How does the tumor microenvironment play a role in hepatobiliary tumors?

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Abstract: The tumor microenvironment (TME) is defined as the structural and dynamic network of cellular and non-cellular interactions between malignant cells and the surrounding non-malignant matrix. Hepatocellular carcinoma (HCC) and pancreatic ductal adenocarcinoma (PDAC) are two of the most challenging gastrointestinal malignancies. Despite clinical advancements in understanding tumor biology and growth of the chemotherapeutic industry, there have been no corresponding improvements in prognosis and overall survival of HCC and PDAC. Both of these cancers have a very intimate relationship with their surrounding environment; the TME is thought to actively participate in initiating and sustaining these malignancies. Individual TME constituents play a vital role in chemoresistance and recurrence after surgery and have been established as independent prognostic factors. This review article will highlight the diverse structural components, key signaling pathways, and extracellular matrices of HCC and PDAC and discuss their crosstalk with tumor cells to promote growth and metastasis. The article will also summarize the latest laboratory and clinical research based on therapeutic targets identified within the TME of both HCC and PDAC.

Keywords: Tumor microenvironment (TME); hepatobiliary cancer, pancreatic ductal adenocarcinoma (PDAC); hepatocellular carcinoma (HCC)

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Introduction

The tumor microenvironment (TME) refers to the structural components and dynamic interactions between malignant cells and the surrounding non-malignant matrix. The TME is thought to influence tumor growth, metastasis, and, ultimately, prognosis. Therefore, understanding the TME is critical in identifying targets for potential therapeutic agents.

Weinberg's Hallmarks of Cancer describe the evolution of benign cells to malignant cells through complex interactions with the surrounding TME through the acquisition of six essential hallmarks (1). These include self-sufficiency in growth signals, the ability to evade anti-growth signals, an escape from apoptosis, unlimited replication, sustained angiogenesis, and metastatic invasion, ultimately leading to an aggressive, immortal cell line.

Further understanding of these six hallmarks precipitated an update to this theory with the addition of two emerging hallmarks of cancer: reprogramming of energy metabolism and evading immune destruction. The metabolic switch in cancer cells promotes less efficient energy production via "aerobic glycolysis" and triggers upregulation of glucose transporters. Another sub-population of tumor cells

alternatively utilizes lactate, which is subsequently secreted by hypoxic cancer cells. Immune surveillance involves the innate and adaptive arms, which include CD8+ cytotoxic T lymphocytes (CTLs), CD4+ Th1 helper T cells, and natural killer (NK) cells. However, tumor cells can evade immune surveillance through deficiencies and malfunction of these cells (2). Current therapies targeting the cell cycle checkpoint pathway such as the anti-programmed cell death-1 (anti-PD-1) and anti-programmed cell death ligand-1 (anti-PDL-1) have exploited the tumor-immunological interactions, but use of these agents has shown mixed success (3).

The TME is composed of distinct groups of cells, including cancer stem cells, immune inflammatory cells, pericytes, cancer-associated fibroblasts, endothelial cells, and local and bone marrow-derived stromal stem and progenitor cells, which carry important functions in tumorigenesis. Cancer stem cells (CSCs) arise from progenitor cells or normal stem cells that undergo malignant transformation or the epithelial-mesenchymal transition (EMT). CSCs have self-renewal capacity and are more resistant to chemotherapy (2). CSCs have been identified by the surface markers CD34, CD24, CD44, CD133, ESA, aldehyde dehydrogenase (ALDH), c-Met, and epithelial cell adhesion molecule (EpCAM) (4). The "angiogenic switch" turns on endothelial cells (ECs) to promote neovascularization as well as lymphatic vessel formation. The surrounding and activated pericytes work symbiotically with tumor-associated ECs, creating a stable infrastructure. The significance of tumor-promoting and tumor-antagonizing immune cells and the variability among different tumor types has been demonstrated *in vivo* and further examined in trials using checkpoint inhibitors. The immune cells previously described are also activated in wound healing, phagocytosis, and tumor destruction but, when re-programmed, can alternatively propagate tumor progression. Tumors heavily infiltrated with CTLs and NK cells tend to have better prognosis than tumors that are depleted of these immune cells. Within the surrounding stroma, a population of cells that include tissue-derived fibroblasts, myofibroblasts, and stromal cells, which are derived from mesenchymal and progenitor cells, are recruited and migrate from neighboring tissue and bone marrow to support the tumor infrastructure. Furthermore, the α-smooth muscle actin (α-SMA) containing myofibroblasts secrete inflammatory mediators in the extracellular matrix (ECM) of the tumor, which play a role in tumor invasion and metastasis (2).

The interactions of tumor cells with the surrounding TME are complex and dynamic. The signaling pathways among these components create the initial fertile soil for a pre-neoplastic lesion to survive and evolve into the primary tumor. The surrounding stroma of the primary tumor provides a pliable environment that encourages further tumor growth and propagation. Moreover, the normal stroma of distant sites also receives signaling factors that induce this same pliable environment, leading to metastatic spread and dissemination (2). Tumorigenesis and tumor progression in the TME thrive in hypoxic, acidic, and leaky conditions. The dysfunctional environment orchestrated by the elements of the TME results in improper vascular networks and metabolic and signaling pathways, which subsequently impairs efficacious drug delivery (4).

In this article, we will review the role of the TME in hepatobiliary malignancies, with a primary focus on hepatocellular carcinoma (HCC) and pancreatic ductal adenocarcinoma (PDAC).

HCC

Liver cancer is the fifth most common cancer in men, the ninth most common cancer in women, and the second most common cause of cancer mortality worldwide. Liver cancer includes cholangiocarcinomas, hepatoblastomas, and HCCs, the latter of which contributes to more than 90% of all liver cancer cases (5). Unlike other gastrointestinal malignancies, HCC is almost always preceded by chronic liver inflammation from liver cirrhosis and/or viral infections such as hepatitis B and C. As a result, HCC was thought to evolve from normal hepatocytes in the setting of long-standing injury and inflammation. However, recent studies have shown that non-hepatic cells within the TME play an important role in initiating and maintaining carcinogenesis (6). Non-hepatic cells include stellate cells, cancer-associated fibroblasts (CAFs), immune cells, ECs, and liver progenitor cells that mature into hepatic and nonhepatic cells. Liver progenitor cells are considered to be stem cells and are currently being investigated in tumorigenesis and the growth of HCC. The TME also includes a dynamic ECM as well as multiple signaling pathways regulated by cells of the TME. It is thought that the TME can favor the survival and growth of cell lines that are able to adapt to changing conditions, making them prime candidates to acquire the hallmarks of cancer (7).

The following sections will review the TME of HCC

and describe the function and tumorigenic capacity of the various components in the hepatic TME. Potential therapeutic targets and current clinical research will also be discussed.

Signaling pathways in TME

Several signaling transduction pathways have been studied in HCC. One of the most crucial is the EGFR-RAS-MAPK pathway, ligands of which are produced by both tumor cells and TME cells. The end result of this cascade is dysregulated cell proliferation. Epidermal growth factor receptor (EGFR) is one of four receptors (others are Her2/neu, ErbB3, and ErbB4) that activate tyrosine kinases once bound to their respective ligands. Consequently, monoclonal antibodies and small molecule inhibitors against the tyrosine kinases involved in this pathway are being studied in clinical trials, particularly erlotinib. However, a phase III trial comparing sorafenib, which is the standard of care for advanced and metastatic HCC, with and without erlotinib showed no difference in overall survival (OS) (8).

The most prominent stromal signaling pathway is transforming growth factor (TGF)-β, which has been analyzed extensively. Therapeutic targets blocking this process are being tested in clinical trials, with encouraging results. In pre-malignant tissue, TGF-β acts as a tumor suppressor; however, in active malignancy, it promotes the EMT, tumor potency, and invasiveness (9). There are 3 isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) and several known receptors, with 2 main receptors (TGF-β receptor I and TGF-β receptor II). TGF-β activates both SMAD and non-SMAD signaling pathways. Activation of the TGF-β pathway leads to downstream activation of proteins that can ultimately regulate gene transcription. TGF-β contributes to the development of liver fibrosis by manipulating the ECM and promotes the transition of hepatic stellate cells (HSCs) into myofibroblasts. The resulting fibrosis further augments expression of the TGF-β pathway, leading to continuous positive feedback (10). As a result, higher levels of TGF-β have been noted in livers with HCC than in normal and cirrhotic livers (11). Patients with metastatic HCC were also found to have higher levels of TGF-β than those with non-metastatic HCC, indicating a role of TGF-β in invasiveness through manipulation of integrin proteins in the ECM (12).

In vitro and *ex vivo* studies with galunisertib, a novel TGF-β inhibitor, have shown promising results. The close relationship between TGF-β and malignancy led to the development of this agent, which has been successfully tested in pre-clinical, phase I, and several phase II clinical trials in patients with HCC (13-15). A recent study showed that HCC tissue with TGF-β activity demonstrated inhibition of downstream SMAD activation with the addition of galunisertib. Combined sorafenib and galunisertib therapy on HCC samples showed a significant decrease in the Ki67 proliferative index and an increase in apoptotic caspases (16). This provided the rationale for a phase II trial of galunisertib and sorafenib in combination (14). A phase II galunisertib trial of 109 patients who had either progressed on sorafenib or who were unable to start sorafenib demonstrated modest time to progression (TTP) of 12 weeks in the overall population. However, there was increased TTP in sorafenib-naive and non-alcohol etiology patients of 18.3 and 12.1 weeks, respectively. Similarly, the median OS was much higher in alpha fetoprotein (AFP) responders than in non-responders (93.1 *vs.* 29.6 weeks) (15). TGF-β activity is regulated by several of the cellular components of the TME, which will be discussed further below.

Other pathways implicated in HCC tumorigenesis include the Wnt β-catenin and insulin growth factor receptor (IGFR) pathways. Wnt β-catenin can lead to transcription of potent oncogenes such as c-myc, cyclin D and survivin, and IGFR (17). The IGFR pathway can lead to downstream expression of the PI3K/AKT/mTOR and MAPK pathways (18,19). A phase III study of octreotide, a somatostatin analog that inhibits the ligands of the IGFR pathway, showed the agent to be well tolerated but to not improve OS (20).

CAFs

Perhaps most prominent in the TME are CAFs. Several co-cultures of hepatic tumor cells with and without CAFs have shown increased tumor growth and invasion in the presence of these cells (21). CAFs are thought to derive from circulating stem cells or resident fibroblasts. They secrete several proteins involved in the crosstalk between tumor and stroma, such as hepatocyte growth factors (HGFs), inflammatory cytokines, and angiogenic mediators (22,23). CAFs also influence tumor-initiating cells by activating signaling pathways through the production of HGF, leading to inflammation and fibrosis in mouse models. Targeting the CAF-derived HGF cascade might thus be

a therapeutic strategy in HCC treatment (24). There is a growing focus of research in finding targets in CAFs to help decrease HCC growth and spread. HGF binding to c-MET receptors activates the RAS-MAPK and PI3K-AKT signaling pathways, which affects cell proliferation, survival, migration, and angiogenesis and liver development and regeneration. This led to the development of tivantinib, a MET inhibitor, which demonstrates activity in patients with high-MET-expression tumors. The phase II clinical trial evaluating tivantinib versus placebo demonstrated longer median TTP in patients with high MET expression (2.7 *vs.* 1.4 months) (25). However, recent early reports from a phase III trial did not show an OS benefit (26).

Similarly, the phase II results of cabozantinib, a small molecular tyrosine kinase inhibitor of MET and vascular endothelial growth factor receptor 2 (VEGFR2), was promising, with a disease control rate of approximately 68% (27). The results of the phase III CELESTIAL trial of cabozantinib in HCC are pending (ClinicalTrials.gov identifier NCT01908426). Results of recent *in vitro* studies of sorafenib-resistant cell lines (Huh7 and Mahlavu) suggest that the relationship between MET expression in HCC tumors and treatment response may be attributed to upregulation of HGF synthesis, leading to autocrine c-MET activation (28). Moreover, *in vitro* and *in vivo* studies of sorafenib combined with a c-MET inhibitor, DE605, demonstrated a synergistic effect through inhibition of FGFR3/Erk signaling and induction of apoptosis. The combination did not result in gross toxicity; however, further *in vivo* studies are warranted to assess tolerability and safety profiles (29).

In addition, CAFs play a role in angiogenesis and tumor invasion by secretion of TGF-β and SDF-1, which promote the expression of cadherin, which is vital in vascular mimicry, a process in which tumor cells acquire the ability to form vascular channels. Yang *et al.* demonstrated that miR-101, a microRNA that usually suppresses TGF-β and SDF-1 signaling was under-expressed in HCC cells and CAFs. Upregulation of activity of miR-101 could be a potential therapeutic strategy (30). Also, blocking the connective tissue growth factor, which stimulates CAFs, with a TGF-β receptor inhibitor, LY2109761, has been shown to decrease crosstalk between CAF and tumor cells and to lead to decreased HCC growth and invasion (23,31).

Finally, CAFs have been implicated in metastasis: a recent *in vitro* study demonstrated increased secretion of chemokines CCL2, CCL5, CCL7, and CXCL16 in co-inoculated CAF and HCC cells compared with peri-tumor fibroblasts and HCC cells in mice. CCL2 and CCL5 stimulated the sonic hedgehog (SHh) pathway and increased HCC cell migration, whereas CCL7 and CXCL16 increased both HCC migration and invasion through the TGF-β pathway. When neutralizing antibodies to these four chemokines were added, the authors noticed decreased activation of the SHh and TGF-β pathways with overall suppressed influence of CAFs over HCC cells (32).

Stellate cells

HSCs, also referred to as "Ito cells," are perisinusoidal cells that line the space of Disse in between the sinusoids and hepatocytes. Inflammatory triggers such as a viral infection and alcohol activate HSCs to gain myofibroblast-like functions, secrete inflammatory cytokines, and increase α-SMA expression and production of ECM proteins, including collagen. High levels of collagen from HSCs are thought to decrease the permeability of the hepatic vasculature to chemotherapy and lead to chemoresistance (33). Unlike chemotherapy, sorafenib appears affect the TME through inhibition of HSCs via suppression of α-SMA expression and inhibition of platelet-derived growth factor (PDGF) signaling and PDGF and TGF-β expression. As a result, the invasive potential and proliferation of HCC is decreased (34).

Moreover, the synthesis of matrix metalloproteinase 9 (MMP-9) was found to be dysregulated by HSCs, leading to facilitated HCC growth and migration. There is evidence that higher levels of activated HSCs have been associated with poorer tumor differentiation, increased portal vein invasion, and higher TNM stage (35). Furthermore, the number of activated HSCs was found to correlate with focal adhesion kinase (FAK) and MMP levels. FAK is a protein tyrosine kinase that plays a key role in cell growth, migration, and invasion and can activate several inflammatory pathways, including MMPs. Upregulation of the FAK-MMP pathway is crucial in HCC carcinogenesis; given the association with HSC activation, this process can likely function as a therapeutic target (36). Repeated activation of HSCs can lead to tissue remodeling, scar formation, and hepatic fibrosis and is thought to be responsible for the pathogenesis of liver cirrhosis.

Immune cells

Given that chronic infection and fibrosis predispose the liver to HCC, the immune microenvironment—including T

helper and regulatory cells, cytotoxic T cells, dendritic cells, macrophages (also known as Kupffer cells), sinusoidal ECs, and hepatocytes—plays a crucial role in inflammation and immune quiescence (37). Immune tolerance and evasion in HCC are thought to be mediated by T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSCs). There are increased levels of Tregs and MDSCs within HCC tumors and circulating blood of patients with HCC. Also, MDSCs, which are immature progenitor cells, are prominent within the spleen, bone marrow, and liver and dampen effector T cell activity and NK cytotoxicity in these patients. The PD-1 and cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) checkpoint pathways and their ligands are principal players in maintaining immune tolerance. T cells, B cells, NK cells, and myeloid cells express PD-1, which interacts with PD-L1 and PD-L2, resulting in suppression of antigen-specific T cell activation. Antigen presentation by MHC molecules on T effector cells mediates the release of cytokines that elicit cancer cells to upregulate PD-L1 expression, further propagating immune escape. Although the mechanism of CTLA-4 is not well understood, activated T cells and Tregs express CTLA-4, which leads to inhibition of T cell activation and may also stimulate the expression of TGF-β and other cytokines (38). Also, tumor-associated macrophages (TAMs) can differentiate into activated M2 cells, which secrete cytokines that promote the growth of Tregs and suppress the growth of effector T cells, leading to a depressed immune response in the TME (37).

The relative populations of Tregs and cytotoxic T cells are thought to be independent predictors of prognosis and survival. It has been reported that low Tregs associated with high cytotoxic T cells lead to higher 5-year survival and disease-free survival (DFS) than in patients with high Tregs and low cytotoxic T cells. Moreover, high Treg cell density was associated with the presence of tumor invasion. Therefore, depleting Tregs and promoting T effector cell activity may be a potential therapeutic target (39). Moreover, IL-12 has been shown to suppress Treg cell activity and has demonstrated promising anti-tumor responses in mouse models (40-42).

The depletion of cytotoxic T cells is thought to be secondary to inhibitory interactions between PDL-1 (also known as B7-H1) on Kupffer cells and PD-1+ CD8+ T cells (43). PD-1 levels have also been demonstrated as a predictor of poor survival, increased invasion, and tumor recurrence after curative surgery (44,45). Overexpression of these checkpoints may lead to cytotoxic T cell exhaustion and a state of immune tolerance to HCC cells. Co-culture studies have demonstrated that PDL-1 or PD-1 blockade through monoclonal antibodies resulted in cytotoxic T cell recovery and improved effector responses (46).

Several clinical trials have studied immunotherapeutic agents in advanced HCC (47). A phase II study of tremelimumab, a monoclonal antibody against CTLA-4, which usually inhibits T cell activation, showed partial responses (PRs) in 17.6% of patients with HCC and hepatitis C virus (HCV) cirrhosis (48). Similarly, phase I/II trials with nivolumab (a PD-1 antibody) in patients with advanced HCC who had been previously treated with sorafenib showed a response rate of 19%, including 2 complete responses (CRs) and 7 PRs (49). The CheckMate-040 dose escalation study by the same authors on a larger population of 214 patients demonstrated that the durability of treatment response in patients with CR was maintained for up to 18 months and that the median duration of response was 23.7 months. The response rate was 16% in the total population and was consistent across all cohorts (uninfected, hepatitis B-infected, and HCV-infected). However, the response rate was highest in the sorafenib-naive population: approximately 20% (50). The overall 9-month survival rate was 70.8%. Combining a CTLA-4 antibody with anti-PD-1 antibody could potentially increase CD8+ T cell infiltration in tumor tissues and is currently being studied in a phase I/II trial of combined nivolumab and ipilimumab (51). Other trials are investigating checkpoint inhibitors in combination with sorafenib, including an ongoing phase III trial of combined nivolumab and sorafenib (52,53).

Locoregional therapies, including transcatheter arterial chemoembolization (TACE) and ablative techniques with cryoablation (CA), microwave, or radiofrequency ablation (RFA), play an important role in the treatment of advanced HCC. Some studies have shown that these therapies may elicit an immune response. A pilot study of 32 patients who were treated with tremelimumab in combination with TACE, CA, or RFA showed median time to tumor progression of 7.4 months. Interestingly, there was an increase in CD8+ T cell infiltration in tumor biopsies among patients showing clinical benefit and a reduction in the HCV viral load. (54). Immunotherapy has been approved for use in many other solid malignancies and to have proven survival benefits. Current preliminary immunotherapy data on HCC is encouraging, and checkpoint inhibitors may soon play a major role in HCC treatment.

ECs

HCC is an incredibly vascular tumor and is heavily dependent on pro-angiogenic cytokines, receptors, and tumor-derived ECs to maintain a rich blood supply. Sorafenib, a multikinase inhibitor of the serine-threonine kinases Raf-1 and B-Raf, the receptor tyrosine kinase activity of VEGFRs 1, 2, and 3, and PDGF receptor β, was one of the first chemotherapeutic drugs to show a survival benefit in the SHARP trial. Sorafenib's anti-angiogenic effects on tumor ECs are mediated through inhibition of VEGFR (55). Several studies have shown that tumor-derived ECs have a different composition than ECs from healthy liver tissue. Tumor-derived ECs have more aberrant chromosomal translocations, leading to leaky vasculature, rapid turnover, and resistance to apoptosis (56). Moreover, ECs from metastatic HCC, compared with those from non-metastatic HCC, expressed higher levels of PDGF-α, which was also associated with high recurrence (57).

Another study evaluated the expression of CD109, a glycoprotein found on ECs, on the growth and metastatic potential of HCC. Cultures with CD109 knockdown showed upregulation of IL-8 through the TGF-β/nuclear factor (NF)-κB pathway and overall poorer prognosis. The authors concluded that low levels of CD109 were associated with increased tumor size, higher TNM stage, and poorer survival (58).

CSCs

HSCs, or liver progenitor cells, have only recently been recognized as part of the TME. Hepatic stem cells typically transform to hepatoblasts, which can further differentiate between hepatocytes and cholangiocytes. Whereas liver regeneration after toxic injury is thought to result from replication of mature hepatocytes, some studies argue that a minority of new hepatocytes may originate from oval progenitor stem cells located within the portal triad (59).

In vitro studies have identified that HSCs express CD90, CD44, and CD133 as well as EpCAM, which is a marker of cholangiocytes (60). The presence of EpCAM+ cells in liver tissue has been associated with a higher AFP level and worse prognosis. Moreover, the presence of systemic circulating EpCAM+ hepatic stem cells were also associated with worse prognosis and early recurrence after curative surgery (61,62). This is thought to be secondary to the stem cells' overexpression of the Wnt β-catenin pathway and was demonstrated to induce chemoresistance to doxorubicin and sorafenib (63). Interestingly, a recent study compared the levels of EpCAM+ HSCs in patients with cancerous versus non-cancerous cirrhosis and found no significant difference in expression of the Wnt β-catenin signaling pathway, indicating an opportunity for pre-emptive screening and early diagnosis of HCC (64). Targeting EpCAM+ cells by inhibition of the Wnt pathways may be a therapeutic target to help decrease tumor stemness and invasiveness (65).

Extracellular matrix

The ECM is composed of proteoglycans, glycoproteins, polysaccharides, and connective tissue proteins such as collagen and fibrin. Increased ECM stiffness correlates to liver fibrosis and cirrhosis, a well-established precursor to HCC (66,67). An *in vitro* study evaluated the effect of different matrix stiffness on the growth and chemoresistance of HCC cells and showed that increased stiffness promoted both outcomes (66). Another *in vitro* study evaluated the signaling pathways associated with stiffer matrices and concluded that stiffer ECMs were associated with upregulation of the TGF-β pathway, along with increased levels of integrins and SMAD proteins (67). Inhibitors of this pathway decreased expression of these proteins and ultimately cellular traction forces.

The TME is an integral part of HCC pathogenesis as demonstrated by the review of all its components and signaling pathways. We will now discuss the structure and function of the TME in PDAC as well as highlight the latest therapeutic targets being tested. PDAC is the epitome of a treatment-resistant malignancy due to its intricate mechanisms between tumor cells and the surrounding microenvironment.

Pancreatic ductal adenocarcinoma

PDAC is a stroma-rich cancer that is the fourth leading cause of cancer-related death in the United States. Over the past 6 years, the 5-year survival rate has only improved to 8% (68). PDAC is distinctly characterized by its intense desmoplastic stroma, which largely contributes to this dismal prognosis (2). The lack of effective treatment strategies in PDAC is attributed to the heterogeneous milieu of cellular elements and tumor cells in the TME, including CAFs, pancreatic stellate cells (PaSCs), ECs, immune cells, and CSCs (69). The complex desmoplastic stroma, which makes up to 90% of the tumor, contributes to resistance to chemotherapy and radiation and is an

obstacle for effective therapies for PDAC, making it even more important for scientists to find new and effective targets within the TME to achieve results (70,71).

Signaling pathways in TME

PDAC is characterized by major cancer signaling pathways that stimulate tumorigenesis. KRAS is one of the most studied oncogenes, and its downstream signaling through the RAF-MEK-ERK pathway is therefore one of the most investigated signaling pathways in PDAC. Nearly all PDAC tumors have KRAS mutations, which are considered to be the initiating step in PDAC pathogenesis. The progression to PDAC has been described from pancreatic intra-epithelial neoplasia (Pan-IN) with varying grade of dysplasia. Accumulation of mutations, including activation of KRAS, has been demonstrated in the pre-neoplastic lesions (72). NF-κB is another downstream transcription factor of KRAS signaling in PDAC whose expression leads to release of proinflammatory cytokines and growth factors that regulate immune response and apoptosis. This NF-κB pathway may also contribute to chemoresistance (73). Another major player in pathogenesis of PDAC involves inactivation of the CDKN2A gene and loss of p53, the "gatekeeper" of the genome, which occurs in approximately 95% and >50% of PDAC cases, respectively. Inactivation of CDKN2A results in loss of p16 protein, which is a regulator of the G1-S of the cell cycle, whereas loss of p53 results in genomic instability (72).

Loss of the SMAD (DPC4) transcriptional regulator is another important event in the progression of PDAC. SMAD is a critical component in the TGF-β signaling pathway, and its loss, either through homozygous deletion or intragenic inactivating mutations of SMAD4 gene or complete loss of SMAD4 protein expression, occurs in approximately 50% of pancreatic cancers (72). An analysis of SMAD status of resected pancreatic cancers obtained from autopsies correlated loss of SMAD with metastatic disease (i.e., 78% patients with hundreds to thousands of metastases had SMAD loss) (73). Further evaluation of SMAD4 as a potential predictive biomarker proposed SMAD4 as predicting benefit for adjuvant therapy rather than prognostic for survival (74,75).

TGF-β modulates cell cycle regulators, including $p15^{INK4b}$ and $p21^{CIP1}$, stimulates apoptosis, and inhibits telomerase (76). TGF-β is also an important driver of the EMT process through upregulation of mesenchymal markers, including vimentin, fibronectin, N-cadherin, snail, and slug and downregulation of epithelial markers, including E-cadherin, and nuclear translocation of β-catenin. This pathway also promotes cell proliferation, immunosuppression, and activation of CAFs (77). A phase II trial of galunisertib, a TGF-β inhibitor, and gemcitabine compared with gemcitabine alone in patients with stage II–IV unresectable PDAC reported promising results, with a median OS of 10.9 for the combination, compared with 7.2 months for gemcitabine alone, and the combination appeared to be well tolerated. Lower levels of TGF-β were associated with higher benefit, so it could be a potential biomarker of activity (78).

The TME of PDAC is characterized by disorganized tumor vasculature. There is overexpression of VEGF, which has been associated with poor prognosis. This disorganized vasculature results in a hypoxic microenvironment. In addition, as discussed earlier, KRAS activation and hypoxia further stimulates VEGF-independent neo-angiogenesis (79). This hypoxic environment leads to a potent response by activating hypoxia-inducible factors, including HIF-1α (80).

Developmental pathways, including SHh, which plays a key role in embryogenesis, and Notch, which directs cell fate and proliferation, are important in PDAC, especially for a subgroup of cancer cells with stem cell–like properties (71,76). The binding to SHh releases Patched's inhibition of Smoothened (Smo), which allows Smo to translocate to the cell surface, a key-activating step in this pathway. Overexpression of the SHh pathway in CAFs contributes to formation of the desmoplastic stroma (76). SHh ligand expression and secretion in cancer cells functions in a paracrine manner (71). SHh signaling promotes tumor metastasis and recurrence through the EMT (4). Early phase trials investigating SHh inhibitors, including vismodegib and saridegib, in combination with cytotoxic chemotherapy (i.e. FOLFIRINOX and gemcitabine) in PDAC patients have shown inconsistent results (81-83) A phase IB/II trial of vismodegib and gemcitabine failed to show a survival benefit and no significant differences in drug delivery or response rates (81). Similarly, a phase II study of saridegib and gemcitabine versus placebo showed shorter median OS and more rapid rate of disease progression in the combination arm, which halted a phase II trial of saridegib and FOLFIRINOX (83). Interim results of saridegib and FOLFIRINOX did show a high ORR of 67% but also did not show any changes in tumor perfusion (82). Preclinical studies of SHh inhibition demonstrated increased intratumoral vascular density and higher chemotherapy delivery (84). Conversely, increased

chemoresistance of S phase—sensitive anticancer drugs (i.e., 5-FU and gemcitabine) has been shown to result from hypoxia-induced increases of Smo, which leads to PDAC proliferation through a hedgehog/Gli1-independent pathway (85).

Notch's activation results in transcriptional activation of a series of target genes and induction of NF-κB pathway. Notch is thought to interact with activated RAS to induce cancer transformation (76). Notch receptors are expressed on the surface of the cell membrane, where they can be cleaved by proteases, including a gamma secretase complex. Several early-phase clinical trials have evaluated various classes of Notch inhibitors, including gamma secretase inhibitors, siRNA, and monoclonal antibodies against Notch receptors or ligands (86).

Other pathways involved in the pancreatic TME include IGF, Met, fibroblast growth factor (FGF), and VEGF. There are high levels of IGF-1 in both tumor cells and stroma. *In vitro* studies have demonstrated autocrine IGF-I signaling that promotes cell proliferation and survival. Met expression is upregulated in Pan-IN lesions and PDAC as well as in stromal cells. FGF signaling is thought to contribute to pancreatic desmoplasia (76).

Further understanding of the importance of the stroma in PDAC has provided potential prognostic information. Erkan *et al.* defined an activated stromal index (ASI) as the ratio of myofibroblasts over collagen deposition and demonstrated poor survival in patients with a high ASI (87). Another recent study evaluated the relationship of stromal abundance with SHh expression as a stromal index (SI), defined as stromal area to proportion of tumor cell area, in 82 resected PDAC tumor specimens and found that low SI was associated with better DFS and OS (88). However, there have been conflicting data that high stromal area was associated with longer DFS and OS. Fokas *et al.* evaluated PD-L1, PD-1, CD8+, and FOXP3 expression and found high PD-1 tumor-infiltrating lymphocyte (TIL) expression to be associated with longer survival on multivariate analysis (P=0.049) (89). Overexpression of secreted protein acidic and rich in cysteine (SPARC) by fibroblasts in the TME has been associated with shorter survival. However, expression of SPARC on tumor cells was not related to prognosis (90). Subanalysis of resected PDAC specimens in the phase III CONKO-001 trial of adjuvant gemcitabine versus nab-paclitaxel with either high SPARC expression in the stroma or tumor epithelium also showed shorter survival (91). The hypothesized activity of nab-paclitaxel is due to binding of albumin to SPARC+ fibroblasts, which results in depletion of tumor stroma (69). However, further investigation of SPARC as a potential predictive biomarker was not supported by results of the phase III MPACT trial, the landmark trial that demonstrated the superiority of nab-paclitaxel and gemcitabine over gemcitabine alone. These conflicting data could be attributed to differences in patients and the origin of tissues obtained (i.e., primary pancreatic lesion *vs.* metastatic lesion) and the methodology evaluating SPARC. SPARC analysis was exploratory in the MPACT trial; therefore, it was not designed to evaluate treatment efficacy (91).

CAFs

CAFs are abundant in the stroma of PDAC. Similar to HCC, the CAFs arise from PaSCs, neighboring fibroblasts, bone marrow–derived cells, ECs, and adipocytes. Recent studies have identified fibroblast-specific protein 1 as a potential marker of CAFs (92). Also, there is a subset of CD10+ cancer CAFs that induces a more invasive phenotype (74). Tumor cells secrete cytokines that activate and recruit CAFs, which then produce signaling factors that mediate CAF to tumor cell cross-talk. Other quiescent fibroblasts become activated through the response of stress stimuli and express α-SMA and transition into myofibroblasts (92). Stromal CAFs also secrete fibroblast activation protein α (FAPα), which induces cell motility and invasiveness, immune suppression, and angiogenesis. FAP-expressing fibroblasts inactivate retinoblastoma protein, an inhibitor of cell cycle progression, leading to a carcinogenic environment (69,93).

PaSCs

PaSCs, also known as activated fibroblasts, play a key role in maintenance of the surrounding stroma and interact with cancer cells, leukocytes, and ECs. The cancer cells induce the PaSCs to increase ECM synthesis (94). Factors produced by cancer cells, such as PDGF, TGF-β1, SPARC, and MMPs activate proliferation of PaSCs and production of the ECM, respectively (95). The pathogenesis of pancreatic fibrosis is thought to be due to increased PaSCs, which are found in areas of high collagen content (69). The molecular signaling between PaSCs and tumor cells perpetuates tumor growth and progression (76). Moreover, recent *in vitro* studies have found that PaSCs augment the "stemness" phenotype of pancreatic cancer cells (70). *In vitro* studies have demonstrated increased expression of

mesenchymal markers, vimentin, and snail when PDAC and PaSCs are co-cultured (95). PaSCs have a unique ability to remain in activated states through autocrine signaling (92). Chronic inflammation is a key feature of PDAC and mediated by activated PaSCs that regulate the inflammatory response including TGF, tumor necrosis factors, and interleukins. Additionally, PaSCs contribute to immune evasion through galectin-1 and FAPα expression (94).

In vitro studies have demonstrated resistance to chemotherapy and radiation may be due to activated PaSCs. The hypoxic environment of PDAC is created in part from PaSC expression of angiogenic factors, including VEGF and angiopoietin-1. Recently, PaSCs have also been found in early Pan-INs, implying that PaSCs may play a role early carcinogenesis of PDAC (94).

Immune cells

PDAC is characterized by an imbalance of pro-tumorigenic immune cells with predominance of myeloid derived suppressor cells (MD-SCs), Tregs, and TAMs and a paucity of CD4+, CD8+, and NK cells (69,76). This immunosuppressive state is sustained by Tregs that secrete cytokines to dampen T effector cell function as well as by MD-SCs that inhibit CD8+ T cells (76). Recent studies have shown that focal FAK plays a role in immune tolerance by upregulating Treg and downregulating CD8+ cells. FAK signaling has also been implicated in pancreatic fibrosis (96). A Japanese study tested FAK inhibitors in mouse models of PDAC, and results showed delayed tumor progression, reduced fibrosis, and lower levels of immunosuppressive T cells (97). Interestingly, murine cancers that were previously unresponsive to immunotherapy with PD-1 antagonists were responsive after FAK inhibition. The authors concluded that addition of FAK inhibitors to immunotherapeutic drugs could alter the TME for a more effective response to immunotherapy. This is being tested in a current phase I clinical trial combining FAK inhibition and immunotherapy with gemcitabine in patients with advanced pancreatic cancer (98).

TAMs are the predominant immune cells in PDAC. M1 TAMs are *pro*-inflammatory macrophages that stimulate T cell antitumor immunity, whereas M2 TAMs are *anti*-inflammatory macrophages produce factors that propagate tumor growth and survival. A recent study has elucidated the role of TIL B cells in pancreatic tumorigenesis (92). These TILs secrete IL-35, which stimulates tumor proliferation and suppresses the antitumor response of CD8+ T cell through the Bruton tyrosine kinase pathway. Also, deletion of HIF-1 results in accumulation of B lymphocytes and pancreatic tumorigenesis (99-101).

Some studies have explored vaccine therapy such as GVAX, a granulocyte macrophage–colony-stimulating factor–based immunotherapy, Reolysin, an oncolytic reovirus, and HLA restricted peptides. A phase 1b study compared GVAX with and without ipilimumab (a CTLA-4 inhibitor) in patients with previously treated advanced PDAC and showed a mild improvement in survival in the combination arm (OS of 5.7 *vs.* 3.6 months) (102). A randomized three-arm trial of neoadjuvant and adjuvant GVAX in combination with either IV or oral cyclophosphamide in resectable PDAC showed formation of intratumoral tertiary lymphoid aggregates within the resected tumors in 33 of 39 patients after GVAX administration. Additionally, there was upregulation of immunosuppressive pathways, including PD-1 and PDL-1, which proposes the hypothesis of creating an "immunogenic" PDAC tumor that is primed for enhanced activity through subsequent checkpoint blockade (103).

Reolysin, an oncolytic virus that selectively replicates in cells harboring an activated RAS pathway, in combination with gemcitabine has been investigated in patients with chemotherapy-naive advanced or metastatic PDAC with promising results and good tolerability with manageable non-hematological toxicities. Upregulation of PD-L1 expression following Reolysin was noted, highlighting the potential of pairing with checkpoint inhibitors (104).

After an encouraging phase I trial using HLA restricted peptides (105), a phase II trial was conducted in which treatment-naive patients were given gemcitabine in combination with a vaccine cocktail of KIF20A (a peptide involved in molecular trafficking) and two angiogenic peptides derived from VEGFR1 and VEGFR2. In the HLA-matched group, patients who demonstrated a peptide induction for these vaccines had a better response than those who did not. In addition, those who experienced stronger injection site reactions had longer survival times than those with a weak or absent site reactions. These results may pave the way for further studies on vaccine immunotherapy (106).

Checkpoint blockade is also being investigated in PDAC. A phase II study of dual blockade of PD-1 and CTLA-4 pathways is being evaluated in metastatic PDAC (107) based on results from a phase 1B study of locally advanced and metastatic non-small-cell lung carcinoma in which there were objective

responses of 23%, irrespective of PD-L1 expression (108). However, single agent ipilimumab did not demonstrate any activity in PDAC (109). Further investigation of combining checkpoint blockade with other agents that may make PDAC more immunogenic is being evaluated.

ECs

ECs are directly and indirectly affected by tumor cells through direct contact or stimulation via FGF, VEGF-A, and PDGF-β or via stromal cells, respectively (92). *In vitro* studies of cultures of ECs and PaSCs have demonstrated increased EC proliferation with introduction of PaSCs. Similar results from cultures of ECs with tumor cells upregulated pro-angiogenic pathways, which could indicate that ECs are potential targets for anti-angiogenic therapies (4). Unlike in HCC, VEGF-targeted therapy has shown no clinical benefit in PDAC. Despite encouraging results in a phase II trial with axitinib (an oral VEGF inhibitor), a phase III trial comparing gemcitabine and axitinib to gemcitabine and placebo showed no significant difference (110). Similarly a phase III trial comparing gemcitabine with and without bevacizumab (a VEGF inhibitor) showed no significant improvements in OS (111).

CSCs

The SHh, Notch, and Wnt/β-catenin pathways play key roles in stem cell self-renewal in normal and CSCs. The first pancreatic stem cell was described in 2007. Subsequently, several studies have been done to identify pancreatic cancer stem cells (PCSCs) and their cell surface markers, which include CD44+ CD24+ ESA+, CD133+ 3, C-Met, and ALDH. These subpopulations vary in tumorigenicity potential and its effects on tumor invasion, metastasis, and survival. CD133+ PCSCs have been found to be resistant to gemcitabine chemotherapy. In addition, CD133+ expression has been significantly associated with lymphatic metastasis, VEGF-C expression, and poor survival. C-met has also been associated with chemoresistance. Tumor cells with high C-met expression and CD44+ have the highest tumorigenicity potential. Clinical trials targeting these pathways are under way (4). There has been increasing attention to the role of miRNAs in mediating EMT. Strategies are focusing on PCSCs, including targeting the EMT markers (112).

Extracellular matrix

In PDAC, there is an increase in ECM that results in a disrupted environment of decreased perfusion and hypoxia (69). The abundance of hyaluronic acid (HA) disturbs the chaotic vascular web and contributes increased intratumoral pressure, resulting in vascular collapse (76). PaSCs are also important in controlling the ECM turnover through synthesis of MMPs and tissue inhibitor matrix proteinases and ECM synthesis through the Hedgehog pathway, as described above (95). Results from a phase II study of pegylated recombinant human hyaluronidase in combination with gemcitabine and nab-paclitaxel showed promising results in tumors with high stromal HA (113). Global phase III studies are ongoing (114).

Another agent, necuparanib, an inhibitor of multiple heparin-binding growth factors, chemokines, and adhesion molecules, which results in decreased stromal activation within the TME, reduced tumor size and fibrosis in preclinical studies (115). However, the phase II trial of necuparanib in combination of gemcitabine and nab-paclitaxel failed to show any clinical benefit and was terminated early (116).

Conclusions

In conclusion, elements of the TME participate in growth and metastasis of HCC and PDAC. They also contribute to chemoresistance and recurrence after curative resection, and certain TME components have been established as independent prognostic factors. CSCs in particular play a role in carcinogenesis and, together with the TME, promote the acquisition of Weinberg's hallmarks of cancer. Increasingly, more clinical research is being done on therapeutic targets located within the TME with promising as well as mixed results. The role of liver cirrhosis as a precursor to HCC as well as the rich vascularity as a channel for metastasis and recurrence highlight the importance of treating HCC as a disease of the liver rather than a population of neoplastic hepatocytes. Similarly, the dense desmoplastic stroma associated with PDAC should be considered just as vital a target as the pancreatic neoplastic cells.

Previously, cytotoxic treatment strategies primarily focused on inducing tumor cell death in HCC and PDAC; however, a better understanding of the TME has shifted to directing therapy against elements of the TME. The interactions of the tumor cells with the TME, particularly

immune cells, have provided a dynamic environment to create novel therapies. The molecular heterogeneity and the etiological differences in HCC could potentially contribute to the failures of certain treatment strategies. Poor understanding of HCC tumor progression and dissemination has been demonstrated, with negative results from MET and SHh inhibitors trials. However, strategies using checkpoint inhibitors have been more successful in HCC, with surprisingly higher response rates. Ongoing HCC studies combining checkpoint inhibitors with other targeted agents are highly anticipated.

Prior PDAC treatment strategies have been only mildly effective, with limited drug delivery due to the dense desmoplastic stroma. Thus, potential PDAC therapies should be directed at expansion of tumor-reactive T cells and vaccination with tumor-specific antigens to activate the immune system. Such therapies should be combined with concomitant reduction of immune inhibitory pathways to decrease immune tolerance. Vaccine trials with GVAX and Reolysin could ideally be combined with checkpoint inhibitors and may offer a novel strategy to combat PDAC. Finally, therapies directed at CSCs are currently being evaluated in both HCC and PDAC; this represents a completely different perspective in treating and understanding these malignancies.

In conclusion, novel therapeutics are moving past traditional cytotoxic therapies and increased understanding of the intricate pathways and mechanisms involving angiogenesis, fibrosis, and immune tolerance between the tumor cells and TME has helped pave the way for tumor destruction.

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Footnote

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