analysis [10]. Briefly, patients with previously untreated, resectable pancreatic cancer were eligible for the trial (supplementary table). After written informed consent, patients were taken to the operating room, and after being deemed resectable by the surgeon, gemcitabine was infused intravenously through a peripheral line at a rate of 10 mg m⁻² min⁻¹ to a total dose of 1000 mg m⁻² for all patients, except the first two. These two initial patients received 500 mg m⁻², based on IRB recommendations to ensure safety of the protocol. The blood supply to the pancreas was maintained until the specimen was ready for complete removal and after all
gemcitabine was infused. The specimen was immediately taken to an adjacent surgical pathology suite for sampling.

**Tumor sampling for gemcitabine incorporation**

The pancreatectomy specimens were evaluated by a pathologist (HW). Multiple biopsy samples from the tumor and adjacent normal pancreas were taken with a 4 mm punch biopsy. The locations of the tumor biopsy samples that were used to quantify gemcitabine incorporation were recorded as either inner or outer tumor (figure 1(A)). Generally, the outer and inner tumor portions were designated by visual inspection and palpation at the time of tumor sampling by the pathologist. Typically, the outer tumor was identified as the outer 3–5 mm of the tumor, and the inner tumor was anything inside this rim. These specimens were then processed as previously described for quantitative analysis of gemcitabine DNA incorporation (Advion Biosciences). Proper calibration of the gemcitabine assay was performed [10].

**CT analysis**

The pancreatic protocol CT scan is a diagnostic test for patients with pancreatic cancer, where iodine-based contrast is injected intravenously at a fixed rate [11]. The test usually consists of a pre-contrast, an arterial phase (35–40 s after starting contrast infusion) and a portal-venous phase (65–70 s after starting contrast infusion). The CT acquisition is usually at a high resolution (0.6–2.5 mm slice thickness), enabling visualization of most pancreatic tumors due to differential contrast enhancement relative to the surrounding normal pancreas tissue. The pre-operative CT images were imported into Pinnacle 9.6 (Varian Medical Systems, Palo Alto, CA) for image registration of the pre-contrast, arterial phase, and portal venous phase of the pancreatic protocol CT scan for each patient. After the registration, three independent medical doctors (EK, FB, AO) delineated the aorta at the level of the celiac artery, normal pancreas, and pancreatic tumor. The general guidelines for radiographic segmentation of the tumor and normal pancreas were to concentrate on the tissue component (i.e., hypodense solid tumor and parenchyma of the normal pancreas, respectively), while avoiding the pancreatic duct, the surrounding fat space of the pancreas, and artifacts from metal. Additionally, the segmentation of the solid tumor avoided enhancing portions of the pancreas parenchyma by 2–4 mm.

The outer portion of the tumor was also segmented by EK to evaluate intra-tumoral heterogeneity in the transport properties, compared to the inner portion of the tumor. The outer and inner radiographic segmentation of the tumors were performed in retrospective review of the patients’ images. The definitions for inner and outer tumor from the CT scan followed the same methodology as the pathology sampling for inner and outer tumor: the outer tumor was identified as the outer 3–5 mm of the visualized tumor, and the inner tumor was anything inside this rim.

**Figure 1.** Heterogeneous delivery of gemcitabine and transport properties. (a) Sampling technique. After intraoperative infusion of gemcitabine during curative-intent resection, human pancreatic tumors were sampled as illustrated. The inner and outer tumor portions were visually identified, and multiple biopsies were taken. (b) Heterogeneity in tumor gemcitabine DNA incorporation. The gemcitabine incorporation into the tumor DNA was assessed for 12 patients. The proportion of the total gemcitabine delivered into the outer and inner portions of the tumor for each patient are shown in gray and black, respectively. The corresponding hENT1 score (low or high) is also shown for reference. The patients are numbered based on the proportions of gemcitabine incorporation, not necessarily their accrual to the trial. (*Denotes the initial two patients who received a total dose of 500 mg m$^{-2}$. All other patients received 1000 mg m$^{-2}$.) (c) Heterogeneity in tumor transport properties. Similar to the gemcitabine sampling of the pancreatic tumors, outer and inner tumor were visually identified on CT scans. The volumetric area under the enhancement curve (AUC) was compared between the outer and inner portions for each patient’s tumor. (Note: patient 5 did not have a pancreatic protocol CT).
The segmented structures (aorta, pancreas, tumor) were analyzed for density, measured in Hounsfield units (HUs), within the delineated volume of interest. The volumetric mean of the HU at each phase of the scan was used to calculate the mass transport properties of each structure, providing an area under the enhancement curve (volumetric AUC), using our previously described mathematical model of transport [10].

Statistics
JMP 10.0 (SAS Institute) was used to perform all statistical analyses. All data were tested for normal distribution using the D’Agostino and Pearson omnibus normality test, where a $p$ value greater than 0.05 was considered to pass the normality test. All data satisfied these tests for normality ($p \geq 0.25$ for all data). The Spearman’s rank-order test was used for non-parametric correlation analysis. Linear regression (analysis of variance [ANOVA]) was used for correlations as long as normal distribution assumptions were met.

Results
Inter-patient and intra-tumoral heterogeneity in gemcitabine delivery and transport properties
The median number of tumor samples was 4 per patient (range 2–6). The median number of samples from the outer portion of the tumor was 2 (range 1–3), and the median number of samples from the inner portion of the tumor was 2 (range 1–3).

As previously described, there was significant inter-patient heterogeneity in gemcitabine DNA incorporation within the tumors [10]. Here, we observed a spectrum of intra-tumoral heterogeneity in the 12 patients, with some patients having a lower proportional gemcitabine delivery to the inner portion of the tumor while others had proportionally higher delivery there (percentage of total gemcitabine DNA incorporation in the inner portion of tumors ranged from 38 to 74%, figure 1(B)). Even within the designated inner and outer portions of each patient’s tumor, we observed variable patterns of gemcitabine DNA incorporation (standard deviation ranging from 6% to 87% of the mean, supplementary table). Similarly, there was a wide range of intra-tumoral heterogeneity in the transport properties. For example, some tumors showed negligible difference between the outer and inner portions of the tumor, while others showed about 200% difference in volumetric AUC between the outer and inner portions of the tumor (figure 1(C)).

Quantitative segmentation to measure mass transport
Three different users independently segmented the aorta at the level of the celiac artery, normal pancreas, and pancreatic tumor (figure 2(A)). There was strong correlation between the volumetric AUC normalized by pancreas and volumetric AUC normalized by aorta ($p<0.0001$ for all observers, figure 2(B)). This was expected since we previously noted high correlation between the AUC of the normal pancreas and the enhancement in the aorta [10]. Additionally, there was high degree of agreement between the observers. For example, at the average value of volumetric AUC normalized by the pancreas for all patients, there was less than a 5% difference across the three observers in terms of the predicted value of volumetric AUC normalized by the aorta (figure 2(B)).

Accounting for heterogeneity with mass transport
For each user, the mass transport properties of the tumors were correlated with gemcitabine DNA incorporation in the
inner and outer portions of the tumor, as well as an average of all of the measurements from the tumor (figure 3). We previously showed that the amount of stroma (extracellular transport) and the expression of human equilibrative nucleoside transporter (hENT1, the cellular membrane transporter of gemcitabine) influenced gemcitabine incorporation, supporting the idea of multi-scale transport dysregulation in pancreatic cancer. Here, we found that the CT-derived transport properties significantly correlated with the gemcitabine incorporation with or without accounting for hENT1 status (figure 3). We also observed high inter-observer agreement in the correlations between transport properties and gemcitabine DNA incorporation (less than 5% differences in the predicted gemcitabine DNA incorporation at the average value of volumetric AUC normalized by aorta). In figure 3, the transport properties are correlated with gemcitabine DNA incorporation for all patients (graphs in the first column), for patients with low hENT1 score (graphs in the second column), and for patients with high hENT1 score (graphs in the third column). Our definitions for low and high hENT1 score were previously explained [10], and were based on the staining intensity of hENT1 using immunohistochemistry. The correlation between CT-derived mass transport and gemcitabine DNA incorporation was seen on linear regression analysis (figure 3) as well as Spearman rank order correlation. For example, observer 1 had a Spearman correlation of $-0.82$ (95% CI $-0.95$ to $-0.41$, $p = 0.003$) between volumetric AUC (normalized by aorta) and the total average tumor gemcitabine incorporation, a Spearman correlation of $-0.91$ (95% CI $-0.98$ to $-0.67$, $p = 0.0003$) between volumetric AUC (normalized by aorta) and the outer tumor gemcitabine incorporation, and a Spearman correlation of $-0.69$ (95% CI $-0.92$ to $-0.14$, $p = 0.02$) between volumetric AUC (normalized by aorta) and the inner tumor gemcitabine incorporation (see first column of figure 3 for corresponding linear regressions). Similar Spearman rank order correlation coefficients were seen for observers 2 and 3. Significant correlations were also seen after accounting for hENT1 expression (second and third columns of figure 3). Indeed, the highest degree of correlation was seen for the outer portion of the tumor in patients with low hENT1 expression (second column, second row of figure 3).

Figure 3. Multi-scale transport and heterogeneous tumor gemcitabine delivery. The averaged gemcitabine incorporation for the entire tumor (top row of graphs), the outer portion of the tumor (middle row of graphs), and the inner portion of the tumor (bottom row of graphs) are shown. The samples were further divided by hENT1 status, as labeled for each column. Each individual patient has three observer measurements for volumetric AUC ($x$-axis) and only one value of gemcitabine incorporation ($y$-axis). We have previously seen correlations between tumor gemcitabine incorporation and the tumor AUC normalized by the pancreas. Here, we compare the gemcitabine incorporation for the entire tumor and the different portions with the tumor volumetric AUC normalized by the aorta for three observers. (*Denotes the initial two patients who received 500 mg m$^{-2}$. All other patients received 1000 mg m$^{-2}$. Note that these two patients are also represented in the first column of the figure, but are not labeled there to avoid confusion with adjacent patients.)
Discussion

A central goal of clinical oncology is a personalized therapeutic approach to each patient. Achieving this goal for pancreatic cancer faces many challenges. There has been limited clinical progress in this deadly disease for over two decades, especially with individualized approaches. Numerous biomarkers have been discovered in both pre-clinical and clinical studies of pancreatic cancer, but these markers have had limited utility in prognosis or prediction for patients [5, 6]. Furthermore, targeted therapies have been shown to have marginal activity in clinical trials of pancreatic cancer.

To deliver on the promise of personalized approaches in pancreatic cancer, it is important to recognize the sources of variability in response to therapy. Here, we highlight considerable inter-patient and intra-tumoral heterogeneity in the delivery of gemcitabine and in the mass transport properties of pancreatic cancer. This heterogeneity can be reproducibly described with the volumetric mass transport methodology that we describe in this study to analyze the CT scans. This volumetric measurement of mass transport improves on our previously reported method of using multiple regions of interest [10]. These data support the view that mass transport properties could be used for patient stratification and therapeutic guidance in this lethal disease.

In addition to having important clinical implications, our data suggest that physical phenomena interact with biological properties of pancreatic cancer to influence heterogeneous drug delivery, as both the CT-derived properties and hENT1 score influenced our correlations and their degree of accuracy (figure 3). These data underscore the Transport Oncophysics view of multi-scale transport dysregulation being a key feature of cancer: the heterogeneity in drug delivery may be due to local differences in the physical microenvironment (e.g., microvascular density, collagen and stromal content) and expression of cellular transporters of gemcitabine (e.g., hENT1). Our proposed mechanism of heterogeneous drug delivery in human pancreatic cancer is also supported by the clinical observation of heterogeneity in the histology of the specimens. For example, the stromal score varied from 10 to 60% in our 12 patients [10].

Though the clinical relevance of these regional variations in physical properties and drug delivery needs further investigation, it is likely that the variable drug delivery that we observed in pancreatic cancer impacts therapeutic outcome; we have shown through a mechanistic mass transport model of cell kill by chemotherapy that the local physical properties of the tumor can describe pathological response in the immediate vicinity [12]. Furthermore, this mechanistic model supported the idea that response is closely tied to drug delivery: more predicted drug delivery translated to more tumor cell kill. This previous work, combined with our current study, indicate that heterogeneity in the pathological response of tumors is due to variations in the biophysical properties that influence drug delivery across the tumor.

Few studies have assessed drug delivery in human cancers, but the observations from these studies appear to consistently demonstrate substantial heterogeneity. For example, for patients with squamous cell carcinomas of the head and neck, gemcitabine incorporation into the primary tumors demonstrated up to five fold differences at a dose of 300 mg m$^{-2}$ [13]. However, the mechanisms of heterogeneity in the drug delivery were not explored in this Phase I trial. Regional variations in doxorubicin delivery were also shown in breast cancer. There was significant intra-tumoral heterogeneity in delivery within the breast tumors of the patients, with some parts of the tumor not receiving any drug [14].

In the current study, we observed heterogeneity in delivery between patients and within pancreatic tumors. Notably, the Advion measurement of gemcitabine DNA incorporation has high inter-assay precision and accuracy [15], making it unlikely that the variability in gemcitabine DNA incorporation was due to the measurement technique. We acknowledge that our data are limited by the fact that we cannot fully know the true extent of heterogeneity, as the designations of outer and inner tumor were defined only by visual inspection and palpation. Furthermore, we note that the proportion of delivery to the inner portion of the tumors was less in the first two patients (figure 1), who received half the dose of the other patients in accordance with IRB recommendations to ensure the safety of the trial. As noted in figures 1 and 3, both of these initial patients had ‘High’ hENT1 expression and may have worsened our correlations between the CT-derived transport properties and gemcitabine incorporation, especially for the inner portion of the tumor. Excluding these two initial patients yielded significant correlations for the applicable analyses in figure 3 (data not shown). Overall, these data are informative in guiding our future efforts to understand drug delivery in pancreatic cancer. In a follow-up drug delivery trial, we plan to carefully map the physical heterogeneity within tumors using imaging, and these mapped areas will direct sampling of tissue for quantification of drug delivery. We also plan to carefully map the areas of vascularity in the tumors to understand how regional variations in this physical property correlate with drug delivery. Using this technique, we may be able to better assess the extent of heterogeneity in drug delivery and the intrinsic multi-scale biophysical properties of each tumor, enabling the development of a more accurate mathematical model of delivery and response to therapy.

In summary, our data highlight the variability in drug delivery for pancreatic cancer between patients and, for the first time, within their tumors. To describe this variability, we have developed a reproducible method to derive transport properties from CT scans. It is conceivable that the variability in drug delivery is directly related to the clinical observation of variable response to treatment in pancreatic cancer. By combining the principles of mass transport with characterization of the molecular drivers of pancreatic cancer, individualized approaches to patients may be achieved to improve outcomes.
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