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Graphical abstract

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Desmoplasia describes the deposition of extensive extracellular matrix and defines primary pancreatic ductal adenocarcinoma (PDA). The acellular component of this stroma has been implicated in PDA pathogenesis and is being targeted therapeutically in clinical trials. By analyzing the stromal content of PDA samples from numerous annotated PDA data sets and correlating stromal content with both anatomic site and clinical outcome, we found PDA metastases in the liver, the primary cause of mortality to have less stroma, have higher tumor cellularity than primary tumors. Experimentally manipulating stromal matrix with an anti–lysyl oxidase like-2 (anti-LOXL2) antibody in syngeneic orthotopic PDA mouse models significantly decreased matrix content, led to lower tissue stiffness, lower contrast retention on computed tomography, and accelerated tumor growth, resulting in diminished overall survival. These studies suggest an important protective role of stroma in PDA and urge caution in clinically deploying stromal depletion strategies.

Introduction

Pancreatic ductal adenocarcinoma (PDA) is among the most lethal solid tumors and is projected to become the second-leading cause of cancer-related death in the United States by 2030 (1, 2). PDA is a heterogeneous and genetically diverse disease. Molecular PDA subtypes have been proposed (3–7) but do not yet drive clinical treatment decision making. Emerging studies implicate the tumor microenvironment as playing a pivotal role in tumor initiation, progression, and response to chemotherapy in conjunction with or independent from intrinsic alterations in tumor cells (4, 8). Identifying key prognostic or predictive features of the tumor stroma will facilitate useful stratification and possibly therapeutic selection tools for precision oncology efforts in this disease.

Notably, one such feature of PDA is the development of extensive fibrosis termed desmoplasia (9), with stromal components being more prevalent than pancreatic cancer cells. Tumor stroma has been considered to contribute to disease progression and therapeutic resistance in PDA and several mechanisms have been suggested (4, 10). However, recent studies have shown that stroma-depletion strategies can lead to more aggressive tumors in transgenic mouse models (11–13). Furthermore, several clinical trials targeting stroma (e.g., depleting hyaluronan) failed to achieve therapeutic benefit over standard-of-care chemotherapeutic approaches and may have been deleterious (14, 15). A more in-depth understanding of the role of stroma within the tumor microenvironment may provide new therapeutic strategies for improving patient survival.

In this study, we objectively assessed the amount of tumor stroma in clinical specimens of primary and metastatic PDA, hypothesizing that differences in tumor composition may translate into differences in patient survival. Further, we investigated the role of stroma in tumor development by depleting the extracellular matrix in experimental mouse models and examined the effects on tumor progression and therapeutic outcomes with standard-of-care agents.
worse overall survival in 431 patients assigned to nab-paclitaxel plus gemcitabine and 430 patients assigned to gemcitabine alone (Figure 1, C and D). Because low stromal content correlates with poor prognosis, we next investigated the stromal signature in liver metastases using automated stroma quantification and collagen I immunofluorescence staining. Tissues from metastatic liver lesions described previously (7) had lower stromal content than did paired primary tumor specimens (mean TSD 23.1% versus 63.6%, \( P = 0.0045 \); Figure 2, A and B).

To further evaluate and characterize the stromal content of primary PDA and liver metastases in an independent patient cohort, we performed collagen I immunofluorescence staining on primary tumor and liver metastasis from samples collected under a rapid autopsy cohort (4). There was a significant correlation between TSD and collagen-positive tumor area across primary and metastatic samples (Spearman’s correlation \( r = 0.69 \), \( P = 0.0155 \); Figure 2C). Analysis of collagen content in paired primary and metastatic liver sites also demonstrated increased collagen tumor area in primary tumors (59.75% collagen-positive tumor area) versus paired liver metastases (33.2% collagen-positive tumor area) (\( P = 0.0383 \), Figure 2D).

Because tumor cellularity reciprocally correlates with stromal content, we next expanded this observation to tumor molecular cellularity in large cohorts. We used mutant allele fractions across a multigene panel to assess tumor cellularity in samples from UCSF, UCLA, UCSD, and UC Davis sent to Foundation Medicine for clinical next-generation sequencing (NGS). Tumor cellularity

**Results and Discussion**

We previously demonstrated an automated method to assess the tumor stromal density (TSD) on hematoxylin and eosin–stained tissue microarray (TMA) slides and validated its use with multiple-pathologist stromal assessments (16). We performed automated TSD quantification on TMAs of primary resected tumors from 92 patients reported previously (17). Patient and clinicopathological characteristics as stratified by observed TSD are listed in Supplemental Table 1 (supplemental material available online with this article; https://doi.org/10.1172/JCI36760DS1). There were no apparent associations between TSD and patient gender, age, tumor grade, or disease stage. We next split the TMA cohort into low (<50% of core composed of stroma) and high (≥50% of core composed of stroma) TSD groups (Figure 1A). We found that the high TSD group enjoyed a longer survival than did the low TSD group using the Kaplan-Meier method (\( P = 0.036 \), Figure 1B). Further, in multivariable Cox regression analyses controlling for patient sex, age, T and N stage, overall stage, tumor grade, and surgical margins, low TSD was associated with impaired overall survival rate (HR = 2.19 [95% CI = 1.11–4.29], \( P = 0.022 \); Supplemental Table 2) in patients undergoing upfront tumor resection.

Liver metastasis is a common feature of PDA. More than 50% of patients with PDA have liver metastases at the time of diagnosis, but the stromal content of liver metastases has not been systematically studied. We analyzed patient characteristics from the MPACT trial that led to the current standard of care for metastatic PDA (18). We found liver metastases to be correlated with worse overall survival in 431 patients assigned to nab-paclitaxel plus gemcitabine and 430 patients assigned to gemcitabine alone (Figure 1, C and D). Because low stromal content correlates with poor prognosis, we next investigated the stromal signature in liver metastases using automated stroma quantification and collagen I immunofluorescence staining. Tissues from metastatic liver lesions described previously (7) had lower stromal content than did paired primary tumor specimens (mean TSD 23.1% versus 63.6%, \( P = 0.0045 \); Figure 2, A and B).

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was scored as a percentage and revealed a trend toward higher cellularity for liver lesions ($n = 73$, median = 35.1) versus primary pancreatic tumors ($n = 150$, median = 20, $P = 0.0008$; Figure 2E).

In line with this finding, tumor cellularity from liver metastases ($n = 173$, median = 30) of an independent Know Your Tumor (19) cohort was significantly higher than that of the primary tumors ($n = 258$, median = 20, $P < 0.0001$; Figure 2F). Here, we observed variability of stromal content according to lesion site and the stromal content in primary tumors and liver metastases showed a strong negative correlation with overall survival in PDA patients.

To investigate the functional contribution of stromal content to PDA progression, we used a monoclonal antibody (mAb) that specifically recognizes lysyl oxidase-like-2 (LOXL2) for in vivo stromal matrix depletion. Collagen cross-linking is an essential process for extracellular matrix stabilization. LOXL2 belongs to the lysyl oxidase (LOX) family of proteins encoding an extracellular copper-dependent amine oxidase that catalyzes the first step in the formation of cross-links in extracellular matrix components, including collagens and elastin. LOXL2 inhibition reduces collagen content and attenuates tissue fibrosis (20). We used an orthotopic murine PDA model to functionally test the effects of experimentally manipulating stromal content on tumor progression (Figure 3A). Hematoxylin and eosin (H&E) staining revealed an extensive reorganization of intratumoral collagen fibers with anti-LOXL2 mAb treatment (Figure 3B). Tumor collagen content assayed by trichrome staining was substantially decreased along with reduced stiffness in anti-LOXL2 mAb–treated tumors compared with control tumors ($P < 0.0001$).

We used an orthotopic murine PDA model to reliably recapitulate clinical and histopathological features of the human disease. We used 2 different mouse PDA lines, p53 2.1.1 (3) and FC1245 (21), that were each isolated from a Kras LSL-G12D/+; Trp53LSL-R172H/+; Pdx1-Cre (KPC) mouse but in FVB and C57BL/6 genetic backgrounds. Anti-LOXL2 mAb AB0023 (30 mg/kg, intraperitoneally 2 times a week, Gilead) started 1 week after the implantation and was continued for another 3 weeks (Figure 3A). Vehicle (IgG control) was administered in parallel. In both orthotopic models, tumors treated with anti-LOXL2 mAb presented with significantly increased bioluminescence signals compared with IgG-treated control tumors ($P < 0.05$ in FC1245 tumors and $P < 0.01$ in p53 2.1.1 tumors; Figure 4, A and B, and Supplemental Figure 1, A and B). Tumor weight was remarkably increased by anti-LOXL2 mAb treatment (Figure 4C and Supplemental Figure 1C).

PDA tissues with abundant stroma commonly show increased contrast enhancement/contrast retention at delayed-time-point CT. For the pattern of enhancement, the tumors were classified as having either low or high enhancement ratios. Figure 4D separately shows a representative image of a tumor in the IgG group classified as having a high normalized enhancement ratio and a representative tumor in the anti-LOXL2 treatment group that was...
in tumors from anti-LOXL2 mAb–treated mice (Supplemental Figure 2C), likely contributing to the aggressive phenotype of tumors upon anti-LOXL2 mAb treatment. As cancer-associated fibroblast (CAF) heterogeneity plays a vital role in PDA tumor biology (23, 24), we next sought to analyze the populations of myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAFs). With flow cytometric analysis of several surface markers that distinguish the subset of iCAFs, we observed no significant difference in the percentage of either αSMAloIL-6hi iCAFs (Supplemental Figure 2D) or Ly6C+ iCAFs (Supplemental Figure 2E) in the tumors isolated from IgG- or anti-LOXL2 mAb–treated mice. Tumor-associated endothelial cells (CD31+) were also equally distributed in tumors from IgG- or anti-LOXL2 mAb–treated mice, as was microvessel density (Supplemental Figure 2F). We then set out to determine the effect of stromal depletion on the immune infiltration. Tumors with stromal depletion presented with no significant changes in overall intratumoral infiltration of CD45+ cells and CD8+ T cells and CD11b+ myeloid cells compared with control tumors (Supplemental Figure 3, A–D).

Figure 3. Anti-LOXL2 treatment abolished extracellular matrix in murine PDA model. (A) Schematic of the animal study. (B and C) H&E and trichrome staining revealed that intratumoral collagen fibers were notably reduced after anti-LOXL2 mAb. Original magnification, ×10 (left) and ×40 (right). (D) Anti-LOXL2–treated tumors had a stiffer extracellular matrix in the periductal region, as measured by atomic force microscopy (AFM).

Stroma likely plays a dynamic and changing role over the course of tumorigenesis in the pancreas, estimated by some to last a decade or more (25), and its heterogeneous cellular and noncellular constituents change in relation to the evolving genetic landscape of cancer cells. In this regard, several studies have suggested that fibroblasts and type I collagen associated with tumor fibrosis are tumor promoting in solid tumors, including PDA (26–28). In this work, we have quantified the tumor stromal content in clinical PDA specimens from primary tumors and liver metastases using a variety of orthogonal methods with a convergent result enabling a generalizable conclusion. We find that low stromal infiltration of tumors with normalized enhancement ratios of 0.32 or greater had significantly higher stromal content compared with those with normalized enhancement ratios less than 0.20 (P = 0.01, Figure 4D). In addition, we observed large amounts of ascites (Figure 4E) in anti-LOXL2–treated mice, indicating that stromal depletion promoted PDA development.

Delivery of small-molecule therapeutics may be hampered by stroma (22). To address the functional sequelae of stromal depletion in drug delivery, we next evaluated the effects of anti-LOXL2 mAb along with gemcitabine. We treated a separate cohort of mice with the combination of gemcitabine plus anti-LOXL2 mAb or gemcitabine plus IgG vehicle. Interestingly, in both orthotopic models, the addition of anti-LOXL2 to gemcitabine unexpectedly accelerated tumor progression (Figure 4, A and B, and Supplemental Figure 1, A and B) and resulted in slightly increased tumor weights at the endpoint (Figure 4C and Supplemental Figure 1C). These results suggest that any hypothetical benefit afforded by possible improved drug availability following stromal matrix depletion is outweighed by protumorigenic effects on the tumor itself.

To elucidate a potential causal link between the tumor biological behavior and the composition of epithelial and fibroblast compartments upon stromal matrix depletion, we flow sorted epithelial cells (CD45 EPSCAM+) and fibroblasts (CD45 EPSCAM–PDGFRα+) from tumors in both orthotopic models. In both FC1245 and p53 2.1.1 xenografts, there was a considerably increased proliferative capacity of tumor epithelial cells with the treatment of anti-LOXL2 mAb, as assessed by Ki67 signal (Supplemental Figure 2, A and B). We also observed a slightly lower percentage of fibroblasts classified as having a low normalized enhancement ratio. Tumors in mice with normalized enhancement ratios of 0.32 or greater had significantly higher stromal content compared with those with normalized enhancement ratios less than 0.20 (P = 0.01, Figure 4D). In addition, we observed large amounts of ascites (Figure 4E) in anti-LOXL2–treated mice, indicating that stromal depletion promoted PDA development.

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As such, conclusions regarding other stroma-depletion techniques are indirect and further studies of other stroma-depleting components might yield differing results.

In summary, we examined variation in tumor stromal content in the primary tumor and liver metastases of PDA. We find important and reproducible differences in PDA tumors based on tumor stromal content as a function of anatomic site and identified low stromal content as an independent, poor prognostic factor. Syn- geneic mouse PDA tumors with decreased stromal content are more aggressive, indicating that tumor stroma is a protective factor for PDA growth. This study highlights the complex interplay of tumor-stroma interactions and provides translational implications for future therapy for PDA patients.

Methods
Refer to Supplemental Methods for details.

Study approval. All animal experiments were performed in accordance with protocols approved by the UCSF Institutional Animal Care and Use Committee (IACUC).

Author contributions
HJ and EAC conceived of and designed the study. HJ, RJT, KS, MK, HH, AJR, DH, SEU, NMJ, KM, MJ, EMB, BL, ML, MH, WW, KV, JIY, and SYMW were responsible for data collection. HJ, RJT, CS, ZJW, MAT, and EAC analyzed and interpreted the data. HJ, RJT, KS, and EAC wrote, reviewed, and/or revised the manuscript.
EAC supervised the study. All authors reviewed and approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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