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Shear wave elastography can differentiate between radiation-responsive and nonresponsive pancreatic tumors: an *ex vivo* study with murine models

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Abstract

Neither contrast-enhanced computed tomography nor magnetic resonance imaging can monitor changes in pancreatic ductal adenocarcinoma (PDAC) microenvironment during therapy. We hypothesized that shear wave elastography (SWE) could overcome this limitation. To test this hypothesis, we measured the shear modulus of two groups of murine pancreatic tumors (KCKO, $n = 30$; PAN02, $n = 30$) treated with stereotactic body radiation therapy (SBRT). The mean shear modulus of KCKO tumors was 7.651 kPa higher than those of PAN02 tumors ($p < 0.001$). SBRT reduced the shear modulus in KCKO tumors by 8.914 kPa ($p < 0.001$). No significant difference in the shear modulus of SBRT-treated PAN02 tumors was observed. Additionally, necrotic and collagen densities were reduced only in the SBRT-treated KCKO tumors. Shear modulus was dependent on collagen distribution and histological texture parameters (i.e., entropy and fractional dimension). Shear wave elastography imaging differentiate between SBRT responsive tumors (KCKO) and unresponsive ones (PAN02).

Keywords

shear modulus; pancreatic ductal adenocarcinoma; tumor microenvironment; stereotactic body radiation therapy (SBRT)

Introduction

To improve therapeutic decisions, clinicians need more comprehensive information about how a patient is responding to therapy. Pancreatic adenocarcinoma (PDAC) is the third most common cause of cancer deaths in the United States (Siegel, et al. 2017). Surgical resection is the primary treatment for pancreatic cancer patients. However, only 30% of pancreatic

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cancer patients have resectable tumors at diagnosis (Mian, et al. 2014). Chemotherapy and radiotherapy (RT) can downstage the disease and enable surgical resection for patients with borderline resectable tumors (Chuong, et al. 2013, Wei, et al. 2015). However, hypovascularity and hypoxia can decrease therapeutic response (Graham and Unger 2018, Lohse, et al. 2016), a limitation that stereotactic body radiation therapy (SBRT) can overcome (Chuong, et al. 2013, de Geus, et al. 2017, Wei, et al. 2015). SBRT delivers a higher radiation dosage over a shorter time frame that can trigger pancreatic cancer cell apoptosis (de Geus, et al. 2017, Wei, et al. 2015) and change the mechanical properties of the collagen matrix (Qayyum and Insana 2012, Qayyum, et al. 2015); however, dose fraction and dose schedules impact SBRT efficacy (Heitmann and Guckenberger 2018, Moningi, et al. 2015). Therefore, to optimize SBRT, clinicians need to know quickly whether a patient is responding to therapy or not.

Computed tomography (CT), magnetic resonance imaging (MRI) and ultrasound (US) do not monitor changes in the tumor microenvironment adequately. Contrast-enhanced imaging (CT, MRI, and US) provides information about blood vessels, soft tissue geometry, and changes in tumor volumes during therapy (Chen, et al. 2017, Cho, et al. 2018, He, et al. 2017, Zhang, et al. 2018). Although clinicians currently use contrast-based imaging to evaluate tumor response to SBRT (Eisenhauer, et al. 2009), an imaging technique that can detect changes in the tumor microenvironment could prove more effective in detecting SBRT-responsive from SBRT-non-responsive patients. Changes in the PDAC microenvironment modulate its mechanical properties (shear modulus); therefore, elastography could prove useful in detecting SBRT non-responsive patients.

Elastography visualizes the spatial variation of shear modulus within soft tissues, information that researchers have used to differentiate pancreatitis from pancreatic cancer in both animal and clinical studies (Iglesias-Garcia, et al. 2010, Payen, et al. 2016, Xu, et al. 2013). Using shear wave elastography (SWE) and two xenograft pancreatic cancer tumor models, we recently demonstrated that shear modulus is inversely related to drug delivery within the PDAC tumor microenvironment in response to increased collagen density and reduced vascular patency (Wang, et al. 2018). We also demonstrated that shear modulus correlates with collagen and hyaluronan distribution (Wang, et al. 2018, Wang, et al. 2017), the stromal components which increase the pressure within the PDAC tumor microenvironment (Provenzano and Hingorani 2013).

In this work, we hypothesize that SBRT changes collagen density and complexity (Nieskoski, et al. 2017) which modulate the shear modulus of pancreatic cancer tumors. To test this hypothesis, we conducted studies with two murine PDAC tumor lines (KCKO and PAN02) which are known to respond differently to RT (Besmer, et al. 2011, Priebe, et al. 1992). To establish the relationship between shear modulus and tumor burden, we performed bioluminescence imaging. We also evaluated the entropy and fractional attributes of collagen distribution, and the densities of necrosis and collagen contents—information that helped us to understand why SBRT influences shear modulus.

MATERIALS AND METHODS

In this section, we describe murine models, radiation treatment protocol, imaging protocol (SWE and bioluminescence imaging), and histological and textual analyses used to test the hypothesis that SBRT changes collagen density and complexity which modulates the shear modulus of pancreatic cancer tumors. The Animal Care and Use Committee of the University of Rochester approved all animal protocols used in this study (#101759/2016–024).

Tumor lines and implantation

Tumors were implanted in six- to eight-week old female C57BL/6 mice (Jackson Laboratory; Bar Harbor, ME, USA). Two murine pancreatic cancer tumor lines KCKO (n = 30) and PAN02 (n = 30) that express firefly luciferase (KCKO-luc, PAN02-luc) were maintained in DMEM/F-12 (Gibco Laboratories, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and 5% CO₂. Tumor cells (1×10^5) suspended in Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and 50% phosphate-buffered saline solution were injected orthotopically into the pancreatic tail of each animal. We used the IVIS bioluminescent imaging system (PerkinElmer, Inc., Waltham, MA, USA) to monitor the growth of KCKO and PAN02 tumors. All tumors were allowed to grow to 100–250 mm³ to facilitate SWE imaging (SWEI). KCKO tumors were harvested between day 24 and day 29, whereas the more aggressive PAN02 tumors were harvested between day 18 and day 22 (Besmer, et al. 2011, Priebe, et al. 1992).

Stereotactic body radiation therapy

We randomly distributed the sixty tumor-bearing mice (30 KCKO and 30 PAN02) into either an untreated (control) group or a SBRT treatment group. Two 4-mm titanium fiducial markers (Horizon™, Teleflex, Morrisville, NC, USA) were placed adjacent to the tumor bubble to assist in CT-visualized SBRT targeting. We used a small animal radiation research platform (SARRP, Xstrahl Inc., Suwanee, GA, USA) with a 5-mm collimator to irradiate mice in the SBRT treatment group (n = 15, KCKO; n = 15, PAN02) daily from days 6 to 9 post injection with 6 Gy radiation in 4 fractions.

Bioluminescent imaging

We measured the tumor growth noninvasively using the IVIS Spectrum *in vivo* imaging system (PerkinElmer Inc.). D-luciferin (75 mg/kg, Invitrogen) in a 100 ml phosphate-buffered saline vehicle solution was injected subcutaneously into tumor-bearing mice while anesthetized under vaporized isoflurane. The mice were placed in a right lateral recumbent position while a series of bioluminescence images were taken every 2 minutes for a total of 12 images. The peak bioluminescence imaging signal (BLI, photons/sec/cm²/sr) of each tumor was recorded after corroborating signal decay with two sequential BLI signals. We used the IVIS to measure tumor growth from day 3 post-injection, which we performed every other day until the animal was sacrificed.

Shear wave elasticity imaging

All tumors were surgically excised from the mice and encased in gelatin (15.24 cm³) for SWEI as described in Wang *et al.* (Wang, et al. 2018). The gelatin phantom consisted of 10% by weight porcine skin gelatin (300 bloom, Type A, Sigma-Aldrich Corporation, St. Louis, MO, USA), 1% by weight corn starch (Wegmans Supermarket, Rochester, NY, USA), and 18 MΩ high purity water. To visualize the shear modulus distribution, we used single tracking location SWEI, which we implemented on a Verasonics Vantage 256 scanner with a ATL 5 MHz L7–4 linear array transducer (Philips/ATL, Bothell, WA, USA) as described previously (Wang, et al. 2018) and shown in Figure 1. We positioned each gelatin-encapsulated tumor onto a tri-axial translation stage to obtain shear modulus maps throughout the entire tumor at 2-mm intervals. Shear modulus elastograms were generated using a cross-correlation-based time-of-flight algorithm (Ahmed, et al. 2018). After SWEI, we highlighted and recorded the locations of each shear wave scan plane and cut the tumors along the same planes of interest to ensure spatial registration between ultrasound scans and subsequent histological analysis.

Collagen density and texture quantification

Tumors were formalin-fixed and embedded in paraffin before sectioning. Each tumor was sliced in 5 μm increments and stained with Masson's trichrome. Stained tissue sections were imaged at 20X with a DP80 Olympus CCD camera (Olympus, Shinjuku, Tokyo, Japan). We used the process described by Wang *et al.* (Wang, et al. 2018) to quantify the collagen density. All analysis was performed in MATLAB (v2016b, The Mathworks Inc., Natick, MA, USA).

To quantify the stromal heterogeneity, we performed a texture analysis of collagen distribution (Nieskoski, et al. 2017). Entropy H of the collagen distribution I measures irregularity as follows (Jones 2012):

$$H(I) = - \sum_{j=1}^N p_j(I) \log_2(p_j(I)), \quad (1)$$

where $p_j(I)$ represents the j^{th} probability of occurrence calculated from its histogram using a fractional area density analysis (Nieskoski, et al. 2017). We divided the segmented collagen pixels into 100 μm × 100 μm windows and obtained the fractional area density of each window by calculating the local collagen density, as shown in Figure 2.

We used the Hausdorff fractional dimension D (Davnull, et al. 2012, Ng, et al. 2013) to quantify collagen distribution complexity. To accomplish this, we implemented the box-counting method, as shown in Figure 3 (a) to (c), where $N = 2^1, 2^2, 2^3, \dots$ is the number of boxes per dimension (Theiler 1990). With increasing N , we counted the number of boxes containing segmented collagen pixels. The Hausdorff fractional dimension D was estimated from absolute slope of Figure 3 as follows:

$$D = - \frac{\log(\text{box counts})}{\log(\text{number of boxes})}. \quad (2)$$

Necrotic area quantification

Normal tissues are generally stiffer than necrotic areas (Bharat, et al. 2005, Reid, et al. 2017, Varghese, et al. 2002). PAN02 tumors contained necrotic regions (see Figure 4(a–c)). To separate the necrotic cells from fibrotic collagen fibers, we performed texture analysis on the necrotic regions. Sixteen 2D texture energy filters were constructed as described in (Laws 1979). The k^{th} texture energy filter was applied to the input image containing both collagen and necrotic area, producing a filtered image F_k . The texture energy E_k at pixel location (r, c) corresponding to filter k was calculated from the local sum of the filtered image at pixel location (i, j) :

$$E_k[r, c] = \sum_{j=c-7}^{c+7} \sum_{i=r-7}^{r+7} |F_k[i, j]|. \quad (3)$$

The resulting sixteen energy maps were combined to produce the nine final energy maps, including E5L5/L5E5 map that measures the total edge contents of the input image (Figure 4(d–e)). We used the combined energy map with the largest difference in texture energy to mask the necrosis from collagen contents. Manual masking of the necrotic regions was also conducted.

Statistical analysis

We performed a linear regression analysis to determine if there was any correlation between the measured biomarkers (BLI, collagen density, necrosis density, entropy, and fractional dimension) and shear modulus. Statistical significance between different treatment groups and tumor lines was analyzed with a one-way ANOVA using Prism (GraphPad Software, San Diego, CA, USA). We used the following significance levels: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

RESULTS

Shear modulus correlates to the SBRT treatment response measured by in vivo BLI

Figure 5(a) shows the mean BLI signal acquired from KCKO control, KCKO SBRT, PAN02 control, and PAN02 SBRT tumors. The BLI intensity of SBRT-treated KCKO tumor was lower relative to the control group; whereas, the BLI intensity of SBRT-treated PAN02 tumor was similar to the PAN02 control group. To understand the relationship between tumor burden and shear modulus, we assessed the endpoint BLI intensities and shear moduli for both groups of tumors (KCKO and PAN02). This analysis revealed a linear relationship between BLI and shear modulus (Figure 5(b)).

KCKO tumors are stiffer than PAN02 tumors and respond more positively to SBRT

We compared the shear modulus of the KCKO control/SBRT-treated and PAN02 control/SBRT-treated tumors. Figure 6(a–d) shows representative shear modulus elastograms obtained from (a) KCKO control, (b) KCKO SBRT-treated, (c) PAN02 control, and (d) PAN02 SBRT-treated tumors. Figure 6(e) shows a box plot of the shear modulus measured for each treatment group. The mean shear modulus of KCKO control tumors was 7.651 kPa higher than their PAN02 counterparts. However, the mean shear modulus of KCKO tumors

decreased by 8.914 kPa after SBRT ($p < 0.001$). The mean shear modulus of PAN02 tumors were not significantly affected by SBRT ($p = 0.5243$).

Increased collagen density and reduced necrosis lead to increased tissue stiffness in KCKO tumors

We performed a quantitative analysis of the Masson's trichrome-stained images for necrosis and collagen contents. Figure 7(a–b) shows box plots of necrotic density and collagen density for the four groups of tumors. KCKO tumors had less necrosis than PAN02 tumors ($p < 0.0001$). SBRT did not influence the level of necrosis incurred in PAN02 tumors (Figure 7(a)). However, the collagen density of SBRT treated KCKO control tumors was 3.323% lower ($p = 0.0210$) than that of the KCKO control group, and 3.852% higher ($p = 0.0014$) than the collagen density of the PAN02 control tumors (Figure 7(b)). We did not observe any statistical significance in necrosis area density and collagen density between the PAN02 control and PAN02 SBRT treated tumors, which was consistent with the shear modulus results shown in Figure 6(e).

Figure 7(c) shows the necrotic density plotted as a function of shear modulus for the PAN02 tumors. A linear regression analysis indicated that necrosis and shear modulus were inversely related ($R^2 = 0.5391$). Figure 7(d) shows collagen density plotted as a function of shear modulus for the two tumor lines. For both tumors, collagen density and shear modulus were not correlated— R^2 of 0.2338 and 0.2731 for KCKO and PAN02 tumors, respectively.

Increased irregularity and complexity of collagen distribution influences shear modulus—Figure 8(a–b) shows box plots of entropy and fractional dimension as a function of shear modulus. The KCKO control tumors had higher entropy and fractional dimension compared to both the KCKO SBRT-treated and PAN02 control tumors. For PAN02 tumors, neither entropy ($p = 0.4986$) nor fractional dimension ($p = 0.5858$) was influenced by the SBRT therapy.

Entropy and fractional dimension are plotted as a function of shear modulus for both tumor lines in Figure 8(c–d). Linear regression analysis revealed that both features correlate with shear modulus. For entropy, $R^2 = 0.5936$ for KCKO tumors and $R^2 = 0.5497$ for PAN02 tumors. For fractional dimension, $R^2 = 0.5490$ for KCKO tumors and $R^2 = 0.5031$ for PAN02 tumors.

DISCUSSION

In this work, we used two murine pancreatic cancer tumor models (KCKO and PAN02) to assess the feasibility of using SWE to differentiate between SBRT responsive and nonresponsive PDAC. Shear modulus of both tumors correlated with BLI intensity (Figure 5(b)). Untreated PAN02 tumors were 32.4% softer than untreated KCKO tumors and were insensitive to SBRT—shear modulus decreased marginally (Figure 6(e)). KCKO tumors had 12.59% less necrosis and 3.852% more collagen contents than PAN02 tumors (Figure 7(a–b)). The irregularity and complexity of collagen distribution had greater impacts on shear modulus distribution than collagen density (Figure 8).

We demonstrated for the first time that shear modulus can be used to differentiate SBRT-responsive tumors (KCKO) from non-responders (PAN02) (Figure 6). The shear modulus results were consistent with the tumor progression both during and after SBRT measured with BLI (Figure 5). The impact of the number of radiation dosages and fractions on the efficacy of SBRT is an ongoing area of research (Arnold, et al. 2018). Qayyum *et al.* (Qayyum and Insana 2012, Qayyum, et al. 2015) demonstrated that increasing fraction size leads to a modest increase in matrix stiffness in mammary stroma. This implies that translating SWE to the clinical setting should enable better assessment of patient response to SBRT. Therefore, we plan to conduct further work with an endoscopic shear wave system to confirm this expectation.

Neoadjuvant therapies reduce cancer fibroblasts, deplete tumor stroma, and reduce collagen contents in both animals and patients with pancreatic cancer (Li, et al. 2018, Miyashita, et al. 2018, Nagathihalli, et al. 2015). Figure 7(b) demonstrates that the collagen density decreased in the responding KCKO tumors after SBRT, accompanied with reduced tissue stiffness measured with SWEI. This implies that the amount of collagen content in tumor stroma is one of several mechanisms working in concert that influence tissue stiffness. We plan to conduct more detailed investigations to identify other potential mechanisms that contribute to the observed change in shear modulus. Linear regression analysis of the data presented in Figure 7(b) suggests that for the KCKO and PAN02 murine PDAC tumors, collagen density is weakly correlated with shear modulus, inconsistent with our previous measurements for human xenograft PDAC tumor models (Wang, et al. 2018, Wang, et al. 2017) and those reported by Payen *et al.* (Payen, et al. 2016). One potential reason for this discrepancy is that the extent of necrosis in AsPC-1 and BxPc-3 tumor lines were considerably less than those observed in KCKO and PAN02 tumors. Kang *et al.* (Kang, et al. 2008) demonstrated that necrotic endothelial cells showed reduced average shear modulus. Necrosis density was indeed correlated with the shear modulus (Figure 7(c)) for PAN02 tumors with large areas of necrosis. Furthermore, Nieskoski *et al.* (Nieskoski, et al. 2017) measured increased total tissue pressure with elevated collagen complexity (fractional dimension) and irregularity (entropy) with a piezoelectric pressure probe in xenograft PDAC tumors. Similarly, Figure 8 demonstrates that collagen complexity and irregularity measurements had larger linear correlation coefficients than the collagen density did with shear modulus, indicating that the spatial distribution of collagen contents within the extracellular matrix had larger impacts on the tissue stiffness.

The current study has two main limitations. First, performing studies with excised tissue samples ensured excellent registration between shear wave measurements and histological images; however, doing this prevented us from assessing the impact of blood pressure on shear modulus, and monitoring changes in shear modulus during and after SBRT treatment. Second, KCKO tumors showed an immediate and significant reduction in BLI intensity when the last fraction of SBRT dosage was administered, and the tumor burden rebounded on day 15 post-injection. To investigate the effect that this has on shear modulus, we plan to conduct serial SWEI measurements of murine PDAC tumors during SBRT treatment.

CONCLUSION

Shear modulus can differentiate between KCKO tumors that are responsive to SBRT and non-responsive PAN02 tumors. SBRT decreases the shear modulus of responsive tumors. The change in shear modulus correlates with tumor progression and the texture attributes of the pancreatic cancer stroma.

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Figure 1.

Experimental setup and examples of tumor shear modulus, BLI tumor growth, and Masson's trichrome images for representative KCKO and PAN02 murine pancreatic cancer tumors. (a) Verasonics Vantage 256 ultrasound system equipped with an L-7 transducer probe, positioned on top of a tumor enclosing phantom where the transducer scan plane was marked with a dotted blue line. (b) *In vivo* imaging system (PerkinElmer) and representative BLI results for monitoring the tumor growth burden in mice with pancreatic cancer tumors. (c) and (d) are the ultrasound sonogram and shear modulus elastogram for two representative KCKO and PAN02 murine pancreatic cancer tumors, respectively. The (e) KCKO and (f) PAN02 tumors were sectioned along the scan plane and stained for Masson's Trichrome for collagen content analysis.

Figure 2. Illustration for collagen entropy calculation. (a) Segmented collagen fibers from the Masson's trichrome-stained images. The segmented collagen was split into $100\ \mu\text{m}$ by $100\ \mu\text{m}$ windows and the collagen fractional area density was calculated and assembled from each window and shown in (b). (c) Histogram of the collagen fractional area density (%) from which the collagen entropy was calculated.

Figure 3.

The box-cutting method for collagen fractional dimension (D) calculation. (a) to (c) illustrate the box-counting method where N is the number of boxes per dimension. (d) Log (box counts) vs. log (number of boxes). The slope of the XY plot represents the fractional dimension and was calculated from least squares estimation method.

Figure 4. The texture energy measures. (a) Masson's trichrome-stained tumor image with regions of (b) collagen fibers and (c) necrotic tissues with similar color features. The L5E5/E5L5 texture energy maps for the corresponding (d) collagen fibers and (e) necrotic tissues.

Figure 5.

Bioluminescence and shear modulus for KCKO and PAN02 tumors at animal sacrifice. (a) The geometric-mean-bioluminescence signal (p/sec/cm²/sr) for the control- and SBRT-treated KCKO tumors taken over 24 days in comparison to those taken over 17 days for the PAN02 mice. The shear moduli at day 24 and 17 after animal sacrifice were plotted for a representative tumor from each group. (b) The BLI signal for each KCKO and PAN02 tumor plotted against the corresponding shear modulus.

Figure 6.

The shear modulus images of SBRT treated KCKO and PAN02 tumors. (a) and (b) are shear modulus elastograms overlaid on sonograms obtained from representative KCKO control and SBRT-treated tumors, respectively. (c) and (d) are the shear modulus elastograms overlaid on sonograms obtained from representative PAN02 control and SBRT-treated tumors, respectively. (e) Boxplot of the average shear modulus distribution for each tumor line.

Figure 7.

Necrosis area and collagen density analyses of KCKO and PAN02 tumors. (a) and (b) are boxplots of necrosis density (%) and collagen density (%) for KCKO control, KCKO SBRT-treated, PAN02 control and PAN02 SBRT-treated groups (**** represents $p < 0.0001$, ** represents $0.001 < p < 0.01$, * represents $0.01 < p < 0.05$, ns represents $p > 0.05$). The relationship between average shear modulus and (c) necrosis density for PAN02 tumors, and (d) collagen densities for KCKO and PAN02 tumors.

Figure 8.

Entropy and fractional dimension analyses for the KCKO and PAN02 tumors. (a) and (b) boxplots of collagen entropy and fractional dimension for the KCKO control, KCKO SBRT-treated, PAN02 control, and PAN02 SBRT-treated groups. The relationship between shear modulus and (c) collagen distribution entropy and (d) fractional dimension for both groups of tumors.