Review Article

Targeting stromal microenvironment in pancreatic ductal adenocarcinoma: controversies and promises

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Contributions: (I) Conception and design: WW Ma, L Mei; (II) Administrative support: WW Ma; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: Pancreatic cancer is a highly lethal disease. Conventional therapeutics targeting pancreas cancer cell compartment using cytotoxics improved patient survival but at the expense of significant toxicity. Microscopically, the tumor is characterized by thick desmoplastic stroma that surrounds islands of pancreatic cancer cells. The tumor microenvironment has been found to play important roles in carcinogenesis, the development of drug resistance, and mediating immunosuppression. The understanding the tumor-stromal interaction has led to the development of novel therapeutic approaches. Here, we review the strategies that are currently in (or, near to) clinical evaluation and the underlying preclinical rationales.

Keywords: Pancreatic cancer; pancreatic adenocarcinoma; stroma; clinical trials; cancer-associated fibroblast (CAF)

Submitted Nov 30, 2015. Accepted for publication Dec 21, 2015.
doi: 10.21037/jgo.2016.03.03
View this article at: http://dx.doi.org/10.21037/jgo.2016.03.03

Introduction

Pancreatic cancer is a devastating disease, which is the fourth leading cause of cancer related death in the Unites States (1). The use of intensive cytotoxic regimen such as FOLFIRINOX has almost doubled the survival but the 2-year survival rate for patients with metastatic pancreatic ductal adenocarcinoma (PDA) is dismally <20% (2). Microscopically, PDA is characterized by thick desmoplastic stromal matrix surrounding islands of cancer cells. It is increasingly realized that the pancreatic stroma plays an active role in carcinogenesis, progression, metastasis, mediating drug resistance, and immunosuppression (3). PDA stroma is highly heterogeneous consisting of stellate cells [pancreatic stellate cells (PSCs)] or carcinoma-associated fibroblasts (CAFs) in activated form, microvasculature, nerves, inflammatory infiltrate and acellular extracellular matrix (ECM). Encouraging results from preclinical studies targeting the PDA stroma, such as using hyaluronidase to improve cytotoxic delivery and CD40 agonist to modulate immune response, renewed the field's enthusiasm and led to a number of clinical trials evaluating stromal-targeting therapy in PDA. However, the failure of hedgehog (Hh) inhibitor to improve patient outcome during clinical evaluation despite positive preclinical results is humbling and demonstrated the paucity in our understanding of the complex PDA biology (4-7). Here, we review the stromal-targeting strategies currently in (or, near-to) clinical evaluation and their preclinical rationales (*Table 1*).

The biology of stromal cells in PDA

The prominent feature of PDA is dense desmoplastic stroma, sometimes comprising up to 80% of tumor mass (42). As early as a decade ago, researchers found that highly heterogeneous components of the stroma, consisting of immune cells, CAF, ECM as well as varieties of proteins, enzymes, growth factors and cytokines, form a sophisticated network interacted with tumor cells (43). Depletion of stromal component has been associated with improved

Table 1 Role of stromal constituents in pancreatic adenocarcinoma

Target	Role	Reference
Cancer-associated fibroblasts (CAFs)	Role of pro-carcinogenesis effect	
	- Promote metastasis	(8,9)
	- Secret growth factors and enhance tumor progression	(10)
	- Cause chemotherapy or radiation therapy resistance by Hh signaling	(11-14)
	- Creating immunosuppressive environment by CXCL12 and IL-17	(15-17)
	Controversies	
	- Genetically or pharmacologically deleted CAF leads to more aggressive tumor growth	(18-20)
	- Vitamin D can reprogram the CAF	(21)
	Clinical trials	
	- Pharmacological ablation of Hh signaling failed to show any clinical benefit	(4-7)
Extracellular matrix (ECM)	Role of pro-carcinogenesis	
	- Collagen I expression is associated with gemcitabine resistance	(22)
	- Fibronectin, laminin, integrin and collagen IV are associated with tumor growth	(23,24)
	- Enzymatic ablated HA by PEGPH20 improved survival in mice models	(25-27)
	Clinical trials	
	- Phase Ib/II trials showed promising effect of PEGPH20 in PDA patients with high HA level	(28-31)
Immune Cells	Immune co-stimulatory factors and checkpoint regulators	
	Role of pro-carcinogenesis	
	- CD40-activated macrophages facilitate the depletion of tumor stroma and induce tmoricidal effect	(32,33)
	- PD-1 antibody showed remarkable response in NSCLC, melanoma and renal cell carcinoma	(34,35)
	- CTLA-4 is involved in disease acceleration and animal survival in PDA	(34)
	Controversies	
	- Only combined with CXCR4 inhibitor, PD-L1 antibody diminishing PDA progression	(16)
	Clinical trials	
	- Antagonizing PD-1 signal showed no responses PDA patients	(35,36)
	- Ipilimumab (CTLA-4 antibody) failed to show any effectiveness for stage III or IV PDA	(37)
	Inflammatory cytokine (IL-17) and cancer vaccine	
	Role of pro-carcinogenesis	
	- CAFs could attract Th17, contributing to immune-suppression in the PDA	(17)
	- Inhibition of IL-17 signaling effectively prevented PDA formation	(38)
	- Combination therapy of GVAX and PD-1 antibody improved PDA murine model survival	(39)
	Controversies	
	- GV1001 in combination with chemotherapy did not find any benefit in advanced PDA	(40)
	Clinical trials	
	- GVAX combined with CRS-207 extended survival in phase II trial for stage IV PDA	(41)

Hh, Hedgehog signal; CXCL12, Chemokine (C-X-C motif) ligand 12; IL-17, interleukin-17; HA, hyaluronan; PEGPH20, PEGylated human recombinant PH20 hyaluronidase; PDA, pancreatic ductal adenocarcinoma; PD-1, programmed cell death 1 receptor; NSCLC, non-small cell lung cancer; CTLA-4, cytotoxic T lymphocyte associated protein 4; CXCR4, chemokine (C-X-C motif) receptor 4; Th17, interleukin-17 secreting CD4+ cells; GVAX, granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transfected tumor cell vaccine; GV1001, simultaneous telomerase vaccination; CRS-207, an attenuated listeria monocytogenes.

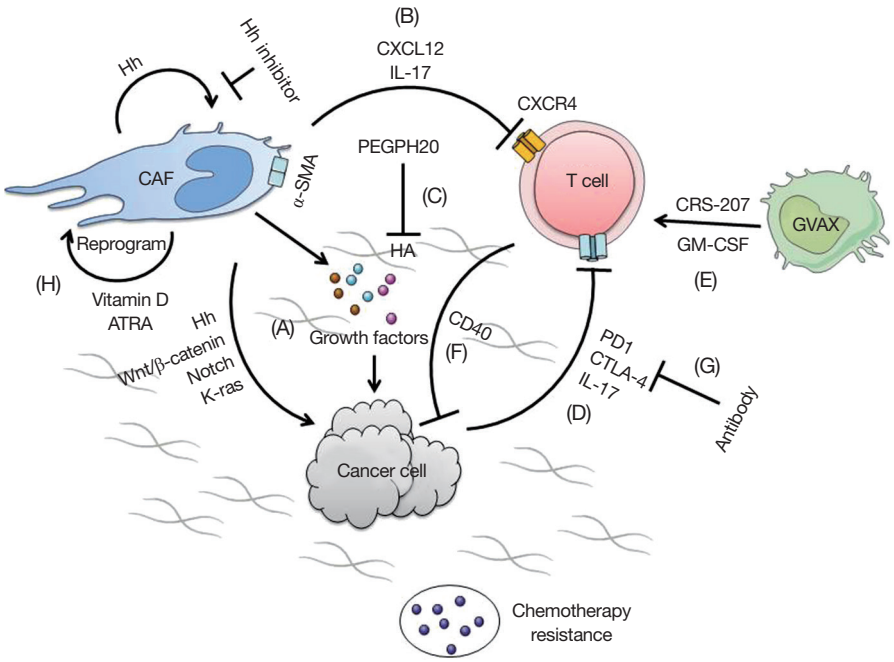

Figure 1 Interaction of stromal constituents with pancreatic ductal adenocarcinoma (PDA). (A) Cancer-associated fibroblasts (CAFs), which expressed α-smooth muscle actin (αSMA), facilitate themselves as well as tumor growth through Hedgehog (Hh), Wnt/β-catenin, Notch, K-ras signaling or production of growth factors; (B) secretion of chemokine (C-X-C motif) ligand 12 (CXCL12), the ligand of chemokine (C-X-C motif) receptor 4 (CXCR4) and interleukin-17 (IL-17) results in suppression of T cells; (C) hyaluronan (HA) reduces the therapeutic indices of chemotherapy agents and enzymatic ablated HA by PEGylated human recombinant PH20 hyaluronidase (PEGPH20) provides promising efficacy in clinical trials; (D) programmed cell death 1 receptor (PD-1), cytotoxic T lymphocyte associated protein 4 (CTLA-4) and IL-17 signaling generated by cancer cell promote to create the immunosuppressive microenvironment; (E) GVAX [granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transfected tumor cell vaccine] combined with CRS-207 (an attenuated listeria monocytogenes) enhances T cell's anti-tumor effect; (F) CD40 stimulation may led to macrophage-mediated cancer cell kill and stromal depletion; (G) targeting of CXCL12 and IL-17 may reverse intra-tumor immune-suppression that enhance the effects of immune checkpoint inhibitors (PD-1, CTLA-4); (H) reprogramming the PDA stroma using vitamin D or all-trans retinoic acid (ATRA) may induce stromal quiescence and reverse tumor progression.

prognosis in some animal models and early-stage clinical trials (11,32,42). α-smooth muscle actin (αSMA) secreted by CAF is confirmed as a negative prognostic factor in PDA (44). Whereas, the current outcome of clinical trials targeting pancreatic stroma does not meet the high expectation. More importantly, recently published several studies provide new explanations for the failure of trials, suggesting ablation of the stroma may lead to poorly differentiated tumors and accelerate PDA progression (18-21). These conflicting evidences prompt us to revisit the role of stromal cell.

Cancer-associated fibroblasts (CAFs)

CAFs have been found to promote tumor progression and metastasis in pancreatic cancer (8,9). The stromal cells mediate the formation of ECM that protects cancer stem cells, secrets growth factors promoting tumor proliferation, and interrupt immune-surveillance resulting in immunosuppressive tumor microenvironment (10,15) (*Figure 1*). In turn, CAFs' biology is positively regulated by PDA cells (43).

The stromal cells were found in preclinical studies to impede the penetration of anti-cancer drugs resulting in inadequate cell kills (11,12). Hh signaling was implicated as a key regulator of tumor-stromal interaction in PDA (13). Using genetic KPC mice model ($Kras^{LSL.G12D/+}$; $p53^{R172H/+}$; $Pdx^{Cretg/+}$), Olive *et al.* (11) found that interrupting Hh signaling using IPI-926 (saridegib) ablate stromal CAFs leading to transient increase in intratumoral vascular density and increased intratumoral gemcitabine level,

resulting in better cytotoxic effect. Similar results were observed using a different Hh inhibitor (AZD8542) (14). However, the clinical trials evaluating Hh inhibitors in PDA failed to demonstrate clinical benefit despite such positive preclinical results. The phase II randomized study using gemcitabine with/without IPI-926 was stopped early due to increased mortality or not showing benefit (4,6,45). Similarly, a single-arm phase II trial (NCT01195415) of GDC-0449 (vismodegib) with gemcitabine was not superior in metastatic PDA compared to gemcitabine alone in historical control (7). The failure of clinical trials to replicate the preclinical success was puzzling and reasons suggested include limitations in the mouse models, chronic versus acute ablation of stromal cells by Hh inhibitors, and off-target effects of the drugs (46). In addition, there was an absence of potential predictive biomarkers such as stromal characteristics to guide clinical trial design (47).

Interestingly, several recent preclinical reports contradicted earlier studies suggesting that Hh-mediated stromal response restrained tumorigenesis and ablation of which was detrimental in PDA. Özdemir *et al.* deleted αSMA myofibroblasts by crossing *Ptfla*$^{Cre/+}$; *Kras*$^{LSL\text{-}G12D/+}$; *Tgfbr2*$^{flox/flox}$ (PKT) mice, demonstrated that the depletion of myofibroblast yielded undifferentiated and more invasive PDA (19). Similar results were also observed in KPC mice crossed with αSMA-transgenic mice (19). The decreased elastic content in PDA did not improve intratumoral gemcitabine concentration. In contrast, it was correlated with reduced survival and confirmed that actually desmoplasia protected the host. Separately, Rhim *et al.* specifically deleted Sonic hedgehog (Shh) ligand expression in mice PDA stroma by crossing *Pdx1*$^{Cre/+}$; *Kras*$^{LSL\text{-}G12D/+}$; *p53*$^{fl/+}$; *Rosa26*$^{LSL\text{-}YFP/+}$ (PKCY) with Shh$^{fl/fl}$ mice. Surprisingly, such Shh-deficent tumors were more aggressive, exhibiting increased vascularity, heightened proliferation and these were recapitulated using Hh inhibitors in KPC mice (20). Lee *et al.* showed that in three distinct genetically engineered mice models, Hh pathway inhibition suppressed stromal desmoplasia and accelerated growth of the epithelial elements; whereas, activation of Hh signaling caused stromal hyperplasia and reduced epithelial proliferation leading restraint on tumorigenesis (18).

Other novel stromal modulating therapies had been explored preclinically. Sherman *et al.* reported activation of vitamin D receptor (VDR) could re-program PSCs to a more quiescent and less tumor-supporting state that potentially countered PDA progression (21). In transgenic mice models, VDR activation reduced inflammatory markers and fibrosis, and increasing intratumoral gemcitabine level, Froeling *et al.* showed that treatment with all-trans retinoic acid (ATRA) induced CAFs quiescence, leading to reduced cancer cell proliferation and invasion, and increased apoptosis via Wnt-β-catenin signaling (48).

Acellular extracellular matrix (ECM)

The acellular part of PDA stroma is composed of proteins, polysaccharides and peptides. Secreted by CAFs, these stromal elements not only provide structural support but are also involved in differentiation, remodeling and carcinogenesis. Collagen I was shown to promote gemcitabine resistance *in vitro* (22,23). It also interacted with collagen IV and integrins on the surface of PDA cancer cells, and is vital for proliferation, maintenance of migratory phenotype, and avoiding apoptosis (24). Other potential ECM remodeling genes differentially expressed in PDA stroma included matrix metalloproteinase 3, collagen type IVα1 and syndecan-2 (49), though their role in PDA tumor-stromal interaction remains unclear for now.

Hyaluronan (HA) is a polysaccharides found in HA stromal matrix. High HA level in PDA increased interstitial fluid pressure (IFP) in tumor, creating substantial barriers to perfusion that attenuate the effects of anti-cancer drugs (25,50). In KPC and KC mice models, treatment using PEGylated human recombinant PH20 hyaluronidase (PEGPH20) ablated stromal HA that led to IFP normalization and re-expansion of collapsed tumor vasculature without increasing the microvessel density (26). When combined with gemcitabine, PEGPH20 significantly enhanced drug penetration throughout the tumor tissues, inhibited tumor growth and extended the mice survival. Similar result was reported by Jacobetz *et al.* (27). Elevated HA level was also found in metastatic PDA lesions, suggesting that HA targeting might also benefit in metastatic disease.

In stage I/IB clinical trials, PEGPH20 in combination with gemcitabine achieved partial metabolic responses by FDG-PET/CT in 4 out of 5 pancreatic cancer patients using PEGPH20 (28), and particularly showed promising activity in those with high HA levels (29,30). The randomized, phase II trials evaluating PEGPH20 in combination with nab-paclitaxel and gemcitabine (NCT01839487) and S1313 trial (NCT01959139) assessing PEGPH20 in combination with modified FOLFIRINOX for previously untreated metastatic PDA are ongoing presently. Preliminary result revealed that PEGPH20

+ nab-paclitaxel + gemcitabine offered greater overall response rate (ORR) and progression free survival (PFS) in patients with high HA status (31).

Immune cells

Broad repertoire of immune cells has been involved in pancreatic cancer stroma. However, PDA creates a hypoxic and highly immunosuppressive environment which is resistant to inhibitory cytokines and immune cells anti-tumor effect (10,51). Recent studies focus on recruiting anti-tumor cells or cytokines to restore their responses.

Immune co-stimulatory factor and checkpoint regulators

CD40 is a cell surface molecule that is a member of the tumor necrosis factor (TNF) receptor family, and participates in immune regulation and mediates tumor apoptosis (52). CD40 was found to be a key regulator in the development of T cell-dependent anti-tumor immunity (53) though recent report showed that CD40-mediated anti-tumor were macrophage dependent (32). Using KPC mice model, Beatty *et al.* showed that both treatments using CD40 monoclonal antibody combined with gemcitabine caused tumor regression in 30% of mice, far superior to gemcitabine alone (32). They further showed that the tumor regression was mediated by macrophage that facilitated the depletion of tumor stroma. CP-870, 893, a monoclonal antibody, is a CD40 agonist that was evaluated in combination with gemcitabine in a phase I trial in patients with advanced PDA (33). Four of twenty-two patients (18%) achieved partial response. Interestingly, after-treatment tumor biopsy showed an absence of tumor-infiltrating lymphocyte and abundant macrophages.

Activation of programmed cell death 1 receptor (PD-1) by binding with PD-1 ligands (PD-L1 and PD-L2) suppresses the T-cell activity and makes cancer cell "invisible" to the immune system. Both PD-1 and PD-1 ligands are expressed in PDA and had been associated with poor prognosis (36). Although early clinical trial of anti-PD-1 antibody achieved response in non-small cell lung cancer, melanoma and renal cell carcinoma (34), but not in PDA (35,36). Treatment against cytotoxic T lymphocyte associated protein 4 (CTLA-4), another costimulatory signaling, improved survival in transgenic mice PDA model (19). A phase II trial using ipilimumab (anti-CTLA-4 antibody) failed to achieve tumor response by RECIST criteria in advanced PDA though delayed shrinkage was observed in one patient with continued dosing (37).

The reasons for the failure of PD-1 and CTLA-4 to achieve tumor response in PDA are unclear. The clue might come from another immune molecule. Chemokine (C-X-C motif) ligand 12 (CXCL12) is a ligand for chemokine (C-X-C motif) receptor 4 (CXCR4). CAFs expressing fibroblast activation protein (FAP) created immunosuppressive environment in PDA via secretion of CXCL12. Administering CXCR4 inhibitor, plerixafor (AMD3100), induced rapid T-cell response and acted synergistically with PD-L1 antibody to greatly diminish cancer cells in KPC mice (16). As such, therapies combining immune checkpoints inhibitors with an agent that reverse the immunosuppression in the tumor microenvironment like CXCR4 inhibitor have potential therapeutic application in PDA. This hypothesis is currently in clinical evaluation (NCT02301130).

Inflammatory factors

Inflammatory cytokines had been shown to promote tumorigenesis. Inflammatory cells such as regulatory T cells (Tregs) and tumor-associated macrophage (TAM) subtype M2 could attenuate intra-tumor immunity by secreting interleukin 10 (IL-10) and transforming growth factor β (TGFβ) (54). In addition, tumor cells and CAFs could attract interleukin-17 (IL-17) secreting CD4+ cells (Th17) via TGFβ secretion, contributing to immune-suppression in the PDA tumor microenvironment (17). McAllister *et al.* illustrated that IL-17 stimulated infiltration of IL-17-expressing T cells drive tumor progression and the disruption of IL-17 signaling prevented PDA formation in preclinical studies (38). Antibodies against IL-17 signaling pathway (brodalumab and ixekizumab) are currently evaluated by clinical trials for treating psoriasis. Therapies utilizing IL-17 inhibitors may hold promise in PDA.

Cancer vaccine

GVAX pancreas is a cancer vaccine generated from pancreatic cancer cell line and has been modified to express granulocyte-macrophage colony-stimulating factor (GM-CSF), which attract dendritic cell (DC) to present tumor antigen to T cells. The effect of GVAX can be amplified by co-administration with CRS-207, an attenuated listeria monocytogenes, as a boost vaccine to express mesothelin (marker of mesothelioma, ovarian and pancreatic cancer) (55). In phase II trial for stage IV PDA patients, GVAX/cyclophosphamide combined with CRS-207 extended 2 months survival compared to GVAX/cyclophosphamide (6 *vs.*

4 months) (41). In murine studies, GVAX treatment significantly upregulate PD-L1 expression and combination therapy of GVAX and PD-1 antibody improved survival (39). In contrast, simultaneous telomerase vaccination (GV1001) in combination with chemotherapy did not find any benefit in patient with advanced PDA in previously untreated patients (40). Accordingly, further evaluation of the efficacy of cancer vaccination is warranted in the future studies.

Conclusions

The increasing understanding of the tumor-stromal interactions in PDA has engendered many novel approaches to targeting the tumor stroma. The complexity and dynamic nature of PDA microenvironment became more apparent following the failure of earlier attempts such as that targeting the Hh signaling, suggesting that more robust preclinical/translational studies and novel clinical trial designs are needed. Currently, there are a number of promising stromal-targeting approaches under clinical investigation that may potentially groundbreaking. Recent report from Moffitt *et al.* utilized elegant bioinformatics methods to distinguish gene signatures from pancreatic tumor cells and stromal cells that independently predict patient outcome (56). Such advances will help tailor personalized treatments in the future.

Acknowledgements

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Cite this article as: Mei L, Du W, Ma WW. Targeting stromal microenvironment in pancreatic ductal adenocarcinoma: controversies and promises. J Gastrointest Oncol 2016;7(3):487-494. doi: 10.21037/jgo.2016.03.03

