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Carcinoma Associated Fibroblast: a Paradoxical Role in Pancreatic Cancer Microenvironment and a Promising Target for Therapy

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Abstract

Pancreatic cancer is a grave malignancy showing an upward trend in morbidity and mortality during the recent decades. For reasons of late diagnosis, chemoresistance, low potential respectable rate and high post-operative recurrence rate, it has been the 6th most common cause of cancer death in China. As one of the most aggressive malignancies and the most common type of pancreatic cancer, pancreatic adenocarcinoma (PDAC) represents a significant therapeutic challenge. Conventional chemotherapeutic cytotoxic agents are proved to be with a poor survival benefit. Though the current first-line therapy FOLFIRINOX increased the median survival time compared with gemcitabine, it has been still unsatisfactory. Another direction enlighted by the treatement experience in other tumors is targeting certain molecules that participate in specific signaling pathways mediating cancer cell proliferating, angiogenesis, chemoresistance or metastasis. Unfortunately, none of the established "targeted" therapy agents that have been approved to be effective in some other tumors has a similar effect on PDAC, suggesting that there are some unique and decisive elements in the microenvironment of PDAC to facilitate its extensive drugresistance.

Thus, the spotlight has been turned on immunotherapy, which is theoretically curative regardless of the complex molecular and cellular heterogeneity while the concrete strategies on PDAC are still in the dark. Revisiting the complex biology of PDAC, three prime characteristics will never be missed: almost 90% of patients with oncogene mutation of KRAS, as well as loss of tumor suppressor genesTP53 and SMAD4; mostly hypovascular; and tumor desmoplasia by persistent activation of fibroblasts/ pancreatic stellate cells (PSC). The last one, which is the defining feature of PDAC, as the target of therapy is the focus of this review.

Keywords: Pancreatic cancer; Pancreatic ductal adenocarcinoma; Drug-resistance; Malignancy

Introduction

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As one of the most aggressive malignancies and the most common type of pancreatic cancer, pancreatic ductal adenocarcinoma (PDAC) represents a significant therapeutic challenge. Conventional chemotherapeutic cytotoxic agents are proved to be with a poor survival benefit. Though the current first-line therapy FOLFIRINOX increased the median survival time compared with gemcitabine, it has been still unsatisfactory [2]. Another direction enlighten by the treatment experience in other tumours is targeting certain molecules that participate in specific signalling pathways mediating cancer cell proliferating, angiogenesis, chemo-resistance or metastasis. Unfortunately, none of the established "targeted" therapy agents that have been approved to be effective in some other tumours has a similar effect on PDAC, suggesting that there are some unique and decisive elements in the microenvironment of PDAC to facilitate its extensive drug-resistance. Thus, the spotlight has been turned on immunotherapy, which is theoretically curative regardless of the complex molecular and cellular heterogeneity while the concrete strategies on PDAC are still in the dark. Revisiting the complex biology of PDAC, three prime characteristics will never be missed: almost 90% of patients with oncogene mutation of KRAS, as well as loss of tumour suppressor genes TP53 and SMAD4; mostly hypo-vascular; and tumour desmoplasia by persistent activation of fibroblasts/ pancreatic stellate cells (PSC). The last one, which is the defining feature of PDAC, as the target of therapy is the focus of this review.

Rationale of Carcinoma-Associated Fibroblast in PDAC

Fibroblasts are the most common cell type in stromal component. In the 1990s, cancer biologists found that neoplasia represented a phenotype of myofibroblasts termed "carcinoma-associated fibroblasts" (CAFs) that documented with aberrant expression of smooth muscle actin (SMA), inappropriate secretion of proteolytic enzymes, and the production of extracellular matrix proteins [3-7]. CAFs are confirmed as the most common cells in a series of tumour stroma that account for the majority of the tumour tissue volume and of important patho-biological functions [8]. CAFs in PDAC are

thought to be derived mainly from PSCs which is a special stromal fibroblast type termed for its stellate shape in healthy exocrine pancreas. They are present mainly in the periacinar, perivascular and periductal regions and play a key role in the pathological process of pancreatic diseases including chronic pancreatitis and pancreatic cancer [9-12]. Once the pancreas is undergoing a disease such as PDAC, PSCs will turn their quiescent state into a myofibroblastic state characterized by loss of fat droplets and highly expression of α-SMA. Moreover they become proliferating and contribute to a collagen-rich, desmoplastic reaction [13]. Thus it makes pancreatic ductal adenocarcinoma a pyknotic and stiff solid tumour. In order to determine the function of PSCs in an *in vitro* model, immortalized PSC cell lines from human, rat and mouse have been established [14-17]. These cell lines all spontaneously retain an activated phenotype and can be induced quiescent with matrigel, N-acetylcysteine (NAC) or all-trans-retinoic acid (ATRA) [14-18]. These allowed the dissection of intercellular cross-talking between CAFs and PDAC cells or other stromal cells.

Besides activation of quiescent fibroblasts into α-SMA positive myofibroblasts, epithelial to mesenchymal transition (EMT) and bone marrow recruitment are also possible mechanisms for the generation of a heterogeneous population of CAFs [19]. Zeisberg et al. [20] found that about 11% of α-SMA + cells express endothelial cell marker CD31 in a Rip1-Tag2 multistage carcinoma model. Further research use Tie2-cre; R26Rosa-lox-Stop-lox-LacZ transgenic mice whose endothelial cells are irreversibly tagged with LacZ that is reactive to β-galactosidase, labelling of β-gal were also observed in α-SMA + and fibroblast specific protein 1 (FSP1+) cells respectively, indicating EMT as a mechanism for the source of CAFs [20]. It may be not likely to be discriminated exactly whether it is EMT or mesenchymal to epithelial transition (MET) that makes these cells double-labelling. However, it demonstrated heterogeneity of CAFs that added to the complexity of tumour microenvironment.

CAFs Activation in the Battlefield of Pancreatic TME

PDAC tumour microenvironment (TME) is now a popular research orientation that cancer biologists think it the key role in process of carcinogenesis and metastasis [21]. The mechanisms of CAFs activation in PDAC TME are multiplex. Firstly, it is similar to inflammation in many ways, for instance the initial event of injury, the mediators of necrosis/apoptosis derived cytokines/growth factors etc., [22-24]. Whereas there are still some specific patterns in which peri-tumoral fibroblasts are stimulated and activated by pancreatic tumour cells. Tumour cell-derived factors such as platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β) and sonic hedgehog (SHH) are elaborated [11-26]. Besides, microRNAs in PDAC tissues have been reported to be independent prognostic factors [27]. Further study proved that pancreatic cancer-secreted miR-155 turns normal fibroblasts into CAFs through an intercellular communication mediator of micro vesicles [28]. KRAS driven expression of IL-1α by PDAC is also an important mediator to activation of CAFs which in turn produce massive amount of inflammatory and immune regulatory factors to maintain an inflammatory microenvironment [29]. Another PDAC-derived mediator is a secreted protein of tissue transglutaminase which moulds the stroma thus leads to activation and proliferation of fibroblasts [30]. The PDAC-activated myo-fibroblasts are of a location-dependent fashion that CAFs immediately adjacent to tumour have a more pronounced expression of fibroblast activation protein (FAP) than those in surroundings [31], supporting that CAFs activation depends on tumour cells. These findings suggest that by changing the fibroblast-activating secretome of PDAC cells, we can protect the naive fibroblasts from pro-tumoral education thereby to normalize the tumour microenvironment to a tumour-maladapted one.

Pancreatic CAFs and Desmoplasia: Soil of Tumorigenesis or Warden of the Cancer?

Tumours were described as wounds that do not heal, so there is one conjecture that desmoplasia in the tumour microenvironment, similar to wound healing and tissue regeneration, is a host defensive process [32,33]. On the other side, desmoplasia associated with tumorigenesis constitutes an immune suppressive response such that is thought tumour promoting [34].

Tumour-educated CAFs are of great expression of fibroblast activation protein (FAP) which was reported to associate with a bad prognosis in PDAC [31-36]. FAP+ CAFs induce desmoplastic reaction through modulating the ECM proteins such as collagen I, fibronectin and cellular α-SMA, thus promote enhanced invasion behaviour of pancreatic cancer cells after co-culturing [37]. FAP inhibitor PT-100 could significantly reduce the accumulation of CAFs and enhance the efficacy of chemotherapeutics to suppress tumour growth and increase survival in xenograft tumour mice [38]. An experiment directly assessed the function of CAFs in primary pancreatic cancer was carried out in the autochthonous LSL-KrasG12D/+; LSL-Trp53R172H/+; Pdx-1-Cre (KPC) model, which faithfully replicates human PDAC in genotype, a PanIN-to-PDAC tumorigenic process, invasive, metastatic feature and resistance to chemo and immunotherapy [39,40]. Feig et al. introduced into the KPC line the bacterial artificial chromosome (BAC) transgene containing a modified Fap gene that drives the expression of the human diphtheria toxin receptor (DTR) selectively in cells that are FAP+. Administration of diphtheria toxin (DTx) leads to conditioned apoptosis of FAP+ cells. The BAC/DTR transgenic model was observed a slowed tumour growth in a CD4+ and CD8+ dependent manner [39]. This is consistent with the former study they have done in the orthotropic models [34]. In addition, and interestingly, FAP+ CAF-derived chemokine CXCL12 was confirmed to attenuate the local IFN-γ secreting T cells infiltration, while administration of AMD3100, a CXCL12 receptor CXCR4 inhibitor, induced accumulation of T cells among cancer cells and had a synergistic effect with anti-PD-L1 therapy [39]. These findings implicated that FAP+ CAFs play a role in the exclusion of T cells from the TME by production of CXCL12 interacted with CXCR4. This newly found mechanism of immune suppression in the PDAC microenvironment has a potential to clinical translation. α-SMA is a selected and classic marker of CAFs. There is evidence supporting that low intra-tumoral α-SMA is correlated with poor micro vessel integrity, early recurrence and decreased survival [41-43]. Ozdemir et al. generated transgenic mice of Ptf1acre/+; LSL-KrasG12D/+; Tgfbr2flox/flox (PKT); α-SMA-tk, which is similar to the KPC; BAC/DTR line mentioned above [41]. The α-SMA+ proliferating CAFs were depleted by daily injection of GCV and subsequent decreased survival and undifferentiated tumours were observed. Depletion of CAFs also displays, interestingly, suppression of angiogenesis, enhanced tumour hypoxia, EMT program, and cancer stem cell-like phenotype [41]. These findings are consistent with the other studies indicating that tumour vessel denormalization-induced hypoxia leads to more malignant tumour behaviour [44-46]. Notably, depletion of α-SMA+ myofibre oblasts starting at the pancreatic

intraepthelia l neoplasia (PanIN) stage leads to a significant reduction of immune cells infiltration except for the FoxP3+ regulator T cells that are account for the immune suppression in the TME, while such immune phonetype cannot be observed in established PDAC with α-SMA+ CAFs depletion [41].

A recent study also demonstrated that targeting FAP+ stromal cells by injection of FAP-CAR T cells could immune-independently inhibit stromagenesis, angiogenesis and restrict tumour growth in KPC models [47]. Taken together, these findings justify conclusions that CAF is a mixed concept that distinct subpopulations may have opposing effects on tumour progression; Inhibition of angiogenesis was observed consistently in different tumour Stroma-depletion strategies indicating a positive correlation ship between CAFs, irrespective of which subset, and tumor angiogenesis; Targeting intra-tumoral FAP should be a potential effective measure to improve survival alone or synergetically enhance the efficacy of chemotherapies and some immune checkpoint antagonists such as α-PD-1, α-PD-L1 and α-CTLA-4. Remarkably, FAP+ cells reside in most tissues like bone marrow, skeletal muscle [48]. Likewise, α-SMA overlaps with a fraction of macrophages, myofibroblasts, pericytes and vascular smooth muscle cells [34-50]. Hence, rough depletion of FAP+ or α-SMA+ stromal cells will lead to a complex change in the microenvironment, because of which, the definite functions of CAFs are not accurately reflected. Depletion of FAP-expressing stromal cells even results in severe systemic alterations such as cachexia and anaemia [48]. Therefore, the functional contribution of CAFs in the progression of PDAC needs further study by improved strategy that targets CAFs more precisely.

Strategies Targeting CAFs in PDAC Therapy

Hedgehog signalling blockade therapy

Considering the heterogeneity of CAFs and overlapping of FAP and α-SMA in PDAC microenvironment, single marker-dependent depletion of CAFs will not be an ideal strategy due to its uncertainty. An earlier study has demonstrated that blockade of hedgehog (Hh) signalling pathway with hedgehog receptor smoothened (SMO) inhibition reduces proliferation of α-SMA+ myofibroblasts, alters the vascular network and thereby facilitates delivery of chemotherapeutic agents [51]. SMO was confirmed up regulated in CAFs [52] while pancreatic cancer cells showed overexpression of Sonic hedgehog (Shh) ligands [53]. The Hh/SMO interaction activates the downstream transcription factor Gli1 expression and subsequently leads to desmoplasia [54]. SMO inhibition demonstrated an inspiring benefit in the preclinical KPC models. However, clinical trials of Hh blockade showed disappointing outcomes that even accelerated disease progression [55]. To investigate the totally paradoxical results between preclinical models and clinical trials, Rhim et al. [56] generated Shhfl/fl; Pdx1-Cre; KrasLSL-G12D/+; p53fl/+; Rosa26LSL-YFP (ShhPKCY) model whose Shh gene is conditionally deleted in the pancreatic epithelial cells. Result, however, showed that Shh is dispensable for tumorigenesis and loss of Shh even results in a shortened survival and poorly differentiated to undifferentiated histology. Likewise, KPC mice administrated with IPI-926 at an early stage of PanIn and ADM (acinar to ductal metaplasia, another precursor of PDAC) exhibited similar properties, and the efficacy of gemcitabine is limited. It may be reasoned that chronic exposure to Hh blockade leads to more aggressive biology which overweighs transient improvement of drug delivery [56]. Interestingly, however, Hh signalling blockade resulted in depletion of CAFs and indirectly increased angiogenesis through stromal cell signals, depending on the existing VEGF rather than de novo VEGF expression.

The anti-angiogenic effect of CAFs here in the context of Shh deletion is at odds with that in other studies depleting CAFs by CAR-T cells or TK/GCV strategy based on markers of FAP or α-SMA [41-47]. The precise mechanism for the different roles of CAF remains unknown. Nevertheless, poorly differentiated tumours with increased vasculature resulted from Hh pathway inhibition are more sensitive to VEGFR2 inhibition [56]. Mathew et al. demonstrated another explanation that the inhibition of CAF-expressed Hh co-receptors to the regulation of pancreatic tumour growth and angiogenesis is dosage dependent [57]. Deletion of all the three Hh co-receptors of GAS1, BOC and CDON almost completely abrogates Hh signalling and results in suppression of tumorogenesis and angiogenesis, while depletion of two co-receptors GAS1 and BOC would reversely promote great tumour growth *in vivo* [57]. These findings may partially explain the clinical failure of Hh pathway blockade in another perspective.

Quiescent CAFs may make differences

To speak of, the SMO inhibition- induced decrease in proliferation of α-SMA+ cells was accompanied with an increase in proliferation of α-SMA negative cells [51], suggesting a general quiescence of CAFs. This stroma-disrupting strategy leads to restored blood vessels which is VEGF independent, indicating a particular mechanism of vasculature dysfunction related to α-SMA+ stromal cells. Associated with the recent study that focused on the pro-angiogenesis strategy of combining cilengitide, verapamil and gemcitabine [58], given that cilengitide can have both pro-and anti-angiogenic effect depending on its concentration, which is hard to assess in the clinic, alternative approaches to promote angiogenesis in a controlled manner should be considered [46]. Therefore, inducing quiescent of CAFs may be an effective approach to remodel the vascular network thereby enhances the efficacy of chemotherapies or immune therapies. It was confirmed recently by Carapuca et al. [59] that chemotherapy along with stroma l co-targeting ATRA resulted in enhanced tumour necrosis, increased vascularity and diminished hypoxia [59]. Moreover, pancreatic CAFs are identified as activated PSCs, which sequester anti-tumour CD8+ T-cell migratory and adhesive function preventing the access to the tumoral cells [60]. Inducing CAFs quiescent by administration of ATRA can increase number of CD8+ T cells in tumour sites, as well as create a physical barrier that prevents tumor cells invasion [18]. Another preclinical study successfully demonstrated that activation of vitamin D receptor (VDR), by administrating vitamin D analogue calcipotriol, renders CAFs a quiescent-like status and thereby inhibits the tumor-supporting cancer-stroma crosstalk [61]. *In vivo* study also evidenced that VDR ligand modulates PSCs into quiescence, and, as expected, enhances drug delivery thus synergizes the efficacy of Gemcitabine in KPC mice [61]. In addition, Given that CAFs are proliferating cells, it is rational to speculate that the proliferating cancer cell-targeting chemotherapeutics like gemcitabine may also target proliferating CAFs. So the proliferating CAFs may help reduce the cytotoxicity of chemotherapeutics to cancer cells [41]. Such compromise function of CAFs may likewise be reversed by calcipotriol, ATRA or other quiescent-inducing agents.

Targeting CAFs to limit PDAC from invasion and metastasis

As is known that metastasis is the major cause of cancer death, thus to prevent the primary tumor from invasion and metastasis should be

an important way to make cancer a controllable chronic disease and a direct path to extend survival of patients. Given that migration and invasion are closely related to the actin skeleton organization, the actin-bundling protein palladin is identified as a major element participating in the process of tumour invasion and metastasis. Palladin has been reported highly expressed in both primary and metastatic pancreatic CAFs [62,63]. Earlier study also introduced a close relationship between palladin mutation and familial ocurrence of pancreatic cancer [64]. Accumulating evidence demonstrated that overexpressed palladin is an indication of poor clinical outcomes in different tumours [62-66]. Chin et al. [67,68] found that the expression of palladin is regulated by signalling's of both Akt1 and Akt2 isoforms for distinct properties in breast cancer migration. Later study found that extracellular signal-regulated kinase (ERK) is involved in the phosphorylation of palladin which exhibits an anti-migratory function [69]. *In vitro* introduction of exogenous 90 kDa palladin activates human dermal fibroblasts into myof ibr oblasts that are phenotypically and morphologicallys imilar with CAFs [70]. Moreover, CAFs expressing palladin are of elevated motility and enhanced invadopodia formation which, in turn, remodel the extracellular matrix to promote invasion of pancreatic cancer [70,71]. A recent study demonstrated that palladin mediated invadopodia formation can be inhibited by expression of special AT-rich sequence-binding protein 2 (SATB2) in HCT116 cells [72]. Another recent study demonstrated that Twist1 activated fibroblasts into a CAF-lik phenotype by up-regulating palladin and collagen α1 [73]. Taken together, the cytoskeleton scaffold protein palladin is majorly expressed in CAFs and promotes tumour migration and invasion, which should be a potential therapeutic target to prevent metastasis of pancreatic cancer.

Targeting CAFs to Overcome Chemo-resistance in Pancreatic Cancer

The role of secreted protein acidic rich in cysteine (SPARC) in pancreatic cancer has been highlighted for years. Albumin-bound paclitaxel is introduced to clinical application for its specific binding to SPARC and depletion of stroma, especially CAFs, that enhances the delivery of chemoagents thus increases the efficacy [74-76]. Neesse et al. further demonstrated in KPC models that nab-paclitaxel only interacts with plasma SPARC in low doses, while in the therapeutic dose, SPARC has no effect on intra-tumoral concentration of Nab-paclitaxel [77]. A recent study, reversely, reported that nab-paclitaxel treatment makes no difference in both SPARC positive and negative patient-derived xenografts, suggesting an indirect interaction between nab-paclitaxel delivery and SPARC expression [78]. Hence, the precise mechanism of SPARC-correlated chemo-sensitivity remains in dark, by looking deep into which we may create more innovative strategies of chemotherapy.

Targeting CAFs to Overcome Immune Suppression in Pancreatic Cancer

CAFs are crucial in mediating the immune suppressive microenvironment of pancreatic cancer. Ene-Obong A demonstrated that CAFs regulate T-cell migration by secreting CXCL12 thereby sequester T cell from juxta-tumoral compartment [60]. The tropism of T cells to CAFs can be reversed by neutralization of CXCL12 or inducing CAFs quiescent [60]. Feig et al. further explored that CXCL12-expressing CAFs help pancreatic cancer cells escape from immune surveillance through CXCR4 signalling [39]. Inhibition of CXCR4 can improve the accumulation of PDAC-specific CD8+ T cells in tumour and can reveal the antitumor efficacy of immune checkpoint antagonist anti-PD-L1 [39]. Another study reported CAFs produce variable cytokines such as IL-6, VEGF, M-CSF and chemokines like SDF-1 and MCP-1 to promote differentiation of myloid-derived suppressor cells (MDSC) through a STAT3-dependent manner, in turn suppress T lymphocytes proliferation [79]. The IL-6/JAK/STAT3 signalling within the tumour stroma thus could be another potential target to overcome immune suppression in pancreatic cancer.

Conclusions

Carcinoma-associated fibroblasts act as a complex role in pancreatic cancer micro-environment [80]. Its heterogeneity resulting from different sources and activation pathways defines the versatility of roles in tumour initiation, progression, invasion, and metastasis and drug resistance. Both its protective and tumorigenic properties are of great potential to develop new therapy strategies to highly improve the outcome of PDAC patients as long as to be well utilized. For instance, activated fibroblasts are involved in the process of wound tissue repair. The tissue-repairing property exhibited by activated fibroblasts in tumour microenvironment correspondingly turn to be a bipolar feature that on the one hand helps tumour cells escape from immune surveillance, on the other hand limits tumour cells from becoming more malignant. Accordingly, to precisely target the immune suppressing, chemo-resistant, pro-metastasis signalling pathways within CAFs to reverse the tumour promoting properties in the tumour microenvironment with the host-protective function preserved, rather than rough depletion of CAFs depending on some general marks, would be an ideal thought. For this consideration, some potential targets has be uncovered, such as CXCL12/CXCR4, palladin, SHH/Gli1, IL-6/STAT3, VDR and so on. It is also worth noting that different subsets of CAFs may play different roles. Recognition of these original and functional subsets has a great practical significance in preclinical research and clinical translation.

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