Investigating the Contribution of Collagen to the Tumor Biomechanical Phenotype with Noninvasive Magnetic Resonance Elastography

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Abstract

Increased stiffness in the extracellular matrix (ECM) contributes to tumor progression and metastasis. Therefore, stromal modulating therapies and accompanying biomarkers are being developed to target ECM stiffness. Magnetic resonance (MR) elastography can noninvasively and quantitatively map the viscoelastic properties of tumors in vivo and thus has clear clinical applications. Herein, we used MR elastography, coupled with computational histopathology, to interrogate the contribution of collagen to the tumor biomechanical phenotype and to evaluate its sensitivity to collagenase-induced stromal modulation. Elasticity (\( G_d \)) and viscosity (\( G_l \)) were significantly greater for orthotopic BT-474 (\( G_d = 5.9 \pm 0.2 \) kPa, \( G_l = 4.7 \pm 0.2 \) kPa, \( n = 7 \)) and luc-MDA-MB-231-LM2-4 (\( G_d = 7.9 \pm 0.4 \) kPa, \( G_l = 6.0 \pm 0.2 \) kPa, \( n = 6 \)) breast cancer xenografts, and luc-PANC1 (\( G_d = 6.9 \pm 0.3 \) kPa, \( G_l = 6.2 \pm 0.2 \) kPa, \( n = 7 \)) pancreatic cancer xenografts, compared with tumors associated with the nervous system, including GTM1/Tp53\textsuperscript{R172H} medulloblastoma (\( G_d = 3.5 \pm 0.2 \) kPa, \( G_l = 2.3 \pm 0.2 \) kPa, \( n = 7 \)), orthotopic luc-D-212-MG (\( G_d = 3.5 \pm 0.2 \) kPa, \( G_l = 2.3 \pm 0.2 \) kPa, \( n = 7 \)), luc-RG2 (\( G_d = 3.5 \pm 0.2 \) kPa, \( G_l = 2.3 \pm 0.2 \) kPa, \( n = 5 \)), and luc-U-87-MG (\( G_d = 3.5 \pm 0.2 \) kPa, \( G_l = 2.3 \pm 0.2 \) kPa, \( n = 8 \)) glioblastoma xenografts, intracranially propagated luc-MDA-MB-231-LM2-4 (\( G_d = 3.7 \pm 0.2 \) kPa, \( G_l = 2.2 \pm 0.1 \) kPa, \( n = 7 \)) breast cancer xenografts, and Th-MYCN neuroblastomas (\( G_d = 3.5 \pm 0.2 \) kPa, \( G_l = 2.3 \pm 0.2 \) kPa, \( n = 5 \)). Positive correlations between both elasticity (\( r = 0.72, P < 0.0001 \)) and viscosity (\( r = 0.78, P < 0.0001 \)) were determined with collagen fraction, but not with cellular or vascular density. Treatment with collagenase significantly reduced \( G_d (P = 0.002) \) and \( G_l (P = 0.0006) \) in orthotopic breast tumors. Texture analysis of extracted images of picrosirius red staining revealed significant negative correlations of entropy with \( G_d (r = 0.69, P < 0.0001) \) and \( G_l (r = -0.76, P < 0.0001) \), and positive correlations of fractal dimension with \( G_d (r = 0.75, P < 0.0001) \) and \( G_l (r = 0.78, P < 0.0001) \). MR elastography can thus provide sensitive imaging biomarkers of tumor collagen deposition and its therapeutic modulation.

Significance: MR elastography enables noninvasive detection of tumor stiffness and will aid in the development of ECM-targeting therapies.

Introduction

Aberrant transitional homeostasis and increased stiffness are hallmarks of cancer. The origin of elevated tumor stiffness is not fully understood, but may often reflect increased mechanical stress associated with rapid tissue expansion and compressed vasculature and lymphatics, and extracellular matrix (ECM) rigidity. There is much evidence showing that increased tissue stiffness contributes to malignant transformation, tumor progression and metastasis (1, 2). The elevated solid stress and interstitial fluid pressure (IFP) that may drive increased tumor stiffness are also two major obstacles to efficient tumor drug delivery (3).

Both breast and pancreatic cancers are characterized by excessive desmoplastic stromal reaction and dense ECM (4, 5). Collagen, the principal component of the fibrillar protein network within the ECM, is the major contributor to ECM stiffening, is
strongly implicated in tumor evolution and progression, and is associated with poor patient prognosis (6–9). Increased collagen deposition and enhanced matrix cross-linking occurs with progressive structural remodeling of the ECM scaffold, which facilitates tumor growth (9). Suppression of collagen synthesis inhibits tumor growth and metastasis, and enhances drug penetration (10).

Significant efforts are currently focused on targeting tumor ECM stiffness for therapeutic gain (2, 4, 11–13). The development of stromal modulating therapies would benefit from noninvasive imaging biomarkers to inform on changes associated with therapeutic efficacy. A number of innovative magnetic resonance (MR) and ultrasound (US) imaging techniques are being exploited to image the viscoelastic and other mechanical properties of tissue in vivo (14–17). One approach, MR elastography, is being used to visualize and measure tissue elasticity and viscosity in vivo. MR elastography yields quantitative images, and therefore imaging biomarkers, that map the absolute value of the complex shear modulus $G'$ in terms of its two components, the elasticity modulus $G_E$ (a measure of the ability of an object to resume a normal shape after being stretched or compressed) and the viscosity modulus $G_I$ (a measure of resistance to gradual deformation by shear or tensile stress). We and others have demonstrated, in preclinical tumor models and cancer patients in vivo, the potential of the MR elastography-derived mechanical phenotype to inform on the underlying tumor microstructure and treatment-induced changes to its integrity (18–25).

Early imaging biomarker development demands close imaging-pathology correlation to understand the biological processes underpinning the imaging measurement, which can be meaningfully studied using animal models (26–28). The systematic evaluation of tumor stromal components and their contribution to tissue stiffness as measured by MR elastography is in its infancy. This study describes the use of MR elastography, coupled with computational histopathology, to interrogate the contribution of the collagen network to the biomechanical phenotype imaged in vivo in a wide range of orthotopically-propagated and spontaneously arising transgenic models with disparate pathologies. The sensitivity of MR elastography to monitor stromal modulation in vivo following administration of collagenase is also demonstrated.

## Materials and Methods

### Cells

All cell lines used in this study tested negative for Mycoplasma infection at the time of tumor implantation and human cells were authenticated by short tandem repeat (STR) profiling. Their origins, provenance, and culture conditions are summarized in Supplementary Tables S1 and S2.

### Animals and tumor models

All animal experiments were approved by the Institute of Cancer Research Animal Welfare and Ethical Review Body, and performed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986, the United Kingdom National Cancer Research Institute guidelines for the welfare of animals in cancer research (29), and reported according to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines (30).

#### Intracranial tumor propagation

Human luc-U-87 MG glioblastoma (5 x 10^5), rat luc-RG2 glioma (5 x 10^5), human luc-D-212 MG pediatric hemispheric giant-cell glioblastoma (5 x 10^5), or human metastatic luc-MDA-MB-231 LM2-4 breast cancer (5 x 10^5) cells were implanted supratentorially in the brains of adult female athymic NCr-Foxn1<sup>nu</sup> mice (Charles River) as described previously (19). Animals were anesthetized using 1% to 2% isoflurane in oxygen (1 l/min). A ~1 cm incision was made in the skin on the top of the head, and a 1 mm hole drilled using a surgical bone microdrill (Harvard Apparatus). Cell suspension (5 µL) was then injected at a depth of 3 mm from the dura, at a rate of 2 µL/minute, using a 10 µL syringe (VWR International) and a nanomite syringe pump (Harvard Apparatus). The needle was removed 3 minutes after completion of the injection and the skin repaired with VetBond Tissue Adhesive (3M Animal Care Products).

Tumor establishment and growth were monitored with bioluminescence imaging (BLI) using a Xenogen IVIS 200 system.

### Table 1. Summary of the in vivo models

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Characteristics</th>
<th>Study</th>
<th>Injection route</th>
<th>Cells injected</th>
<th>$n$</th>
<th>Tumor volume (mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>luc-MDA-MB-231 LM2-4</td>
<td>Highly malignant human triple-negative breast cancer cells isolated from a lung metastasis</td>
<td>MRE-histology correlation</td>
<td>i.c.</td>
<td>5 x 10^3</td>
<td>5</td>
<td>29 ± 8</td>
</tr>
<tr>
<td>luc-MDA-MB-231 LM2-4</td>
<td>MRE-histology correlation</td>
<td>o.t.</td>
<td>2 x 10^6</td>
<td>6</td>
<td>406 ± 47</td>
<td></td>
</tr>
<tr>
<td>luc-RG2</td>
<td>Rat glioma cells</td>
<td>MRE-histology correlation</td>
<td>o.t.</td>
<td>5 x 10^3</td>
<td>5</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>luc-U-87 MG</td>
<td>Human glioblastoma cells</td>
<td>MRE-histology correlation</td>
<td>i.c.</td>
<td>5 x 10^5</td>
<td>8</td>
<td>26 ± 4</td>
</tr>
<tr>
<td>luc-D-212 MG</td>
<td>Derived from a pediatric hemispheric giant-cell glioblastoma</td>
<td>MRE-histology correlation</td>
<td>i.c.</td>
<td>3 x 10^5</td>
<td>6</td>
<td>616 ± 14</td>
</tr>
<tr>
<td>luc-D-212 MG</td>
<td>MRE-histology correlation</td>
<td>s.c.</td>
<td>1.5 x 10^5</td>
<td>7</td>
<td>12 ± 3</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Highly malignant human triple negative breast cancer cells</td>
<td>Collagenase treatment</td>
<td>o.t.</td>
<td>2 x 10^6</td>
<td>14</td>
<td>585 ± 49</td>
</tr>
<tr>
<td>BT-474</td>
<td>Invasive ductal breast carcinoma cells</td>
<td>Collagenase treatment</td>
<td>o.t.</td>
<td>5 x 10^6</td>
<td>7</td>
<td>438 ± 36</td>
</tr>
<tr>
<td>luc-PANC-1</td>
<td>Pancreatic ductal epithelial carcinoma cells</td>
<td>Collagenase treatment</td>
<td>o.t.</td>
<td>5 x 10^6</td>
<td>7</td>
<td>287 ± 51</td>
</tr>
<tr>
<td>Transgenic mouse models</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th-MYC</td>
<td>High-risk neuroblastoma</td>
<td>MRE-histology correlation</td>
<td>Spontaneously arising tumors</td>
<td>5</td>
<td>1369 ± 254</td>
<td></td>
</tr>
<tr>
<td>GTML/Thp52&lt;sup&gt;sh&lt;/sup&gt;</td>
<td>Medulloblastoma</td>
<td>MRE-histology correlation</td>
<td>Spontaneously arising tumors</td>
<td>7</td>
<td>27 ± 4</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: i.c., intracranial; o.t., orthotopic; s.c., subcutaneous.

*Tumor volumes at the time of the MR elastography experiment and determined using segmentation from regions of interest drawn on each tumor-containing T2-weighted MRI slice.*
coupled with LivingImage software (Caliper Life Sciences). Lucif\-erin (150 mg/kg; Caliper Life Sciences) was administered intra- peritoneally 10 minutes before imaging. MR elastography was performed when the BLI photon flux reached a threshold value previously determined to represent a tumor of approximately 30 to 40 mm$^3$, a volume considered of sufficient size to acquire MR elastography data but not large enough to cause neurologic effects in the mice. The average time from implantation to imaging was 18 days for the luc-U-87 MG and luc-MDA-MB-231 LM2-4 tumors, 22 days for the luc-RG2 tumors, and 45 days for the luc-D-212 MG glioblastomas.

**GTML/Trp53$^{K^{1/l}}$ transgenic model of medulloblastoma.** The generation of the GTML/Trp53$^{K^{1/l}}$ mouse has been reported previously (31). Mice were genotyped to detect the presence of human MYCN and Trp53 transgenes. Male and female mice were monitored twice weekly for the development of a BLI signal from the midbrain. MR elastography was performed when the photon flux reached a threshold value previously determined to represent a tumor of approximately 20 to 30 mm$^3$.

**Th-F-MYC transgenic model of neuroblastoma.** Transgenic Th-F-MYC mice were genotyped to detect the presence of the human MYCN transgene (27). Both male and female hemizygous mice were used, which spontaneously developed palpable abdominal tumors between 50 and 130 days with a 25% penetrance. Tumor progression was monitored weekly by palpation by an experienced technician until the tumor reached a diameter greater than ~5 mm, at which point they underwent MR elastography.

**Subcutaneous U87-MG xenografts.** Adult female NCr-Foxn1$^{TM}$ mice were injected subcutaneously in the flank with 2 $\times$ 10$^6$ luc-U-87 MG cells. Tumor development was monitored weekly by caliper measurements, and MR elastography performed when tumors reached a diameter of ~7 mm.

**Orthotopic models of breast and pancreatic cancer.** Human luc-MDA-MB-231 LM2-4 or BT-474 breast cancer cells (5 $\times$ 10$^6$) were injected into the third abdominal fat pad of adult female NCr- Foxn1$^{TM}$ mice [100 $\mu$L cell suspension in PBS and Matrigel (1:1)]. A 17$\beta$-estradiol pellet (60-day release; Innovative Research of America) was implanted in the neck nape 1 day before implantation of BT-474 cells. Tumor development was monitored weekly by caliper measurements, and MR elastography performed when tumors reached a diameter of ~7 mm.

For the orthotopic propagation of pancreatic cancer xenografts, a small incision was made on the left flank of adult female athymic CD1-Foxn1$^{TM}$ mice (Charles River) through the skin and peritoneum, the pancreas exteriorized, and human luc-Panc-1 [1 $\times$ 10$^7$ cells in suspension in PBS and Matrigel (1:1)] injected using a Hamilton syringe. The pancreas was then returned into the abdominal cavity and the incision sutured. Successful engraftment and tumor progression were confirmed using BLI, and MR elastography performed ~30 days postimplantation.

**Response to collagenase**

Orthotopic parental MDA-MB-231 and BT-474 breast cancer xenografts were propagated as described previously. MR elastography was performed on established tumors 24 hours prior to and 5 hours after intravenous treatment with either collagenase (62 U/kg, bacterial collagenase from *Clostridium histolyticum*, Sigma-Aldrich) or saline.

**MRI and MR elastography data acquisition and analysis**

MRI was performed on a 7T horizontal bore MicroImaging system (Bruker) using a 3 cm birdcage volume coil. Tumor-bearing mice were anesthetized with a 10 mL/kg intraperitoneal injection of fentanyl citrate (0.315 mg/mL) plus fluanisone (10 mg/mL; Hypnorm; Janssen Pharmaceutical Ltd.), midazolam (5 mg/mL; Hypnovel; Roche), and sterile water (used at a ratio of 1:1:2). The mouse core temperature was maintained at 37°C with warm air blown through the magnet bore.

Anatomical T$_2$-weighted images [using a rapid acquisition with refocused echoes (RARE) sequence, with TE = 36 milliseconds, TR = 4.5 seconds, RARE factor = 8, 40 contiguous 1-mm-thick transverse slices, 1 average, matrix size 128 $\times$ 128 over a 3 $\times$ 3 cm field of view (FOV)] were used to localize and determine the tumor volume, plan the MR elastography acquisition, and optimize the local field homogeneity over the region of interest (ROI) using the FASTMAP algorithm.

MR elastography was performed as described previously (18, 19). The mechanical vibrations, generated by an electromagnetic shaker (Brüel & Kjaer), were transmitted through a flexible nylon rod to either (i) a square piston with a concave curved face positioned on the mouse head for intracranial tumors, or directly on the skin over subcutaneous luc-U87 MG tumors and orthotopically propagated breast cancer xenografts, or (ii) a round flat-faced piston placed on the abdomen above a palpated tumor (Th-F-MYC or orthotopic pancreatic tumor), all positioned within the volume coil at the isocenter of the magnetic field. MR elastography was performed using mechanical excitations at a vibration frequency of 1,000 Hz, exciting the shaker with a voltage that generated mechanical waves inside the tumor with amplitude greater than 0.5 $\mu$m. A 2D spin-echo sequence incorporating sinusoidal motion-sensitizing gradients synchronized to the mechanical excitation was used. Data were acquired in three orthogonal directions from 10 contiguous transverse slices (300 $\mu$m thick), using 2 averages of 64 phase encoding steps over a 1.92 $\times$ 1.92 cm FOV, with TE = 27 milliseconds, TR = 1001 milliseconds, and 8 time sampling steps, giving an isotropic spatial sampling of 300 $\times$ 300 $\times$ 300 $\mu$m of the mechanical wave propagation displacement inside the tumor. The total acquisition time was ~51 minutes. Finally, high-resolution T$_2$-weighted RARE images were acquired from the same 10 contiguous transverse slices (TE = 36 milliseconds, TR = 4.5 seconds, RARE factor = 8, 300 $\mu$m thick, 10 averages, matrix size 128 $\times$ 128 over a 1.92 $\times$ 1.92 cm FOV).

**Image reconstruction and analysis.** Parametric maps of the absolute value of the complex shear modulus $|G|^*$, elasticity $G_0$ and viscosity $G_1$ (where $|G|^* = G_0 + iG_1$) were reconstructed using in-house software from the 3D displacement vector measured as described previously, and using the following equation (32):

$$-\rho \omega^2 \vec{q} = G^* \nabla^2 \vec{q}, \quad \nabla = \nabla \times \vec{u} \in C^3,$$

where $\vec{q}$ is the complex-valued curl of the measured displacement field $\vec{u}$, $\rho$ is the density of the material, and $\omega$ is the angular frequency. For each slice, $G_0$ and $G_1$ (kPa) were determined pixelwise from an ROI covering the whole tumor delineated from the high resolution T$_2$-weighted images.
Computational histopathology

Tissue preparation. Guided by the $T_2$-weighted MR images, tumors were carefully excised and oriented for subsequent histopathologic processing. Adjacent formalin-fixed paraffin embedded sections (3 $\mu$m) were cut and tinctorially stained with picrosirius red (for collagen I and III), hematoxylin and eosin (H&E, for cellularity), or immunohistochemically processed for detection of the murine vascular endothelial marker Cd31 (rabbit EP13095; Millipore), using diaminobenzidine (DAB) as the chromogen.

Digitized histology. Whole-slide images were digitized using a NanoZoomer XR scanner ($\times 20$ magnification, 0.46 $\mu$m resolution; Hamamatsu). ROIs of viable tumor and necrosis for each sample were independently provided. Histology images were subsequently split into tiles of 2,000 $\times$ 2,000 pixels (jpeg).

Picrosirius red staining segmentation. A macro was written in Fiji (https://fiji.sc/) to segment picrosirius red staining from each tile using ImageJ/Fiji plugins (Java 8). Images were first converted from the RGB color space into the green-red ($a'$) color channel of the CIELAB color space (lightness, green–red, and blue–yellow), and subsequently thresholded to segment the picrosirius red staining from the background. Following appraisal of automatic and manual thresholding, two manual thresholds were chosen to achieve the optimal picrosirius red staining segmentation across all samples, while compensating for variations in staining intensity and complex background values associated with the different cancer pathologies. A threshold value of above +17 was used in the $a'$ color channel for the majority of tumor types, increased to +23 for medulloblastomas and neuroblastomas arising in the GTML/Tp53$^{+/-}$ and Th-MYCN transgenic mice respectively, and subcutaneous luc-U-87 MG tumors. The segmentation algorithm was tested using independent annotation, from a single observer blinded to the algorithm’s result, of stained/nonstained points (3920/3478) on 17 different samples across all cancer types, giving an accuracy of 95% with 91% sensitivity.

Cell segmentation from H&E-stained sections. Images were processed using the EBImage Bioconductor package (33). Cell nuclei were extracted from each image tile using the Otsu thresholding algorithm, followed by morphologic opening to delete the noisy structures and the Watershed algorithm to separate clustered nuclei (34).

Cd31 segmentation from Cd31-stained IHC sections. A macro was written in Fiji to extract DAB staining from each tile by applying color unmixing to extract the brown color channel, followed by application of the maximum entropy threshold detection method, both using ImageJ/Fiji plugins (Java 8) as described previously (27).

Generation of collagen fraction, cellularity, and vascular density parametric maps. Whole-slide images of picrosirius red stained, segmented cells, and Cd31 staining were converted into binary and processed to match MR elastography resolution (300 $\times$ 300 $\mu$m), with the fraction of pixels occupied by the center of each cell nucleus, picrosirius red, and Cd31 staining within 664 $\times$ 664 pixel-regions representing a single pixel in the final calculated maps. Quantitative analysis of each stain was performed from 1 histologic tumor section aligned with the central slice of the elastogram. Necrotic areas visible on both $T_2$-weighted images and corresponding histopathology slides were subtracted from the viable tumor by manual regional segmentation and excluded from the quantitative analysis.

Texture analysis. We evaluated the 2D heterogeneity of collagen distribution by quantifying entropy and fractal dimension (FD), as described by Nieskoski and colleagues (35), on the extracted collagen parametric maps. The texture analysis was implemented in Matlab (R2018b; Mathworks). Necrotic areas were subtracted from the viable tumor and excluded from the analysis. Entropy was quantified to measure the irregularity of collagen distribution by applying the “entropy” function of Matlab, which uses the equation:

$$\text{Entropy} = - \sum_{i=1}^{N} p_i \log_2 p_i,$$

where $p_i$ represents the normalized histogram count of the collagen fraction. FD defines the complexity (textural roughness) of collagen distribution within the tumor samples. The Hausdorff (box-counting) method was applied in the histology images within the 664 $\times$ 664 pixel-regions described earlier, using the equation:

$$\text{FD} = \log(N(\epsilon))/\log\left(\frac{1}{\epsilon}\right),$$

where $\epsilon$ represents the box size set to the size of the image and $N(\epsilon)$ corresponds to the number of boxes of size $\epsilon$, which contain collagen. The median value of the generated FD maps (excluding necrotic areas) was used as the tumor’s FD value.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc.). Unless stated otherwise, data are presented as mean $\pm$ 1 SEM. Significant differences in quantitative MR elastography parameters between tumor types, and in relative treatment-induced changes, were identified using the non-parametric Mann–Whitney U test with a 5% level of significance, while significant changes in MR elastography parameters with treatment were identified using the Wilcoxon matched-pairs signed ranks test with a 5% level of significance. Significant correlations were determined using linear regression analysis with a 5% level of significance using the robust regression and outlier removal approach (36).

Results

MR elastography was successfully performed in all mice, yielding an intratumoral coefficient of variation (CoV) of 13.0% and 15.4% for repeated measurements of $G_d$ and $G_v$, respectively (Supplementary Materials and Methods). MR elastography revealed a heterogeneous distribution of elasticity and viscosity across the 9 orthotopic and transgenic models of cancer investigated (Fig. 1A). Pronounced contrast between the established tumor and the surrounding brain was clearly evident in the intracranial models, with the lesion boundaries aligning with those seen in the high-resolution $T_2$-weighted images. Quantitative analysis of the MR elastography data demonstrated a wide range in $G_d$ and $G_v$ values across the models, from 3.5 $\pm$ 0.2 and 2.2 $\pm$ 0.2 kPa respectively, in the GTML/Tp53$^{+/-}$ transgenic mouse model of medulloblastoma, to 7.9 $\pm$ 0.4 and 6.0 $\pm$ 0.2 kPa in the orthotopic luc-MDA-MB-231 LM2-4 mammary carcinomas.
Collectively, elasticity and viscosity were significantly greater for the orthotopically-propagated breast and pancreas models, compared with the tumors associated with the central or peripheral nervous system.

Intravenous injection of collagenase resulted in a clear overall reduction in the elasticity and viscosity of orthotopic MDA-MB-231 and BT-474 mammary tumors, as measured by MR elastography, 5 hours after administration (Fig. 2A). Tumor regions exhibiting relatively high $G_d$ and $G_l$ pretreatment were typically reduced following challenge with collagenase. No similar response was evident in the vehicle-treated mice. Collectively, collagenase resulted in a significant reduction in both $G_d$ ($6.0 \pm 0.4 \text{kPa to } 4.9 \pm 0.4 \text{kPa, } P = 0.001$) and $G_l$ ($3.8 \pm 0.6 \text{kPa to } 3.0 \pm 0.5 \text{kPa, } P = 0.001$), which was not observed in the vehicle-treated cohort ($G_d: 5.1 \pm 0.4 \text{kPa to } 5.4 \pm 0.3 \text{kPa, } P = 0.3; G_l: 3.0 \pm 0.6 \text{kPa to } 3.4 \pm 0.5 \text{kPa, } P = 0.13$). Relative changes in both $G_d$ and $G_l$ were significantly different between the collagenase and vehicle-treated cohorts ($P = 0.0008$ and $0.0004$, Fig. 2B).

To investigate the pathologic determinants of the regional variations in tumor viscoelasticity seen in vivo, parametric maps of $G_d$ and $G_l$ were compared with maps of picrosirius red (collagen I and III), H&E (cellularity), and CD31 (vascular density) staining, automatically segmented from high-resolution images of aligned tissue sections from the same tumor (Fig. 3A). In the GTML/Trp53$^{K1/K1}$ transgenic mouse model, which exhibited the lowest mean $G_d$ and $G_l$, of all the models investigated, tumors presented with a thin layer-like region of elevated $G_d$ and $G_l$ spatially associated with strong picrosirius red staining adjacent to the skull, consistent with tumor invasion into the collagen-rich meninges (Fig. 3B). In general, but especially in breast and pancreatic tumors, regions demonstrating high values of $G_d$ and $G_l$ spatially corresponded to cellular regions with higher deposition of collagen. In some tumors, histologically-defined regions of extensive tissue damage (e.g., necrosis) were spatially associated with areas of markedly lower elasticity and viscosity. It is important to note the relative softness of these necrotic regions irrespective of their also having high collagen content. These regions were excluded from the subsequent quantitative analysis. Quantitative analysis identified statistically significant positive intertumor correlations of both tumor-mean elasticity $G_d$ ($r = 0.72, P < 0.0001$) and viscosity $G_l$ ($r = 0.78, P < 0.0001$) with...
tumor-mean collagen fraction, but not with tumor-mean cellularity and vascular density (Fig. 3C).

The irregularity of collagen distribution and deposition, and its relationship to tumor viscoelasticity *in vivo* was further investigated using texture analysis of the extracted images of picrosirius red staining. Significant negative correlations of entropy with $G_d$ ($r = -0.69, P < 0.0001$) and $G_l$ ($r = -0.76, P < 0.0001$), and positive correlations of FD with $G_d$ ($r = 0.75, P < 0.0001$) and $G_l$ ($r = 0.78, P < 0.0001$) were found (Fig. 4A and B). Entropy values close to 0 and relatively high values of FD determined in the BT-474, luc-PANC-1, and luc-MDA-MB-231 LM2-tumors are consistent with the presence of a homogeneous and dense collagen network. Note that in the models investigated, increasing collagen content was associated with both increasing density and uniformity of its distribution, as shown by the mono-exponential relationship of entropy ($y = 3.86e^{-0.24x} r^2 = 0.76$) and logarithmic relationship of FD ($y = 0.15 \ln x + 1.16, r^2 = 0.98$) with collagen fraction, respectively (Fig. 4C).

**Discussion**

ECM stiffening is increasingly recognized as a major mechanical signal, which alters cell behavior and in part confers cancer cell hallmark capabilities including sustained growth, invasion, and metastasis (6, 7, 37–42). ECM stiffening is also associated with increased solid stress and IFP, two other hallmarks of tumor mechanobiology that induce blood and lymphatic vessel compression and reduce transcapillary transport, respectively, and that impair effective drug delivery (3). Disrupting the cross-talk between cancer cells and the ECM, as well as reversing ECM stiffness, solid stress or IFP, thus represents a promising therapeutic strategy (11). The clinical development of stromal modulating therapies would be facilitated and accelerated by noninvasive imaging methods to longitudinally image and quantify tumor mechanical properties *in vivo* (15–17).

In this preclinical study, our quantitative MR elastography tumor data, combined with aligned computational histopathology, showed that elevated tumor $G_d$ and $G_l$ correlated with increased collagen deposition across a wide range of clinically-relevant tumor models with disparate pathologies. Our data particularly highlight the relative softness of tumors arising in the nervous system, and the predicted elevated stiffness of orthotopic breast and pancreatic models. Furthermore, modulation of the collagen network with bacterial collagenase in two relatively stiff orthotopic models of breast cancer revealed a marked reduction in both $G_d$ and $G_l$ compared with vehicle control.

**Figure 2.**

MR elastography can inform on tumor stromal modulation induced by collagenase. **A**, Representative T2-weighted anatomical MRI images and parametric maps of $G_d$ and $G_l$, scanned from mice bearing orthotopic BT-474 breast cancer xenografts 24 hours prior to and 5 hours after intravenous administration of either vehicle or collagenase. **B**, Relative changes (%) in tumor median $G_d$ and $G_l$ measured in orthotopic MDA-MB-231 (blue symbols) and BT-474 (red symbols) breast cancer xenografts measured 5 hours after administration of either vehicle (○) or collagenase (△). Data are the individual changes from each tumor and the combined cohort mean ± 1 SEM. Collagenase induced a significant reduction in both $G_d$ and $G_l$ compared with vehicle control.
mechanopathology, our data show they provide a very moderate contribution to the observed range of mechanical phenotypes, in contrast to differences in ECM characteristics.

Intracranial tumors were typically at the softer end of the spectrum of viscoelastic properties measured in this study (Gd ~4 kPa and Gl ~2 kPa; refs. 19, 22). The high compliance (inversely related to elastic modulus) of human brain tumors relative to the surrounding brain parenchyma has been reported, and in the case of glioblastoma, stiffness has been shown to decrease with tumor grade, measured as part of clinical MR elastography-embedded prospective studies (44, 45). Brain tumors share the unique composition of the healthy brain, characterized by the absence of a fibrillar network (which resists shear deformation) and a reliance on hygroscopic hyaluronic acid (the main mechanical support against compressive forces that act to cause a change in volume but offer little resistance to shear) for mechanical support. The lack of collagen, a major facilitator of tumor cell intravasation, one of the earliest stages of metastasis, is consistent with the fact that intracranial tumors rarely disseminate outside the brain (46). Note also the relatively low viscoelastic...
properties of the intracranially grown luc-MDA-MB-231 LM2-4 tumors compared with those propagated in the mammary fat pad, highlighting the contribution of implantation site to the resulting biomechanical phenotype. Abdominal neuroblastomas spontaneously arising in Th-MYCN transgenic mice, and which are derived from the sympathetic nervous system, were also relatively soft with little collagen, consistent with their general clinical presentation (47). Interestingly, increased collagen III (reticulin) deposition helps to define an ultra-high-risk group of patients in which increased stiffness relates to metastatic potential, the major cause of mortality for children with neuroblastoma (48, 49).

MR elastography revealed an acute reduction in breast tumor elasticity and viscosity following systemic administration of collagenase. A similar biomechanical response was recently reported following intratumoral injection of collagenase, measured using US-based elastography (16). Collagenase has also been shown to rapidly decrease tumor IFP in breast cancer xenografts (50, 51). Cleavage of collagen with collagenase produces large peptide fragments, which remain trapped within the ECM, making the acute effects of collagenase challenging to detect on conventional histology at such an acute time point (12). Having established their dependence on relative tumor collagen fraction, quantitation of tumor Gd and Gv can also provide early MRI biomarkers of response to collagen degradation in vivo. In this way, MR elastography could thus be exploited for monitoring direct enzymatic degradation of the ECM and/or targeted inhibition of collagen synthesis, potent strategies being actively investigated to improve drug penetration in solid tumors, and showing promising results in clinical trials (10, 12, 52).

Solid stress has been shown to correlate with collagen deposition and be the major contributor to total tumor pressure in models of pancreatic cancer (53). ECM stiffening is a marker of poor prognosis in breast cancer and pancreatic ductal adenocarcinoma (6, 37, 38, 40). Given its sensitivity to collagen deposition, MR elastography may thus potentially provide prognostic information, and through its sensitivity to collagen modulation, provide biomarkers of response to collagen-targeted approaches designed to alleviate solid stress for improved drug delivery. Tumor shear modulus, measured by US elastography, has been shown to positively correlate with collagen deposition and inversely correlate with functional vasculature and drug delivery (17). However, MR elastography cannot measure pressure directly, and as such may not be directly informative for strategies designed to decrease fluid stress (e.g., hyaluronidase), for which direct measurements of IFP are required. Reconstruction of tumor viscoelastic parameters from the properties of propagating waves...
or strain visualized by MRI or US has often used a simple monophasic linear viscoelastic mechanical model, which does not take into account any mobile fluid component (54). Measuring IFP and solid stress in itself represents a major clinical challenge, as current approaches for measuring both are invasive. Clinical measurement of IFP with invasive wick-in-needles only permits very discrete sampling of the tissue and is unreliable, and innovative preclinical MRI methods shown to correlate with IFP in vivo would be difficult to routinely implement in a clinical setting (51, 55).

Finally, the utility of MR elastography is being actively evaluated clinically in a wide range of health conditions including neuro- and cardiovascular pathologies, with applications including diagnosis, staging, surgical planning, and intraoperative guidance. For example, the integration of MR elastography into the management of patients with chronic liver disease is now becoming well established for its excellent diagnostic accuracy, superior to that of US-based techniques, and ability to discriminate the different stages of liver fibrosis, characterized by increased deposition and crosslinking of collagen (56). Although less widely available and more costly than US-based techniques, MR elastography does allow the 3D investigation of large tissue areas and deep-seated organs. MR elastography can uniquely afford the noninvasive investigation of the mechanical properties of the brain and its pathology including, as recently demonstrated, neuronal activity (57). Finally, MR elastography can be incorporated into multiparametric MR imaging protocols to enable comparison with other MRI-derived biomarkers of tumor structure and function in a single clinical scanning session.

In conclusion, we have shown that quantitation of elasticity $G_\alpha$ and viscosity $G_\eta$ are sensitive imaging biomarkers of tumor collagen deposition, and response to direct enzymatic degradation of the collagen network. Given the importance of elevated ECM stiffness in tumor progression, and the continuing need for new technologies for faster and more accurate detection, diagnosis and monitoring, MR elastography has the potential to inform noninvasively on prognosis and improve risk stratification of patients with cancer with dense stroma, and accelerate the development of stromal targeting/modulating treatment strategies.

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

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References


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