Biology of Human Tumors

Clinical Cancer Research

Use of Angiotensin System Inhibitors Is Associated with Immune Activation and Longer Survival in Nonmetastatic Pancreatic Ductal Adenocarcinoma

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Abstract

Purpose: Angiotensin system inhibitors (ASI) can improve prognosis in multiple cancer types, including pancreatic ductal adenocarcinoma (PDAC). However, no study has examined the effect of ASIs alone or combined with adjuvant chemotherapy in resected PDAC patients.

Experimental Design: We performed an analysis of the records of ASI users and nonuser patients with PDAC seen at Massachusetts General Hospital (Boston, MA) between January 2006 and December 2010. To identify mechanisms of ASIs in PDAC, we performed RNA sequencing (RNA-Seq) of resected primary lesions.

Results: A total of 794 consecutive patients were included. In 299 resected patients, ASI users experienced longer overall survival (OS) in both univariate (median OS, 36.3 vs. 19.3 months, $P = 0.011$) and adjusted multivariate [HR, 0.505; 95% confidence interval (CI), 0.339–0.750; $P = 0.001$] analyses. Propensity score–adjusted analysis also showed a longer median OS for chronic ASI users. In unresected patients, the beneficial effect of ASIs was significant in patients with locally advanced disease, but not in metastatic patients. RNA-Seq analysis revealed in tumors of ASI users (lisinopril) a normalized extracellular matrix, a reduced expression of genes involved in PDAC progression (e.g., WNT and Notch signaling), and an increased expression of genes linked with the activity of T cells and antigen-presenting cells. Finally, chronic use of ASI was associated with a gene expression signature that is predictive of survival in independent validation cohorts.

Conclusions: In patients with nonmetastatic PDAC, chronic ASI use is associated with longer OS independently of chemotherapy. Our RNA-Seq analysis suggests that ASIs reduce the malignant potential of cancer cells and stimulate the immune microenvironment in primary PDAC. *Clin Cancer Res; 23(19); 5959–69. ©2017 AACR.*

Introduction

The renin–angiotensin–aldosterone system (RAAS) is a well-studied hormone system. It was first recognized as a master regulator of blood pressure homeostasis and electrolyte balance. The subsequent discovery of local RAAS in various organs and tissues highlighted its significant role in basic cell biological processes, such as proliferation and migration, as well as pathophysiologic processes like inflammation (1). In solid tumors,

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Translational Relevance

Angiotensin system inhibitors (ASI) are widely used to treat hypertension. ASIs have additional cardiac and renal protective effects, due to their modulation of the immune system and wound healing. In this study, we examined the effect of long-term ASI use on the survival of patients with pancreatic ductal adenocarcinoma (PDAC) and explored its potential mechanisms. Our findings indicate that chronic ASI use is independently associated with longer overall survival in nonmetastatic PDAC patients. Unbiased gene expression profiling suggested that the improved survival associated with ASI therapy might be due to normalization of the extracellular matrix, inhibition of tumor progression, and enhanced antitumor immunity. Our results advocate for prospective clinical trials to assess specific ASIs as adjuncts to primary tumor resection in PDAC. With the increasing use of neoadjuvant chemotherapy in PDAC, reprogramming of the extracellular matrix and immune microenvironment with ASIs has a great potential.

angiotensin II (Ang II) enhances tumor cell proliferation and growth by acting as a paracrine and/or autocrine signal in the tumor microenvironment. It promotes the growth of stromal cells, such as fibroblasts, endothelial cells, neutrophils, and macrophages, leading to an increased secretion of tumor growth factors (2). Some cancer cells also utilize Ang II signaling for survival (3).

Cancer–microenvironment interactions shape the pathophysiology of pancreatic ductal adenocarcinoma (PDAC). In our attempt to understand the role of physical barriers to cancer treatment, we discovered that the collagen matrix forms a formidable obstacle that hinders the penetration of nanotherapeutics into solid tumors (4). We later found that losartan, an angiotensin receptor blocker (ARB), decreased collagen and enhanced the intratumoral distribution of nanoparticles and efficacy of nanotherapeutics in breast and pancreatic cancer mouse models (5). We subsequently showed that losartan and lisinopril, an angiotensin converting enzyme inhibitor (ACEi), decreased not only collagen but also hyaluronan, and enhanced the efficacy of low molecular weight chemotherapeutics in desmoplastic mice tumors (6). This benefit was in part due to the decompression of blood vessels resulting from decreased solid stress (7, 8). The safety and low cost of ARBs and ACEis, together called angiotensin system inhibitors (ASI), along with their potentiation of conventional chemotherapy make a strong case for repurposing ASI as adjuncts in cancer treatment. These insights formed the basis of a prospective trial at Massachusetts General Hospital (MGH, Boston, MA) evaluating the efficacy of losartan combined with the FOLFIRINOX cocktail and radiotherapy in patients with locally advanced PDAC (ClinicalTrials.gov identifier: NCT01821729). The preliminary results ($N = 25$ patients) suggest that the addition of losartan to neoadjuvant FOLFIRINOX followed by chemoradiation increases the resection rate and R0 resection. Also, the median overall survival (OS) and 2-year survival rates are longer than historically observed (9). These encouraging results agree with our preclinical findings and should be tested in independent prospective trials.

Several studies have associated the use of ASI with longer survival in various tumor types (2). However, no study has determined the effect of ASI use in PDAC patients independent of stage and treatment received, including surgery, chemotherapy, or radiotherapy. Furthermore, no unbiased effort has been undertaken to understand the mechanisms through which ASIs confer survival benefits. To determine the role of ASI use in pancreatic cancer, we conducted a retrospective analysis of all PDAC patients treated and followed at MGH during a 5-year period. Through unadjusted univariate, adjusted analysis with multivariate modeling, and propensity score methods, we correlated chronic ASI use with OS and recurrence-free survival (RFS) of PDAC patients. Chronic ASI use is associated with longer OS in patients with resected primary tumors as well as in locally advanced cancer patients. To gain insight into the mechanisms, we conducted RNA sequencing (RNA-Seq) analysis in resected tumors from treatment-naïve patients with or without chronic lisinopril use. Results suggested that ASI changed the tumor microenvironment, normalized the stroma, and enhanced the activity of immune cells. Together, our results enable a deeper understanding of the biological mechanisms underlying the survival benefit associated with ASI use in PDAC.

Materials and Methods

Patient cohorts

Patient data were acquired through MGH super-database, a research patient data registry created by Partners Healthcare. The Partners Healthcare Internal Review Board and local ethics committee approved the retrospective analysis of patient data. Diagnosis, pathology, body mass index (BMI), treatment, medications, progression, and survival were confirmed and collated by reviewing medical records. For resected patients, BMI information was collected at the time of diagnosis. BMI was only available for a fraction of patients with locally advanced cancer and metastasis. Therefore, BMI was not included in the analysis of these patients. All the patients included in the analysis had a tissue diagnosis of PDAC. We used the date of tissue collection as the time of diagnosis. OS was calculated from the time of diagnosis to the time of death or last contact. RFS was calculated from the time of diagnosis to the time of recurrence in any organ or death. Organ-specific time to metastasis was calculated from the time of diagnosis to the occurrence of metastasis in that organ, while occurrence to other organs was censored. Competing risk analysis was performed, with first organ metastatic recurrence in liver, lung, local site, other metastases, or death treated as competing events.

Propensity score–adjusted analysis

We calculated propensity score using all parameters available: in metastatic patients: age, tumor site, chemotherapy, and hypertension; in locally advanced patients: age, tumor site, tumor size, radiotherapy, chemotherapy, and hypertension; and in resected patients: age, tumor site, tumor size, BMI, grade, lymph node ratio, perineural invasion, vascular invasion, neoadjuvant treatment, adjuvant chemotherapy, adjuvant radiotherapy, and hypertension. We calculated Cox proportional hazards ratio with the propensity score as well as the ASI status.

Fresh tumor samples

Patient samples were obtained from the Departments of Surgery and Pathology at MGH. All patients gave signed informed consent for the collection of excessive tumor samples and

molecular analysis. The protocol was approved by the Internal Review Board of Partners Healthcare. All patients had a resectable PDAC and were not treated with chemotherapy or radiation prior to surgery, but the ACEi group was treated with lisinopril for their hypertension. Fresh tumor samples were collected after surgical resection. Each fresh tumor chunk was sampled from the center of the pancreatic tumor and was snap frozen in liquid nitrogen for later use.

RNA extraction and RNA-Seq

Whole tissue RNA was extracted using a standard phenol–chloroform protocol. RNA integrity numbers (RIN) were obtained using a Bioanalyzer 2100 (Agilent), and samples with RIN >7.5 were used for subsequent steps. Sequencing libraries were prepared from 100 to 500 ng of total RNA using the TruSeq RNA Sample Preparation Kit v2 (Illumina). Read alignment and junction mapping were accomplished using TopHat2 v2.0.4, using a 25-bp 50-segment seed for initial mapping to map reads to the reference genome annotation, NCBI human build 37.2 (10), followed by differential gene expression analysis using Cuffdiff v2.0.2. Data were expressed as fragments per kilobase of exon per million fragments mapped (FPKM). To identify functional gene categories enriched in our differentially expressed genes, we used the Gene Ontology (GO) and REACTOME databases. We also performed gene set enrichment analysis (GSEA) in preranked analysis mode with classic enrichment method, ordering genes by their fold changes in lisinopril versus control PDAC tumors. We used FDR q-value 0.05 as a threshold to determine significantly changed gene sets.

ASI induced gene signatures and validation cohorts

We searched for validation cohorts that have: (i) genome-wide expression measurements; (ii) OS data; (iii) normal pancreatic tissue control; and (iv) greater than 50 tumor samples. GEO dataset GSE71729 (11) as well as The Cancer Genome Atlas (TCGA) dataset were selected. Survival data and normalized expression matrices were downloaded and analyzed. RNA-Seq expression profiling was performed on fresh tumor samples as described above. Significantly differentially higher or lower expressed genes in tumors from patients with chronic ASI use versus no ASI use were identified as two separate gene sets. These genes are PDAC-specific ASI response gene signatures and were used for pathway deregulation analysis. Briefly, we used a previously described algorithm called Pathifier to calculate a pathway deregulation score for either significantly higher or lower expressed genes in each individual patient (12). This single score is a continuous variable that represents the expression level of the entire gene set as a pathway. Patients were divided into high, medium, or low expression categories based on this deregulation score, and the association of expression categories with OS was calculated. Kaplan–Meier and Cox proportional hazards analyses were performed using SPSS v22.

Results

A total of 944 PDAC patients visited MGH from January 2006 to December 2010. Patients were followed for periods ranging from 1 month to 9 years, with a median follow-up of 11 months. We excluded 150 patients who did not have complete follow-up information because they received follow-up treatment outside of MGH. The remaining 794 patients with complete oncological history were divided into three groups: group A patients ($n = 310$) did not receive resection because they already presented with metastatic disease, group B patients ($n = 185$) did not receive resection due to locally advanced disease, while group C patients ($n = 299$) had resectable disease and successfully underwent primary tumor resection (Supplementary Fig. S1). A total of 480 out of 794 patients had hypertension, among which 289 patients were chronic ASI users. Eight patients were on chronic ASI for congestive heart failure. Non-ASI users included all other patients, including patients who were normotensive and never used ASI ($n = 306$), short-term ASI users [less than 1 month or less than 50% of follow-up time in medical record ($n = 21$)], and patients treated for hypertension with non-ASI antihypertensives ($n = 170$).

ASI use is not associated with survival in patients with metastatic disease

The characteristics of patients with metastasis are shown in Supplementary Table S1. Patients on ASI were significantly older (70.6 vs. 64.8) and had a higher proportion of tumor in the pancreatic head (42% vs. 27%). In the unadjusted univariate model, factors that were associated with better survival were younger age and chemotherapy treatment (Supplementary Table S1). The median survival of ASI^+ and ASI^- patients is 4.4 [95% confidence interval (CI), 3.3–5.5] and 6.3 (95% CI, 5.2–7.4) months ($P = 0.561$), respectively (Fig. 1A). In the multivariate model with adjustment for potential confounders (e.g., hypertension), the tumor located in the head of the pancreas was also associated with better survival (Supplementary Table S1). However, chronic ASI use (HR = 0.944; 95% CI, 0.705–1.263) was not significantly associated with OS in metastatic patients.

ASI use is associated with increased survival in patients with locally advanced disease

In patients with locally advanced disease, all parameters besides hypertension were comparable between chronic ASI users and ASI nonusers (Supplementary Table S2). In the unadjusted univariate analysis, younger age, radiotherapy treatment, and chemotherapy treatment were associated with better survival (Supplementary Table S2). The median OS of ASI^+ and ASI^- patients was 11.3 (95% CI, 8.1–14.5) versus 9.3 (95% CI, 8.4–10.2) months ($P = 0.091$), with a 5-year survival rate of 3.3% ± 3.0% versus 1.9% ± 1.7%, respectively (Fig. 1B). In the adjusted multivariate model, chronic ASI use was independently associated with significantly increased survival (HR = 0.572; 95% CI, 0.386–0.847; $P = 0.005$) after adjusting for other covariates.

ASI use is independently associated with longer OS in resected patients

The tumor resection rate of ASI users (130/297, 43.8%) is significantly higher than for ASI-naïve patients (169/497, 34%; $P < 0.05$). Resected ASI users are older (69.1 vs. 64.6), have a higher rate of being hypertensive, and have a higher BMI compared with resected non-ASI users. All other parameters were distributed equally between chronic ASI users and nonusers (Table 1). In the unadjusted univariate model, ASI use, as well as smaller tumor size, negative surgical margin, lower lymph node ratio (LNR), lower histologic grade, absence of lymphovascular invasion, and absence of perineural invasion were associated with better survival. Median survival of ASI^+ and ASI^- patients was

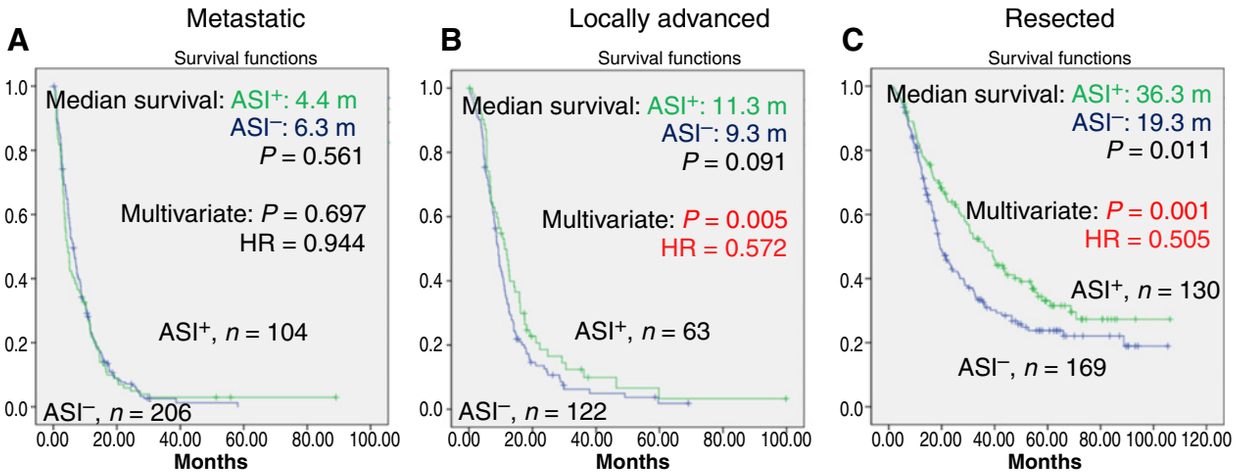

Figure 1.
Unadjusted Kaplan–Meier curves for OS in metastatic (**A**), locally advanced (**B**), and resected (**C**) patients. HR, hazard ratio.

36.3 months (95% CI, 28.3–44.3) versus 19.3 months (95% CI, 15.7–22.9; $P = 0.011$), respectively (Fig. 1C), with a 5-year survival rate of 31.6% ± 4.6% versus 23.8% ± 3.7%. ASI use is associated with lower risk for overall death (HR = 0.69; 95% CI, 0.52–0.92; $P = 0.012$). There was no difference in survival between patients on ARB versus ACEi ($P = 0.9$; Supplementary Fig. S2).

Cox proportional hazards analysis (Table 1) adjusted for potential confounding covariates showed that ASI use (HR = 0.505; 95% CI, 0.339–0.750; $P = 0.001$) as well as smaller tumor size, negative surgical margin, lower LNR, lower grade, and adjuvant chemotherapy were associated with increased survival. Notably, our adjusted analysis with multivariate modeling revealed ASI use as an independent factor associated with OS with the lowest HR, followed by chemotherapy. Chronic ASI use, alone or combined with chemotherapy, provided unique survival benefit after adjustment for all parameters in the Cox model.

We then performed stratified analysis based on treatment. In patients that did not receive adjuvant chemotherapy after resection, most commonly because patients refused chemotherapy, the median survival was 39.2 for ASI users and 9.3 months for ASI-naïve patients ($P = 0.01$). In patients treated with chemotherapy, median OS of ASI users and ASI-naïve patients was 28.7 ± 9.8 and 12.3 ± 4.9 months ($P = 0.048$), respectively (Supplementary Fig. S3; Supplementary Table S3). Additional stratified analysis showed that ASIs provide significant survival benefits in patients with BMI at diagnosis <25 ($n = 139$, median survival ASI 44 vs. non-ASI 17 months, $P = 0.005$), while in patients with BMI >25 ($n = 160$) the median survival of ASI users (34 months) versus ASI-naïve patients (21 months) was also longer but not significant (Supplementary Fig. S4). In summary, our data suggest a strong independent benefit of ASI use in patients with resected disease.

Propensity score analysis

We performed propensity-adjusted analysis and inverse probability–weighted analysis in all three groups of patients included in our analysis. There was no benefit of ASI use in patients with metastasis, whereas ASI use correlated with longer survival in locally advanced and resected patients (Supplementary Table S4A). We also performed the inverse probability–weighted analysis, which showed similar results indicating a significant benefit of ASI use in nonmetastatic patients (Supplementary Table S4B).

In resected patients, ASI use is associated with longer time to recurrence

In addition, we found that chronic ASI use correlated with a longer time to recurrence. The prolongation of time to recurrence is mainly due to longer time to distant metastasis, rather than to a reduction of local recurrence (Supplementary Fig. S5). We performed competing risk analysis, treating the local recurrence, recurrence in the liver, lung, other distant site (peritoneal and ascites) as well as death as competing risks. We found that the HR of lung recurrence was not affected with ASI use (Supplementary Table S5). We continued with cause-specific survival analysis, treating recurrence in each site as a separate event in distinctive analysis. Chronic ASI use was strongly correlated with longer time to recurrence in the liver in both unadjusted univariate and multivariate analyses (Supplementary Fig. S5C; Supplementary Table S6). Time to recurrence in the lung and other sites was not correlated with ASI use, likely because liver is the most common site for PDAC metastasis, and thus there are high censor rates for these other sites.

RNA-Seq identifies pathways altered by chronic ASI use

To gain mechanistic insight into the association between ASI use and longer survival in resected PDAC patients, we prospectively collected treatment-naïve PDAC samples (4 lisinopril-treated patients vs. 4 controls) and performed RNA-Seq. We chose lisinopril because it is the most commonly used ASI in our cohort. With 4 samples in each condition, we estimated a 70% power to detect 50% of the genes that are differentially expressed with a 2.5× fold change (13). A total of 148 genes were differentially expressed (FDR q-value < 0.05) in PDAC lesions of lisinopril users versus non-ASI users (Supplementary Table S7). The expression of 80 genes was higher and 68 genes lower in the PDAC lesions of lisinopril users. To systematically analyze our RNA-Seq results, we

Table 1. Patient characteristics, unadjusted univariate model, and adjusted analysis with multivariate model, of OS in patients with resected primary tumor

		Patient characteristics					Unadjusted univariate model					Adjusted analysis with multivariate model			
		No ASI	ASI	Total	*P*	FDR q-value	*n*	Median survival (months)	95% CI	*P*	FDR q-value	HR	95% CI	*P*	FDR q-value
Age	Y	64.6 ± 10.7	69.1 ± 10.0	299	<0.001	0.007	299	1.002 (HR)	0.99–1.02	0.758	0.816	0.999	0.999–1.014	0.911	0.911
Size	cm	2.9 ± 1.1	3.0 ± 1.5	299	0.625	0.813	299	1.14 (HR)	1.06–1.23	<0.001	0.005	1.161	1.061–1.271	0.001	0.005
BMI	kg/m^2	25.4 ± 4.6	27.4 ± 5.7	299	0.02	0.0867	299	0.984 (HR)	0.956–1.012	0.261	0.332	0.99	0.960–1.021	0.533	0.622
Site	Head	40 (24%)	33 (25%)	73 (24%)	0.732	0.848	73	26.7	16.9–36.6	0.941	0.941	0.96	0.663–1.389	0.828	0.892
	Other	129 (76%)	97 (75%)	226 (76%)			226	26.5	20.6–32.4						
Margin	−	137 (81%)	107 (82%)	244 (82%)	0.783	0.848	244	29.9	23.3–36.6	0.002	0.006	1.554	1.068–2.261	0.021	0.049
	+	32 (19%)	23 (18%)	55 (18%)			55	18.8	15.9–21.7						
LNR	0	49 (29%)	45 (35%)	94 (32%)	0.393	0.754	94	49.6	33.8–65.3	<0.001	0.005	1.503	1.209–1.869	<0.001	0.005
	≤0.2	65 (39%)	52 (40%)	117 (39%)			117	24.9	17.4–32.4						
	>0.2	54 (32%)	33 (25%)	87 (29%)			87	16.3	12.9–19.7						
Grade	1 & 2	103 (61%)	75 (58%)	178 (60%)	0.57	0.813	178	32.5	22.7–42.3	0.002	0.006	1.524	1.133–2.050	0.005	0.018
	3 & 4	66 (39%)	55 (42%)	121 (40%)			121	18.3	14.5–22.1						
LVI	−	72 (43%)	48 (37%)	120 (40%)	0.3	0.754	120	39.6	23.9–55.4	<0.001	0.005	1.138	0.810–1.600	0.456	0.58
	+	96 (57%)	82 (63%)	178 (60%)			178	19.8	16.1–23.6						
PNI	−	20 (12%)	15 (12%)	35 (12%)	0.908	0.908	35	54.1	NA	0.004	0.009	1.519	0.892–2.587	0.124	0.193
	+	147 (88%)	115 (88%)	262 (88%)			262	23.5	17.9–29						
Naj Tx	−	128 (76%)	107 (82%)	235 (79%)	0.17	0.553	235	25.4	19.4–31.4	0.22	0.308	0.796	0.530–1.196	0.272	0.381
	+	41 (24%)	23 (18%)	64 (21%)			64	28.7	20.8–36.6						
Adj RT	−	112 (66%)	82 (63%)	194 (65%)	0.566	0.813	194	21.7	15–28.4	0.124	0.217	0.712	0.503–1.007	0.055	0.096
	+	57 (34%)	48 (37%)	105 (35%)			105	31.7	23–40.4						
Adj Chemo	−	36 (21%)	33 (25%)	69 (23%)	0.406	0.754	69	16.2	8.8–23.6	0.202	0.308	0.597	0.398–0.896	0.013	0.036
	+	133 (79%)	97 (75%)	230 (77%)			230	28.3	23.1–33.5						
HTN	−	114 (67%)	2 (2%)	116 (39%)	<0.001	0.007	116	21.6	13.8–29.4	0.535	0.624	1.507	1.010–2.249	0.044	0.088
	+	55 (33%)	128 (98%)	183 (61%)			183	28.7	21.3–36.1						
ASI	−						169	19.3	15.6–22.9	0.011	0.022	0.505	0.339–0.750	0.001	0.005
	+						130	36.2	28.2–44.3						

NOTE: Significant values highlighted in red.

Abbreviations: Adj, adjuvant; Chemo, chemotherapy; CI, confidence interval; HTN, hypertension; LVI, lymphovascular invasion; Naj Tx, neoadjuvant treatment; PNI, perineural invasion; RT, radiotherapy.

Table 2A. PANTHER overrepresentation test, using GO Ontology database, on genes that are differentially downregulated with ACEi use in human PDAC tissue

Gene Ontology enrichment analysis		
GO cellular component complete	***P***	**FDR q-value**
Intermediate filament (GO:0005882)	0.0053	0.0092
Intermediate filament cytoskeleton (GO:0045111)	0.0181	0.0253
Proteinaceous extracellular matrix (GO:0005578)	0.0254	0.0296
Extracellular matrix (GO:0031012)	0.0486	0.0486
Extracellular space (GO:0005615)	<0.0001	0.0002
Extracellular region part (GO:0044421)	<0.0001	0.0002
Extracellular region (GO:0005576)	<0.0001	0.0002

performed gene annotation enrichment analysis, GO (http://geneontology.org/page/go-enrichment-analysis). In the cellular component category, gene sets associated with intermediate filaments and ECM remodeling were less expressed in lisinopril versus non-ASI lesions (Table 2A). Similarly, analysis of REACTOME gene sets (http://www.reactome.org) revealed that differentially expressed genes were involved in ECM remodeling and organization (Table 2B). For example, ECM (*TNC*, *COL4A5*, *HAPLN1*) and matrix-degrading enzyme (*MMP10*, *MMP13*) genes were expressed at significantly lower levels in the lesions of lisinopril than non-ASI patients (Table 3). In lesions of lisinopril patients, the WNT signaling ligand WNT10a, which is known to enhance fibrosis (14), was also less expressed, whereas *WISP2*, which plays a role in the WNT-1 signaling pathway, and inhibits fibrosis and invasion, was highly expressed (Table 3). Complete results of the GO and REACTOME analyses are presented in Supplementary Tables S8 and S9. Our results indicate that ASI/lisinopril can induce a normalization of the tumor stroma.

Finally, we performed GSEA using the complete expression dataset. The GSEA results in PDAC lesions of lisinopril users versus ASI-naïve patients showed that gene sets linked to integrin signaling, Notch, WNT, and the cell cycle were underexpressed, whereas pathways linked to oxidative phosphorylation, PPAR signaling, normal pancreas function, and antitumor immune response were overexpressed (Fig. 2). In lesions of lisinopril users, we found enrichment for gene sets linked to T-cell activity, and antigen processing and presentation (Fig. 2; Supplementary Table S10). Several genes that were differentially expressed are also associated with the functional activity of T cells and antigen presenting cells (APC). In PDAC lesions of ASI users, we found a higher expression of gene transcripts for *CCL4*, a chemokine that stimulates the recruitment of immature dendritic cells (DC) and Th1-polarized T cells (15), the DC marker *CD209*, and *CCL21* and *IRF8*, two genes that play significant roles in the differentiation/maturation of DCs (Table 3; refs. 16, 17). Furthermore, lisinopril increased the expression of the *WT1* gene, a tumor-associated antigen, and MHC class II gene *HLA-DQB1* expressed by APCs (Table 3). The increased DC/APC activity in lisinopril-treated PDAC lesions was associated with a higher expression of *TNFRSF8*, expressed by activated T cells and B-cells, which promotes the survival of memory T cells (18). The complete GSEA analysis, including GO, BIOCARTA, KEGG, PID, and REACTOME pathways, is included in Supplementary Table S11. Collectively, our results suggest that lisinopril use normalizes the PDAC microenvironment, reduces PDAC progression, and increases antitumor immunity.

Table 2B. REACTOME pathway enrichment analysis

REACTOME pathway analysis		
Pathway name	***P***	**FDR q-value**
Keratinization	1.11E−16	7.77E−16
Formation of the cornified envelope	1.11E−16	7.77E−16
Developmental biology	1.53E−12	7.14E−12
Collagen degradation	3.03E−06	1.06E−05
Oxidative stress Induced senescence	7.11E−06	1.99E−05
Activation of matrix metalloproteinases	1.65E−05	3.42E−05
Assembly of collagen fibrils and other multimeric structures	1.71E−05	3.42E−05
Extracellular matrix organization	2.74E−05	4.80E−05
Oncogene Induced senescence	3.49E−05	5.17E−05
Collagen formation	3.69E−05	5.17E−05
Degradation of the extracellular matrix	4.72E−05	6.01E−05
Regulation of pyruvate dehydrogenase (PDH) complex	6.67E−05	7.78E−05
Cellular senescence	3.64E−04	3.92E−04
Pyruvate metabolism	7.77E−04	7.77E−04

Expression signature induced by ASI use alone is associated with longer OS

The survival advantage associated with chronic ASI use in nonmetastatic patients as well as the gene expression changes induced by lisinopril prompted further analysis in independent patient cohorts. We intersected our RNA-Seq results with publicly available primary PDAC gene expression data that also included survival information. Two datasets are used in our study: TCGA (n = 178) and UNC datasets (n = 125; ref. 11). First, we investigated in our RNA-Seq data the genes with a significantly lower expression in PDAC lesions of lisinopril users (Supplementary Table S7), in these two independent cohorts. Using the algorithm Pathifier (12), we calculated a deregulation score, collapsing the expression level of all lower expressed ASI genes into one measurement, for each patient. Next, we divided patients in each cohort into three groups (low, medium, and high) based on their deregulation score. In the UNC (Fig. 3A) and TCGA (Fig. 3B) cohorts, patients in the low category, those with the lowest expression of genes that also had lower expression in lisinopril-using patients, lived significantly longer than patients with high- or mid-level expression. In the TCGA dataset, which was the only dataset to also provide other clinical parameters, the low expression category remained significant after correcting for tumor site, lymph node status, and other potential confounders (Supplementary Table S12A). Stratification of patients based on genes that were expressed higher in ASI treatment in our RNA-Seq dataset did not reach statistical significance (Supplementary Table S12B, C). This indicates that genes associated with pancreatic tumor progression and extracellular matrix production, which has a lower expression in lisinopril-treated patients, likely play a significant role in the observed survival benefit.

Discussion

We retrospectively analyzed the effect of chronic ASI use on survival in patients with all stages of PDAC. Adjusted analysis with multivariate modeling showed that chronic ASI use was an independent factor associated with longer OS in PDAC patients with resected disease or locally advanced disease, but not in patients with metastatic disease. The benefit provided by ASI is independent of chemotherapy or radiotherapy, as well as other

Table 3. Changes in gene expression in human PDAC with lisinopril use

Name	Descriptor	Control	Lisinopril	Fold	FDR q-value
TNC	Tenascin C	21.5	3.7	−5.8	0.0093
HAPLN1	Hyaluronan and proteoglycan link protein 1	9.56	0.27	−35.4	0.009
WNT10A	Wnt family member 10A	16.87	2.6	−6.5	0.0345
COL4A5	Collagen type IV alpha 5 chain	8.4	1.6	−5.3	0.0093
MMP10	Matrix metalloproteinase-10	13.7	1.1	−12.5	0.0093
MMP13	Matrix metalloproteinase-13	11	0.4	−27.5	0.0145
CCl4	Chemokine ligand 4	5	35.5	7.1	0.028
CD209	DC-SIGN	0.7	5.4	7.7	0.0145
CCL21	Chemokine ligand 21	5.2	42.1	8.2	0.0145
IRF8	IFN-regulatory factor 8	6	28.4	4.7	0.0093
WT1	Wilm tumor protein	0.8	8.7	10.9	0.0093
TNFRSF8	TNF Rc superfamily member 8	0.39	4.12	10.6	0.028
WISP2	WNT1-inducible signaling pathway protein 2	1.64	64.73	39.5	0.0093

pathologic features known to be associated with survival. Propensity score–matched analysis showed longer OS in chronic ASI users as well. To determine the biological mechanisms of ASI action, we performed RNA-Seq and identified functional gene categories altered by ASI. Our results reveal that the ASI/ACEi lisinopril therapy is associated with normalization of the tumor stroma, reduced tumor progression, and antitumor immunity. We also identified a signature of genes downregulated by lisinopril, which is significantly associated with patient survival in independent validation cohorts.

Previous retrospective analyses of patients with locally advanced and metastatic PDAC treated with gemcitabine revealed that ASI therapy increased OS from 8.9 months to 15.1 months (19). A prospective phase I trial comparing gemcitabine with or without candesartan in patients with locally advanced or metastatic PDAC reported encouraging survival data (20). However, the subsequent phase II trial did not show a survival advantage for candesartan combined with gemcitabine versus gemcitabine alone (21). These conflicting results might have resulted from heterogeneous patient cohorts, which included patients with locally advanced as well as metastatic disease. Our results do not conflict with those studies, showing that patients with locally advanced PDAC benefit from RAAS inhibition, while there is no survival advantage for patients with metastasis.

In patients who underwent primary tumor resection, ASI use is also associated with a significant increase in median survival of 17 months (19.3 vs. 36.3 months) in unadjusted univariate analysis, and an HR as low as 0.505 (0.339–0.750) in adjusted analysis with multivariate modeling. Furthermore, our subgroup analysis suggests that ASI therapy with or without chemotherapy improves the survival of PDAC patients who underwent pancreatectomy. In a small number of hypertensive patients using ASI ($N = 22$) that did not receive adjuvant chemotherapy following surgery, we found that median survival was of 39.2 months, which is surprising given that the other recorded parameters between ASI users and naïve patients were comparable (Table 1). In comparison, the median survival for patients who underwent surgery alone has been reported to vary between 13.0 and 20.2 months (22, 23), whereas in patients who received adjuvant chemotherapy after surgery, median survival can vary between 22.6 and 23.6 months (22, 23).

Our transcriptome analysis suggests that the ASI lisinopril can induce changes in ECM remodeling and organization, increase oxidative phosphorylation, and reduce the activity of profibrotic pathways like RAAS and WNT. Profibrotic pathways are known to promote tumor desmoplasia, which acts as a physical barrier to drug delivery and immune cell infiltration (24–26), thus reducing the efficacy of chemotherapy and immunotherapy. Desmoplasia compresses blood vessels and promotes a hypoxic tumor microenvironment, which further aggravates immunosuppression (6, 27, 28). In murine models, we have shown that inhibition of the RAAS reduces tumor desmoplasia and improves vascular perfusion and drug delivery (6). Consequently, hypoxia is relieved and the efficacy of chemotherapeutic drugs, oncolytic viruses, or vaccine therapy is significantly enhanced (5, 6, 29). Thus, our findings suggest that changes in ECM remodeling and organization could explain the increase in oxidative phosphorylation and the improved efficacy of chemotherapy in ASI users.

The survival advantage of ASI use by itself, revealed by our adjusted multivariate model analysis, could also be related to antitumor immunity, and altered proliferation or aggressiveness of cancer cells. Our GSEA analysis showed that cell-cycle gene sets were underrepresented in PDAC lesions of lisinopril users versus ASI-naïve patients. A reduction in cell proliferation induced by ASI is consistent with previous reports that the angiotensin II receptor type 1 (AT1R) blocker losartan inhibits the growth of tumor cells overexpressing AT1R (3). Angiotensin receptors are also expressed in human PDAC and pancreatic cancer cell lines (30), and ASI can directly inhibit pancreatic cancer growth *in vitro* and *in vivo* (31). Other studies suggest an antiangiogenic mechanism of ASI in PDAC models. In PDAC lesions of lisinopril users, our GSEA results also showed a lower expression of gene sets associated with integrin-mediated interaction of cells with the ECM, and WNT and Notch signaling. In experimental PDAC models, WNT and Notch signaling promote early tumorigenesis (32, 33). Furthermore, the expression of WNT signaling proteins (β-catenin, WNT2) in PDAC lesions correlates with reduced survival of patients with a pancreatectomy (34). In addition, the activation of WNT signaling increases the formation of lymph node and liver metastases, but does not induce metastatic seeding in the lung (34). Thus, ASI inhibition of WNT signaling could inhibit metastatic spread to the liver. This is consistent with our data showing that metastatic recurrence in the liver, but not in the lung, was significantly reduced in chronic ASI users.

In PDAC, the intratumoral infiltrations of myeloid cells, macrophages, B cells, neutrophils, and Tregs contribute to the immunosuppressive microenvironment (35–37), while the poor recruitment of CD8-positive T cells and antigen-presenting DCs correlates with poor prognosis (38). The RAAS can stimulate the immunosuppressive function of monocytes, tumor-associated macrophages, and neutrophils (39–41). Our results suggest that ASI therapy stimulates antitumor immunity. The resected PDAC

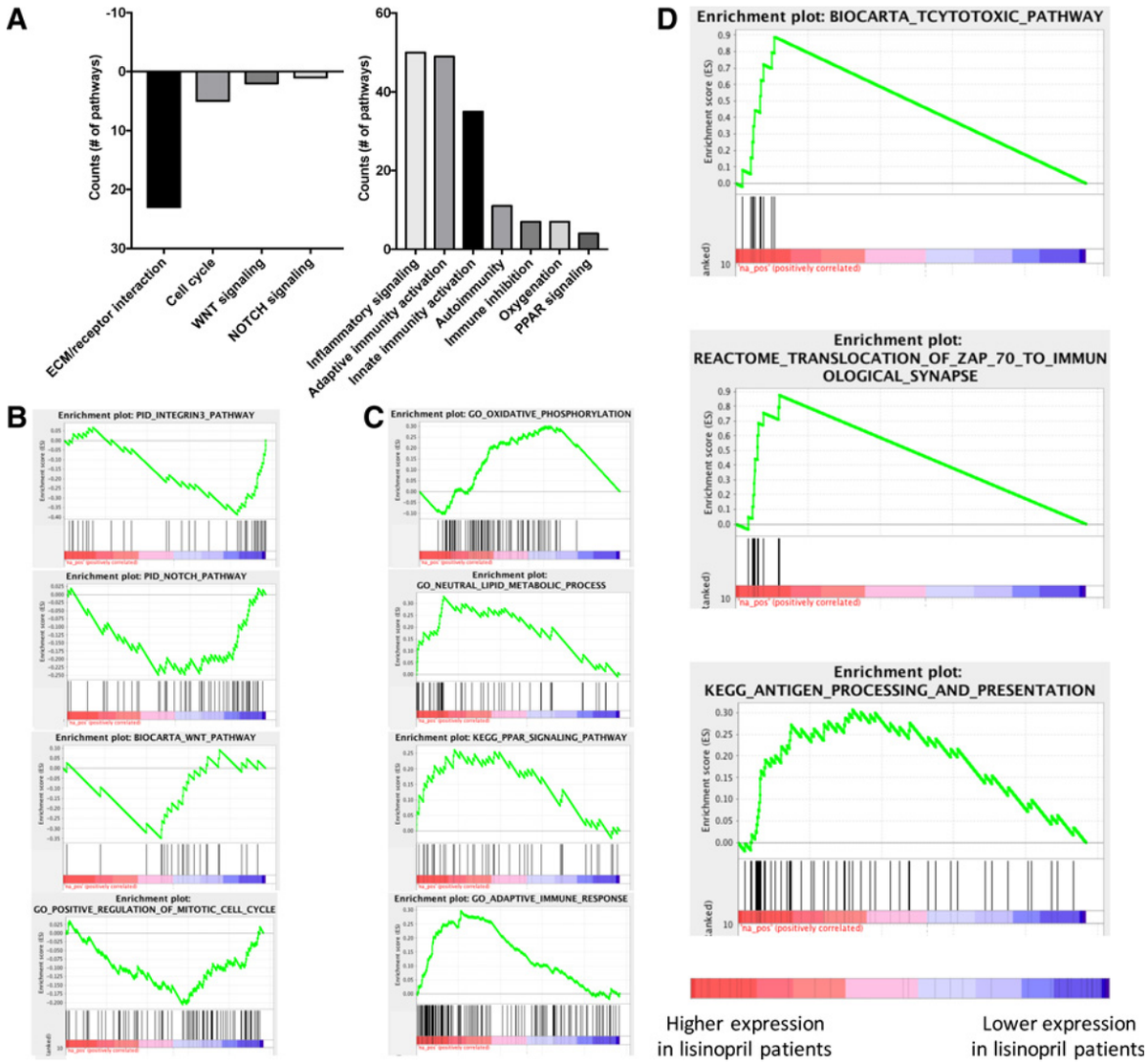

Figure 2.

Number of pathways in GO, BIOCARTA, KEGG, PID, and REACTOME gene sets that are significantly changed via GSEA analysis, grouped in biological functions, at a threshold of FDR q-value < 0.05. GSEA of human PDAC comparing ACEi-treated tumors versus control tumors. Downregulated pathways included, for example, the activity of integrin beta 3, Notch, WNT, and the cell cycle. Upregulated pathways included oxidative phosphorylation, improvement in lipid metabolism, PPAR signaling, and various adaptive immune response pathways, including cytotoxic and antigen presentation pathways (detailed enrichment score is provided in Supplementary Table S11).

samples of lisinopril users were enriched for genes sets linked to antigen processing and presentation, and activity of T cells. We have recently shown in obese mice that losartan decreases the recruitment and immunosuppressive effects of neutrophils and increases the recruitment of CD8-positive T cells in PDAC (40). In another study, Vanpouille-Box and colleagues showed that local radiation and blockade of TGFβ, which is downstream of RAAS pathway, can be used to enhance autovaccination in PDAC (42). These findings suggest that inhibition of TGFβ or RAAS signaling could stimulate the efficacy of immunotherapy in PDAC.

Hypertension is a common comorbidity for this disease. ASIs are also widely used for the treatment of hypertension. Other commonly encountered medical conditions or medications have been shown to correlate with survival in PDAC patients. For example, long-term but not recent diabetes (less than 4 years before diagnosis) has a negative impact on survival (43). Similarly, the use of metformin, a drug used to treat diabetes, increased survival in preclinical PDAC models (44) and correlated with increased survival in retrospective analyses (45). However, in other retrospective studies, metformin use did not show a survival advantage (46). A randomized controlled phase II trial testing metformin in patients with advanced PDAC treated with gemcitabine and erlotinib showed no benefit (47). Diabetes in PDAC is more complicated compared with

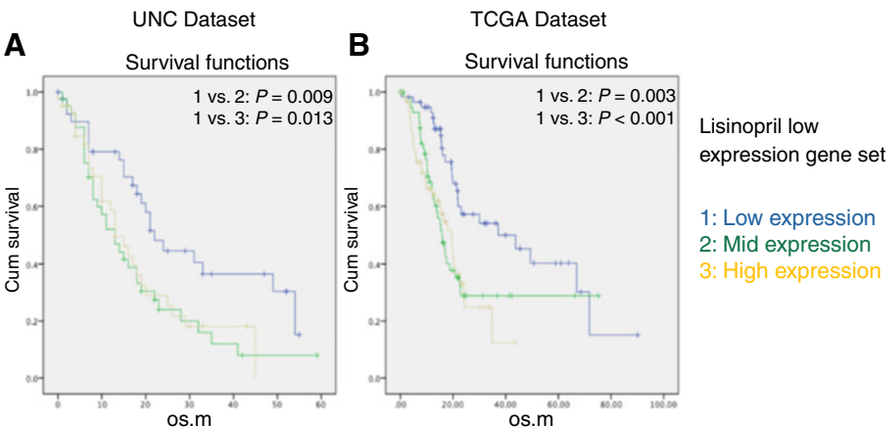

Figure 3.
Kaplan–Meier analysis on ASI downregulated gene pathway and OS. ASI pathway expression level: 1 = low, 2 = med, 3 = high. **A,** UNC dataset. **B,** TCGA dataset.

other cancer types, as the pancreas is a vital organ that maintains blood glucose levels. Diabetes in PDAC patients can be unrelated to pancreatic cancer, caused by pancreatic cancer, or caused by pancreatectomy.

Because of the known effect of obesity on PDAC progression and survival, we also addressed the combined effect of BMI and ASI on PDAC survival. Our stratified analysis showed that ASIs provide a significant survival benefit in patients with BMI <25, whereas in ASI users with a BMI >25, the survival was longer but not significant. ASI may still have beneficial effects in obese patients. In a mouse model, we found that losartan inhibition of the RAAS decreased obesity-induced inflammation, fibrosis, and tumor growth (40, 48). In our preclinical study on obesity, we did not assess the effect of losartan on survival. The time of BMI assessment could also affect our findings. In other patient cohorts, baseline BMI measured 2 to several years prior to PDAC diagnosis correlates with overall survival (49). However, BMI at the time of diagnosis or after treatment is less clearly associated with survival, especially as low body weight associated with cachexia is also a known risk factor of poor survival. Further analysis of how obesity, diabetes, and metformin affect the treatment outcome of ASI users should be evaluated in prospective pancreatic cancer trials.

Our results advocate for an early and prolonged angiotensin system inhibition in PDAC patients. With the changing paradigm in favoring early systemic treatment, and the promising efficacy of neoadjuvant FOLFIRINOX and chemoradiation therapy in locally advanced/borderline disease (50), ASI could be added to the preoperative cytotoxic therapy. In our ongoing phase II clinical trial at MGH (ClinicalTrials.gov identifier: NCT01821729), we are currently testing the efficacy of the AT1R blocker losartan combined with FOLFIRINOX, followed by chemoradiation in patients with locally advanced PDAC.

For patients with resectable disease, prospective trials should be designed to test the benefit of the addition of ASI to neoadjuvant chemotherapy and the continuous administration of ASI following surgical resection. The results of our retrospective analysis suggest that ASI improves survival in nonmetastatic patients but not in metastatic patients. Thus, it will be critical to determine in experimental models of primary versus metastatic PDAC how ASIs alone or combined with cytotoxic agents or immunotherapies affect RAAS signaling, the tumor microenvironment, adaptive and innate immune cells, and tumor response. A translational bench to bedside approach will likely identify how to best combine ASI with other agents for the treatment of PDAC patients.

Limitations

Our retrospective analysis is based on unselected single-institute experience combined with other independent retrospective cohorts. The retrospective nature of this study means it is prone to selection bias. In our cohort, the percentage of patients who presented with resectable disease was higher compared with national statistics. For the adjusted multivariate modeling, we included all available parameters. We also performed propensity score analysis. However, there may still be other unrecognized confounders.

Second, the duration of ASI usage before the time of diagnosis was difficult to assess. However, a significant fraction of patients was on ASI because of hypertension, which is a chronic condition. Very few patients switched medications during the follow-up. We thus assumed that all patients who repeatedly had ASI in their active medication were long-term ASI users. We also used propensity score–weighted analysis to adjust for the potential bias regarding the probability of patients receiving ASI treatment. Our conclusions remained the same after propensity score analysis.

Third, the sample size of the prospective clinical tumor collection for RNA-Seq analysis is relatively small. During the time of prospective sample collection, a neoadjuvant chemotherapy trial was ongoing, which explains in part the relatively low number of treatment-naïve PDAC cases included in our analysis. Even though the sample size was small, our transcriptome analysis identified key gene sets and pathways that confirmed known effects of ASI on RAAS inhibition and revealed novel pathways/mechanisms, which we will explore in greater depth in future studies.

Conclusions

In summary, our retrospective analysis shows that the chronic use of ASI is associated with significantly longer OS in PDAC patients with nonmetastatic disease, independent of anticancer treatment or tumor characteristics. ASI use likely has microenvironment normalizing and immunostimulatory effects in PDAC patients and is also associated with an expression signature predictive of patient survival. Prospective randomized trials are needed to confirm the efficacy of ASI use in PDAC.

Disclosure of Potential Conflicts of Interest

M. Pinter reports receiving travel grants and speaker fees from Bayer and is a consultant/advisory board member for Bayer and Bristol-Myers Squibb. D.P. Ryan is an advisor of MPM Capital. Y. Boucher reports receiving consultant fees from XTuit. R.K. Jain reports receiving consultant fees from Ophthotech, SPARC, SynDevRx, and XTuit, owns equity in Enlight, SPARC, SynDevRx, and XTuit, and serves on the Board of Directors of XTuit and Boards of Trustees of Tekla Healthcare Investors, Tekla Life Sciences Investors, Tekla Healthcare Opportunities Fund, and Tekla World Healthcare Fund. No potential conflicts of interest were disclosed by the other authors.

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References

1. Benigni A, Cassis P, Remuzzi G. Angiotensin II revisited: new roles in inflammation, immunology and aging. EMBO Mol Med 2010;2:247–57.
2. George AJ, Thomas WG, Hannan RD. The renin–angiotensin system and cancer: old dog, new tricks. Nat Rev Cancer 2010;10:745–59.
3. Rhodes DR, Ateeq B, Cao Q, Tomlins SA, Mehra R, Laxman B, et al. AGTR1 overexpression defines a subset of breast cancer and confers sensitivity to losartan, an AGTR1 antagonist. Proc Natl Acad Sci U S A 2009;106:10284–9.
4. Netti PA, Berk DA, Swartz MA, Grodzinsky AJ, Jain RK. Role of extracellular matrix assembly in interstitial transport in solid tumors. Cancer Res 2000;60:2497–503.
5. Diop-Frimpong B, Chauhan VP, Krane S, Boucher Y, Jain RK. Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors. Proc Natl Acad Sci U S A 2011;108:2909–14.
6. Chauhan VP, Martin JD, Liu H, Lacorre DA, Jain SR, Kozin SV, et al. Angiotensin inhibition enhances drug delivery and potentiates chemotherapy by decompressing tumour blood vessels. Nat Commun 2013; 4:2516.
7. Stylianopoulos T, Martin JD, Chauhan VP, Jain SR, Diop-Frimpong B, Bardeesy N, et al. Causes, consequences, and remedies for growth-induced solid stress in murine and human tumors. Proc Natl Acad Sci U S A 2012; 109:15101–8.
8. Nia HT, Liu H, Seano G, Datta M, Jones D, Rahbari N, et al. Solid stress and elastic energy as measures of tumour mechanopathology. Nat Biomed Eng 2016;1:0004.
9. Murphy JE, Wo JY, Ferrone CR, Wenqing J, Yeap B, Blaszkowsky LS, et al. TGF-B1 inhibition with losartan in combination with FOLFIRINOX (F-NOX) in locally advanced pancreatic cancer (LAPC): preliminary feasibility and R0 resection rates from a prospective phase II study. 2017 Gastrointestinal Cancers Symposium, Virtual Meeting, Meeting Library [Internet]. J Clin Oncol 35, 2017(suppl 4S; abstract 386).
10. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 2012;7:562–78.
11. Moffitt RA, Marayati R, Flate EL, Volmar KE, Loeza SGH, Hoadley KA, et al. Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma. Nat Genet 2015;47:1168–78.
12. Drier Y, Sheffer M, Domany E. Pathway-based personalized analysis of cancer. Proc Natl Acad Sci U S A 2013;110:6388–93.
13. Busby MA, Stewart C, Miller CA, Grzeda KR, Marth GT. Scotty: a web tool for designing RNA-Seq experiments to measure differential gene expression. Bioinformatics 2013;29:656–7.
14. Kuma A, Yamada S, Wang KY, Kitamura N, Yamaguchi T, Iwai Y, et al. Role of WNT10A-expressing kidney fibroblasts in acute interstitial nephritis. PLoS One 2014;9:e103240.
15. Mukaida N, Sasaki SI, Baba T. Chemokines in cancer development and progression and their potential as targeting molecules for cancer treatment. Mediators Inflamm 2014;2014:170381.
16. Marsland BJ, Bättig P, Bauer M, Ruedl C, Lässing U, Beerli RR, et al. CCL19 and CCL21 induce a potent proinflammatory differentiation program in licensed dendritic cells. Immunity 2005;22:493–505.
17. Murphy TL, Grajales-Reyes GE, Wu X, Tussiwand R, Briseño CG, Iwata A, et al. Transcriptional control of dendritic cell development. Annu Rev Immunol 2016;34:93–119.
18. Sabbagh L, Snell LM, Watts TH. TNF family ligands define niches for T cell memory. Trends Immunol 2007;28:333–9.
19. Nakai Y, Isayama H, Ijichi H, Sasaki T, Sasahira N, Hirano K, et al. Inhibition of renin-angiotensin system affects prognosis of advanced pancreatic cancer receiving gemcitabine. Br J Cancer 2010;103:1644–8.
20. Nakai Y, Isayama H, Ijichi H, Sasaki T, Kogure H, Yagioka H, et al. Phase I trial of gemcitabine and candesartan combination therapy in normotensive patients with advanced pancreatic cancer: GECA1. Cancer Sci 2012;103:1489–92.
21. Nakai Y, Isayama H, Ijichi H, Sasaki T, Takahara N, Ito Y, et al. A multicenter phase II trial of gemcitabine and candesartan combination therapy in patients with advanced pancreatic cancer: GECA2. Invest New Drugs 2013;31:1294–9.
22. Horowitz DP, Hsu CC, Wang J, Makary MA, Winter JM, Robinson R, et al. Adjuvant chemoradiation therapy after pancreaticoduodenectomy in elderly patients with pancreatic adenocarcinoma. Int J Radiat Oncol Biol Phys 2011;80:1391–7.
23. Oettle H, Neuhaus P, Hochhaus A, Hartmann JT, Gellert K, Ridwelski K, et al. Adjuvant chemotherapy with gemcitabine and long-term outcomes among patients with resected pancreatic cancer. JAMA 2013;310:1473.

24. Jain RK. Normalizing tumor microenvironment to treat cancer: bench to bedside to biomarkers. J Clin Oncol 2013;31:2205–18.
25. Watt J, Kocher HM. The desmoplastic stroma of pancreatic cancer is a barrier to immune cell infiltration. Oncoimmunology 2013;2: e26788.
26. Whatcott CJ, Han H, Hoff Von DD. Orchestrating the tumor microenvironment to improve survival for patients with pancreatic cancer: normalization, not destruction. Cancer J 2015;21:299–306.
27. Jain RK, Martin JD, Stylianopoulos T. The role of mechanical forces in tumor growth and therapy. Annu Rev Biomed Eng 2014;16:321–46.
28. Jain RK. Antiangiogenesis strategies revisited: from starving tumors to alleviating hypoxia. Cancer Cell 2014;26:605–22.
29. Huang Y, Yuan J, Righi E, Kamoun WS, Ancukiewicz M, Nezivar J, et al. Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy. Proc Natl Acad Sci U S A 2012;109:17561–6.
30. Arafat HA, Gong Q, Chipitsyna G, Rizvi A, Saa CT, Yeo CJ. Antihypertensives as novel antineoplastics: angiotensin-I-converting enzyme inhibitors and angiotensin II type 1 receptor blockers in pancreatic ductal adenocarcinoma. J Am Coll Surg 2007;204:996–1005.
31. Fujimoto Y, Sasaki T, Tsuchida A, Chayama K. Angiotensin II type 1 receptor expression in human pancreatic cancer and growth inhibition by angiotensin II type 1 receptor antagonist. FEBS Lett 2001;495: 197–200.
32. Avila JL, Kissil JL. Notch signaling in pancreatic cancer: oncogene or tumor suppressor? Trends Mol Med 2013;19:320–7.
33. Zhang Y, Morris JP, Yan W, Schofield HK, Gurney A, Simeone DM, et al. Canonical wnt signaling is required for pancreatic carcinogenesis. Cancer Res 2013;73:4909–22.
34. Sano M, Driscoll DR, DeJesus-Monge WE, Quattrochi B, Appleman VA, Ou J, et al. Activation of WNT/β-catenin signaling enhances pancreatic cancer development and the malignant potential via up-regulation of Cyr61. Neoplasia 2016;18:785–94.
35. Clark CE, Hingorani SR, Mick R, Combs C, Tuveson DA, Vonderheide RH. Dynamics of the immune reaction to pancreatic cancer from inception to invasion. Cancer Res 2007;67:9518–27.
36. Clark CE, Beatty GL, Vonderheide RH. Immunosurveillance of pancreatic adenocarcinoma: insights from genetically engineered mouse models of cancer. Cancer Lett 2009;279:1–7.
37. Gunderson AJ, Kaneda MM, Tsujikawa T, Nguyen AV, Affara NI, Ruffell B, et al. Bruton tyrosine kinase-dependent immune cell cross-talk drives pancreas cancer. Cancer Discov 2016;6:270–85.
38. Ino Y, Yamazaki-Itoh R, Shimada K, Iwasaki M, Kosuge T, Kanai Y, et al. Immune cell infiltration as an indicator of the immune microenvironment of pancreatic cancer. Br J Cancer 2013;108:914–23.
39. Cortez-Retamozo V, Etzrodt M, Newton A, Ryan R, Pucci F, Sio SW, et al. Angiotensin II drives the production of tumor-promoting macrophages. Immunity 2013;38:296–308.
40. Incio J, Liu H, Suboj P, Chin SM, Chen IX, Pinter M, et al. Obesity-induced inflammation and desmoplasia promote pancreatic cancer progression and resistance to chemotherapy. Cancer Discov 2016;6:852–69.
41. Engblom C, Pfirschke C, Pittet MJ. The role of myeloid cells in cancer therapies. Nat Rev Cancer 2016;16:447–62.
42. Vanpouille-Box C, Diamond JM, Pilones KA, Zavadil J, Babb JS, Formenti SC, et al. TGFβ is a master regulator of radiation therapy-induced antitumor immunity. Cancer Res 2015;75:2232–42.
43. Yuan C, Rubinson DA, Qian ZR, Wu C, Kraft P, Bao Y, et al. Survival among patients with pancreatic cancer and long-standing or recent-onset diabetes mellitus. J Clin Oncol 2015;33:29–35.
44. Incio J, Suboj P, Chin SM, Vardam-Kaur T, Liu H, Hato T, et al. Metformin reduces desmoplasia in pancreatic cancer by reprogramming stellate cells and tumor-associated macrophages. PLoS One 2015;10:e0141392.
45. Amin S, Mhango G, Lin J, Aronson A, Wisnivesky J, Boffetta P, et al. Metformin improves survival in patients with pancreatic ductal adenocarcinoma and pre-existing diabetes: a propensity score analysis. Am J Gastroenterol 2016;111:1350–7.
46. Ambe CM, Mahipal A, Fulp J, Chen L, Malafa MP. Effect of metformin use on survival in resectable pancreatic cancer: a single-institution experience and review of the literature. PLoS One 2016;11:e0151632.
47. Kordes S, Pollak MN, Zwinderman AH, Mathôt RA, Weterman MJ, Beeker A, et al. Metformin in patients with advanced pancreatic cancer: a double-blind, randomised, placebo-controlled phase 2 trial. Lancet Oncol 2015; 16:839–47.
48. Incio J, Tam J, Rahbari NN, Suboj P, McManus DT, Chin SM, et al. PlGF/VEGFR-1 signaling promotes macrophage polarization and accelerated tumor progression in obesity. Clin Cancer Res 2016; 22:2993–3004.
49. Yuan C, Bao Y, Wu C, Kraft P, Ogino S, Ng K, et al. Prediagnostic body mass index and pancreatic cancer survival. J Clin Oncol 2013;31: 4229–34.
50. Ferrone CR, Marchegiani G, Hong TS, Ryan DP, Deshpande V, McDonnell EI, et al. Radiological and surgical implications of neoadjuvant treatment with FOLFIRINOX for locally advanced and borderline resectable pancreatic cancer. Ann Surg 2015;261:12–7.