Elastography Can Map the Local Inverse Relationship between Shear Modulus and Drug Delivery within the Pancreatic Ductal Adenocarcinoma Microenvironment

Hexuan Wang, Reem Mislati, Rifat Ahmed, Phuong Vincent, Solumtochukwu F. Nwabunwanne, Jason R. Gunn, Brian W. Pogue, and Marvin M. Doyley

Abstract

Purpose: High tissue pressure prevents chemotherapeutics from reaching the core of pancreatic tumors. Therefore, targeted therapies have been developed to reduce this pressure. While point probes have shown the effectiveness of these pressure-reducing therapies via single-location estimates, ultrasound elastography is now widely available as an imaging technique to provide real-time spatial maps of shear modulus (tissue stiffness). However, the relationship between shear modulus and the underlying tumor microenvironmental causes of high tissue pressure has not been investigated. In this work, elastography was used to investigate how shear modulus influences drug delivery in situ, and how it correlates with collagen density, hyaluronic acid content, and patent vessel density—features of the tumor microenvironment known to influence tissue pressure.

Introduction

High tissue pressures prevent effective treatment of pancreatic ductal adenocarcinoma (PDA), the most common form of pancreatic cancer, which is a lethal disease with a 5-year survival rate of less than 7% (1). Currently, surgical resection is the most effective treatment, but only 20% of patients have a resectable tumor at diagnosis (2, 3). For patients with borderline resectable tumors, neoadjuvant chemotherapy can sometimes enable downstaging of disease for surgical resection (4, 5), but high tissue pressures (on the order of 10–100 mm Hg) can prevent chemotherapeutic agents from reaching the tumor core (6–8). Leaky vasculature, high stromal density, and high hyaluronic acid content all increase tissue pressure heterogeneously (9–12). Consequently, several groups are now developing targeted therapies to reduce pressure. These therapies include the ablation of hyaluronic acid with PEGPH20 (6, 13–15), losartan (16–18), and stromal targeting (19–21). PEGPH20 in combination with gemcitabine is currently undergoing phase I/II clinical trials. Early results indicate that patients with metastatic PDA suffered no adverse reaction when PEGPH20 was administered in combination with gemcitabine (15, 22). However, choosing the most appropriate pressure-reducing therapies is difficult because no technique can measure tissue pressure in vivo.

Researchers have used invasive pressure catheters (23) to evaluate the effectiveness of different pressure-reducing therapies either with animal models or, to a lesser extent, in the clinic. Current catheters measure tissue pressure by using either the piezoelectric effect or the wick-in-needle method. Piezoelectric catheters measure the stress (solid stress and interstitial fluid pressure) exerted on the active element at the probe’s insertion point, while catheters based on the wick-in-needle method measure the interstitial fluid pressure transmitted through saline solution within the catheter. Provenzano and colleagues (6, 24) used a piezoelectric catheter to measure the interstitial fluid pressure within murine pancreatic tumors and to demonstrate that depleting the tumor microenvironment of hyaluronic acid reduces interstitial fluid pressure. Researchers have also used a wick-in-needle pressure catheter to predict the outcome of patients with cervical cancer (25). Despite these important
Translational Relevance

Surgical resection has prolonged life of a small percentage of patients with pancreatic cancer (<20%). For a subset of patients with borderline resectable tumors, neoadjuvant therapy can work to downstage the tumor and enable surgical resection, but for many patients, high tissue pressure prevents neoadjuvant chemotherapeutics from reaching the tumor interior. Although no imaging technique can measure tissue pressure, ultrasound elastography can measure shear modulus, an excellent surrogate for tissue pressure. Understanding the relationship between shear modulus and features of the tumor microenvironment (collagen density, hyaluronic acid content, and patent vessel density) known to impact tissue pressure and drug delivery would allow us to understand how well elastography can monitor changes in the tumor microenvironment of pancreatic patients undergoing systemic therapies.

Preclinical and clinical results, the invasive nature of pressure catheters and their inability to measure the spatial variation of tissue pressure (23, 26) have limited their clinical use. Elastography could overcome these limitations by providing a surrogate measure to tissue pressure. Elastography uses a three-step process to visualize shear modulus, an intrinsic property of soft tissues that is defined as the ratio of shear stress to shear strain. First, motion is induced within the tissue using either an external or internal mechanical stimulation. Second, ultrasound measures the spatial variation of the mechanical response. Third, shear modulus is inferred by applying either a simplified or continuum mechanical model to the measured mechanical response (27). Although elastography cannot measure tissue pressure directly, the steep pressure gradient at the tumor margin exerts mechanical stress on the extracellular matrix (ECM) and the stromal cells, which in turn increases shear modulus (28). Our long-term goal is to establish that shear modulus distribution measured noninvasively and in real-time with elastography is a good alternative to tissue pressure measurements.

The goal of this work was to use elastography, mapping point by point within spatially registered images, to assess (i) the relationship between shear modulus and drug delivery in PDA, and (ii) the relationship between shear modulus and patent vessel density, hyaluronic acid content, and collagen density. To achieve these goals, we used mice bearing two very different phenotypic PDA tumor models, AsPC-1 (n = 25) and BxPC-3 (n = 25), imaging drug uptake from verteporfin, a fluorescent drug FDA-approved for human use, and then mapping the same tumors for shear modulus. AsPC-1 tumors grow much faster and have higher chemotherapy drug uptake (29) than their BxPC-3 counterparts. Both groups of tumors were derived from human PDA. Specifically, we obtained BxPC-3 cells from the primary tumor and AsPC-1 cells from ascites of a metastatic site.

Materials and Methods

This section describes the tumor models, imaging protocols (shear wave elasticity imaging and fluorescence), and histologic and statistical analysis used in this study. The Animal Care and Use Committees of the University of Rochester and Dartmouth College approved all animal protocols, and the procedures used here followed these approved protocols (#101759/2016-024).

Tumor models

A total of 25 AsPC-1 (ATCC, catalog no. CRL-1682) and 25 BxPC-3 (ATCC, catalog no. CRL-1687) tumors were grown orthotopically in the pancreas of female nude mice (4-months old, 225 g, athymic nude mice, Charles Rivers Laboratories). We injected $10^6$ tumor cells in Matrigel (BD Biosciences) and 50% media orthotopically in the pancreas of each animal. All tumors were allowed to grow until their volume was between 100 and 200 mm$^3$. AsPC-1 tumors reached the desired volume range within 4–6 weeks; however, BxPC-3 tumors took 10 weeks to reach the same volume (30, 31).

Shear wave elasticity imaging

We used the single-track-location–shear wave elastographic imaging (STL-SWEI) method to visualize the shear modulus distribution of excised tumors, which we recently implemented on a Vantage 64 Ultrasound Scanner (Verasonics Inc.) equipped with an L7-4 (Phillips Healthcare) linear transducer array (32). During STL-SWEI, we used ultrasound beams to both induce and track shear wave propagation in tissues. We estimated the speed of shear waves produced with a pair of laterally spaced push beams at a fixed location with a single-tracking beam. To create an image, we transmit several pairs of push beams within the tissue. We then combine local tissue density ($\rho$) with shear wave speed (SWS) to get regional shear modulus estimates ($\mu = \rho \times \text{SWS}^2$). All tumors were surgically removed and encased in a 216-mm$^3$ gelatin block as described previously (33) to minimize the impact of respiration and cardiac motion on shear wave estimates (see Supplementary Fig. S1A and S1B). Each gelatin block consisted of 10% by weight porcine skin gelatin (300 bloom, Type A, Sigma-Aldrich Corporation), 1% by weight corn starch (Sigma-Aldrich Corporation), and 89% by weight high purity water (18 MΩ). All SWEI was performed using 5 MHz pushing and tracking beams. The focal lengths of both beams (pushing and tracking) were placed at the center of each tumor, which corresponded to scan depths ranging from 20 to 30 mm depending on the tumor volume. A 400-µs pulse was used to induce shear waves in the gelatin-encapsulated tumors, at a pulse repetition frequency of 7 kHz. For each transmitted pulse, we acquired multiple tracking A-lines (a lateral line density of ~4 beams/mm) from a 2-cm wide region of interest (ROI). For each cancer, shear wave elastographic images were acquired from multiple cross-sections (n = 10), corresponding to slice intervals of approximately 2 mm (see Supplementary Fig. S2). To minimize registration errors between the shear wave and histologic images, the central scan plane was marked as illustrated in Supplementary Fig. S1C–S1F.

Fluorescence imaging

To assess how shear modulus relates to drug delivery, an FDA-approved fluorescent drug was used (1 mg/kg verteporfin, MW 719 DA; Sigma-Aldrich) for intravenous injection into each mouse via the tail vein, 1 hour before sacrifice (34–36). To determine the relationship between blood vessel density and drug uptake, 1 mg/kg of lectin (Vector Laboratories,
catalog no. FL-1211), a fluorescence stain, was injected intravenously into all tumor-bearing mice 1 minute prior to sacrifice. After tissue prep and sectioning, slices of tissue were scanned on a flatbed scanner (GE Typhoon 9410, GE Healthcare) and a PerkinElmer Vectra 3.0 fluorescence slide scanner to acquire verteporfin and lectin fluorescence images.

**Histologic analysis**

To quantify collagen density, patent vessel density, and fluorescence uptake, quantitative histology was calculated from all excised tumors. All tumors were formalin-fixed and embedded in paraffin for thin sectioning (2 μm) and stained with Masson trichrome stain, hyaluronic acid–binding protein (HABP-1; ref. 37), and lectin, after imaging. The fluorescence slide scanner was used to image each section, and a three-step color segmentation process (see Supplementary Fig. S3) was implemented in MATLAB (v2016b, The Mathworks Inc.) to estimate collagen density from the Masson trichrome stained images (26). Collagen density (%) was calculated by dividing the number of blue collagen pixels by the total number of pixels in the tumor. We used a similar analysis to quantify hyaluronic acid density (%).

A global thresholding algorithm was used to isolate pixels stained with lectin fluorescence images, and so the patent vessel density (%) was estimated by dividing the number of lectin-stained pixels by the total number of pixels in the tumor. Verteporfin perfusion was estimated from the fluorescence maps, by defining ROI within the fluorescence images and computing the mean fluorescence intensities within these.

**Statistical analysis**

We used a linear regression analysis to determine the correlation between the measured biomarkers (e.g., collagen density, patent vessel density, hyaluronic acid content, drug uptake, and shear modulus). Statistical significance was determined from the differences in measured biomarkers of each tumor type using a Welch t test. All statistical analysis was performed with Prism v7.0 for Mac (GraphPad).

**Results**

**Drug uptake is lower in stiffer tumors than softer ones**

To understand how shear modulus affects drug uptake, the fluorescence intensity of verteporfin was measured and compared with the mean shear modulus of the two groups of excised tumors (AsPC-1 and BxPC-3). We performed a quantitative analysis of Masson’s trichrome and lectin-stained histologic images to quantify differences in collagen and patent vessel density, factors known to impact drug delivery, and tissue pressure.

Figure 1 shows box plots of fluorescence intensity (verteporfin uptake), shear modulus, collagen density, and patent vessel density for the two groups of tumors. Fluorescence intensity in AsPC-1 tumors was three times higher than that measured in BxPC-3 tumors (Fig. 1A). The mean shear modulus of AsPC-1 tumors was 36.07 kPa lower than their counterparts (Fig. 1B). Univariate ANOVA revealed that this difference was significant ($P = 0.0008$; $F$-number = 11.87). ANOVA also revealed a within-group variance of 7.46 kPa and 7.35 kPa for AsPC-1 and BxPC-3 tumors, respectively. The observed difference in the shear modulus reflects differences in the total tissue pressure (solid stress and interstitial fluid pressure). The observed differences in shear modulus and
fluorescence intensity were statistically significant ($P < 0.0001$ and $P < 0.001$). BxPC-3 tumors had higher collagen density (Figs. 1C and 2). AsPC-1 tumors had higher patent vessel density than the BxPC-3 tumors (Fig. 1D).

**Patent vessel density is linearly related to drug delivery and inversely related to shear modulus**

To understand the relationship between patent vessel density to both drug uptake and shear modulus, we assessed the mean properties (shear modulus, patent vessel density, and fluorescence intensity) measured for each tumor. This analysis revealed that patent vessel density and verteporfin uptake were linearly related (Fig. 3A). In addition, there was a strong inverse correlation between patent vessel density and shear modulus (Fig. 3B). This suggests that the high shear modulus of the ECM, which compresses tumor vasculature, is the primary mechanism that impedes drug delivery.

**Shear modulus and drug delivery are inversely related**

Figure 4A–D shows representative shear modulus elastograms and verteporfin uptake maps obtained from AsPC-1 and BxPC-3 tumors. For both tumor groups, shear modulus was heterogeneously distributed, which is consistent with our previous reported results (33). Fluorescence intensity was also heterogeneously distributed, which suggests that drug uptake in the tumors varied spatially (Fig. 4C and D). Localized regions of high fluorescence intensity corresponded to localized region of low shear modulus; whereas, regions with low fluorescence intensities corresponded to high shear modulus. Further analysis revealed that shear modulus and verteporfin fluorescence intensity were inversely related (Fig. 4E). The fluorescence intensities of AsPC-1 tumors were consistently higher than those obtained from BxPC-3 tumors, a trend that was consistent with our previous study performed with a pressure probe (26).

**Hyaluronic acid density is weakly associated with shear modulus**

The pancreatic cancer tumor microenvironment often contains high levels of hyaluronic acid, which increases interstitial fluid pressure (9–12, 38). For both groups of tumors, hyaluronic acid density was lower than collagen density (Fig. 5B and C) but was distributed heterogeneously throughout the tumors. Global assessment of hyaluronic acid density revealed no significant difference in hyaluronic acid between the two groups of tumors or any correlation between hyaluronic acid density and shear modulus (Fig. 5E). Localized assessment of the tumors...
identifying areas of different hyaluronic acid density on the HABP-1–stained images and relating these to the corresponding areas in registered shear modulus elastograms) revealed a weak correlation between hyaluronic acid density and shear modulus (Fig. 5F).

**Discussion**

In this work, imaging of elastography and drug fluorescence within two xenograft pancreatic cancer tumor models [AsPC-1 and BxPC-3] was used to study how the shear modulus correlates with drug delivery, patent vessel density, collagen density, and hyaluronic acid content. AsPC-1 tumors were perfused with more fluorescent verteporfin than their BxPC-3 counterparts (Fig. 1A) due to differences in tissue pressure that were measured indirectly using shear modulus (Fig. 1B). Solid stress and interstitial fluid pressure both influence tissue pressure; therefore, to understand which of these two sources are responsible for the observed difference in shear modulus, we measured collagen density and hyaluronic acid content of all tumors. The BxPC-3 tumors had higher collagen density than AsPC-1, but their hyaluronic acid content was similar. Stiffer tumors were more collagen-rich than softer ones (Fig. 2). Drug uptake increased linearly with patent vessel density (Fig. 3A) but decreased linearly with increasing shear modulus (Fig. 3B). Patent vessel density was inversely related to shear modulus, suggesting that increasing total tissue pressure may cause vessels to collapse and impede drug delivery (Fig. 5).

Clinical trends predict that pancreatic cancer will be the second leading cause of all cancer-related deaths in the United States (39). Consequently, developing therapies for either treating the disease or allowing more patients to qualify for surgery is an area of active research (4, 5). High tissue pressure can impede the delivery of therapeutic agents and encourage tumor progression (40, 41).
Tissue pressure is difficult to measure with currently available invasive pressure catheters. No imaging technique can measure tissue pressure in vivo today, but we have previously demonstrated that shear modulus is a good surrogate. Using a modified invasive pressure catheter, it was shown that drug delivery in both AsPC-1 and BxPC-3 tumors was inversely related to pressure, which is consistent with Fig. 3. Our current work is the first demonstration that shear modulus imaging correlates with drug uptake. Specifically, drug delivery is inversely related to shear modulus, and hence tissue pressure. This implies that imaging could be used to evaluate how well a given therapy reduces shear modulus, and this information could serve as a surrogate for drug penetration.

Preclinical studies demonstrate that depleting the tumor microenvironment of hyaluronic acid lowers interstitial fluid pressure, restores vasculature, and improves permeability to chemotherapeutics (42, 43). Figure 5E demonstrates that globally there is no correlation between hyaluronic acid and shear modulus. This would imply that SWEI could prove to be an ineffective method for monitoring the efficacy of hyaluronic acid ablation. Although consistent with the results reported in ref. 44, it contradicts our previous results that showed that shear modulus correlated with hyaluronic acid content. The discrepancy in the current results and our previous results is shown in Fig. 5F and is due to differences in the analysis in the two reports. Specifically, in (33), local analysis of hyaluronic acid and shear modulus was performed, whereas in our current work the analysis was performed globally. Hyaluronic acid concentrates in localized regions within many cancerous tissues. Thus, when hyaluronic acid absorbs water and swells, it exerts pressure on the collagen-rich ECM that produces an equal but opposing force in the localized region. The magnitude of the resulting pressure depends on the amount of water absorbed and the shear modulus of the ECM (i.e., the higher the shear modulus, the higher the localized pressure) as we have demonstrated previously (33). We expect the global stiffness of the ECM will increase when the number of hyaluronic acid pockets exceeds a critical value or when the localized hyaluronic acid pockets are located in areas of abnormally high collagen density. We observed neither of these phenomena when HABP-1 stain histologic slides were analyzed; therefore, the observation that global estimates of shear modulus and hyaluronic acid density (Fig. 5E) were not related. Figure 5F revealed a weak but significant correlation between the local estimates of hyaluronic acid and shear modulus. However, because of differences in the frequency of the transducers used (10 MHz vs. 5 MHz), there was higher variability in measured shear modulus. Therefore, higher frequency probes and methods such as plane-wave-single-track location elastography (32).
stiffness are dependent on collagen density, this was not necessarily the case for hyaluronic acid. Specially, a strong correlation was shown between the local estimate of shear modulus and hyaluronic acid content for the two groups for tumors, but global estimation showed no such correlation. This suggests that elastography could, in principle, monitor local changes in tissue pressure during antistromal therapy and hyaluronic acid depletion, but monitoring hyaluronic acid depletion could prove more challenging because this will require local assessment whose accuracy will depend on the resolution of the resulting elastograms.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H. Wang, B.W. Pogue, M.M. Doyley
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Wang, R. Mislati, R. Ahmed, S.F. Nwabunwanne, J.R. Gunn, B.W. Pogue
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Wang, R. Mislati, P. Vincent, B.W. Pogue
Writing, review, and/or revision of the manuscript: J.R. Gunn
Study supervision: B.W. Pogue, M.M. Doyley
Other (contributed in the experiments for the study): S.F. Nwabunwanne

Acknowledgments
This work is supported by NIH grants R56EB024320 and P01CA084203.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement
in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 16, 2018; revised October 5, 2018; accepted October 19, 2018; published first October 23, 2018.

References
16. Losartan and Nivolumab in Combination with FOLFIRINOX and SRT1 in Localized Pancreatic Cancer; [about 3 screens]. Available from: https://ClinicalTrials.gov/show/NCT03563248.
17. Proton w/FOLFIRINOX-Losartan for Pancreatic Cancer; [about 3 screens]. Available from: https://ClinicalTrials.gov/show/NCT01821729.

Downloaded from clincancerres.aacrjournals.org on April 9, 2020, © 2019 American Association for Cancer Research.