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Emerging Frontiers in Pancreatic Cancer Research: Elaboration of Key Genes, Cells and the Extracellular Milieu

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Abstract

Purpose of review—We review recent literature with a view to forge an integrative understanding of the molecular, cellular and extracellular milieu of pancreatic cancer, and discuss them in the context of development of novel, personalized therapeutic options.

Recent findings—Pancreatic tumorigenesis, examined using genetically engineered mouse models, appears to be driven by local inflammation, in concert with the ‘big four’ mutations involving oncogenic KRAS, SMAD4, CDKN2A, and TP53, through induction of EMT and cancer stem cells, and accompanied by metastasis. High throughput sequencing of pancreatic ductal adenocarcinoma (PDAC) as well as neuroendocrine tumors and rarer subtypes of cancers of the pancreas have revealed several novel mutations in genes like PALB2, GNAS, DAXX, ATRX, SWI/SNF pathway related, and in genes in the ubiquitin dependent pathways such as USP9X. Therapeutic targeting of the tumor-stroma axis by cytokines and immune response modulators and the role of autophagy in pancreatic cancer are some other salient themes explored in the recent publications.

Summary—Recent publications shed new light on the mutational landscape of pancreatic cancer and further delineate the distinctive pancreatic cancer-stroma ecosystem as determined by the dynamic interplay of inflammation, hallmark mutations, epithelial to mesenchymal transition (EMT) and cancer stem cells.

Keywords

pancreatic ductal adenocarcinoma (PDAC); next generation sequencing; cancer stem cells; genetically engineered mouse models (GEMM) of pancreatic cancer; desmoplastic stroma

Introduction

While not the most prevalent of malignancies, pancreatic cancer continues to be a leading cause of cancer related deaths worldwide, due to late detection and lack of specific therapeutic targets and ineffective therapies. With the next generation sequencing of tumors, development of primary tumor xenograft models, elucidation of early events in tumorigenesis and the interplay between tumor and stroma using genetically engineered

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mouse models, the field of pancreatic cancer research is poised for major breakthroughs in early detection and more effective treatment modalities. Here we review salient, sometimes representative, reports in pancreatic cancer published over the last year, encompassing the broad areas of high throughput sequencing, cancer cell and stromal biology, and cell fate related studies using genetically engineered mouse models (Figure 1) (1).

High throughput sequencing of pancreatic cancers reveals intratumoral heterogeneity

High throughput sequencing has quickly emerged as the primary modality of genomic characterization of cancers (2), with mutational profiles of several major carcinomas being reported over the past several years (3–7), including pancreatic cancer. In a high throughput Sanger sequencing analysis of more than twenty thousand genes from 24 pancreatic cancers, Jones et. al. have identified an average of 63 genetic alterations per tumor, and assigned each of these alterations to a core set of 12 distinct cellular pathways (1). While most tumors had genomic alterations in each of these pathways, individual tumors each seemed to harbored a distinct set of mutations affecting these conserved core pathways. Analyzing genomic rearrangements in pancreatic cancer, Campbell et. al. described evidence of telomere dysfunction and dysregulated G1-to-S-phase transition in early cancer development (8). They also noted ongoing evolution among metastases, and interestingly, phylogenetic trees across metastases showed organ-specific branches. In an independent study analyzing clonal relationships among primary cancer and metastatic cells, Yachida et. al. reported that clonally distinct subpopulations within the primary pancreatic carcinoma presumably gives rise to distant metastases, suggesting that the genetic heterogeneity of metastases is engendered in the primary tumor itself. Recent studies using mouse models of pancreatic cancer progression also appear to support this premise (discussed below). Further, comparing the mutation profiles in the initiating lesions with those in the metastatic tissues, Yachida et. al. estimated that it takes at least 15 years from the initiating mutation in a benign cell to reach a full blown metastasis. This inference, if validated in independent studies, will imply that early detection of pancreatic cancer could potentially afford a broader therapeutic window (9).

Apart from global genomic analyses, recent studies have identified several novel mutations in pancreatic cancers. Through exome sequencing of familial pancreatic cancer patients, a truncating mutation in the BRCA2 interacting protein, PALB2 (Partner and localizer of BRCA2), was identified as a pancreatic cancer susceptibility gene (10). Mutations in PALB2 have since been reported in familial breast and pancreatic cancer in independent cohorts (11–13). Likewise, recurrent mutations in GNAS (guanine nucleotide binding protein, alpha stimulating) were identified in intraductal papillary mucinous neoplasms (IPMN), pancreatic cysts frequently associated with malignant growth (14, 15). Activating mutations in GNAS have been previously described in colorectal cancer(16), thyroid carcinomas(17), adrenocortical lesions(18–21), pituitary tumors(22), and Leydig cell tumors(23), among others. Exomic sequencing of pancreatic neuroendocrine tumors (PanNETs) identified novel recurrent mutations in the transcription/chromatin remodeling complex comprising of DAXX (death-domain-associated protein) and ATRX (α thalassemia/mental retardation syndrome X-linked), as well as mutations in genes in the mTOR (mammalian target of rapamycin) pathway that could potentially be amenable to treatment with mTOR inhibitors (24). Interestingly, as speculated, tumors with ATRX and DAXX mutations were found to harbor altered telomeres in a follow up study (25), and have been associated with good prognosis (26). Focusing on functional grouping of somatic mutations identified in genomic sequencing of pancreatic cancers, Shain et. al. have identified loss of function mutations in genes encoding components of the SWI/SNF chromatin remodeling complex that includes putative DNA binding subunits (ARID1A,

ARID1B, and PBRM1) and the enzymatic subunits (SMARCA2 and SMARCA4), that, while rare individually, together are present in one-third of all pancreatic cancers (27).

Mutations identified through high throughput sequencing of tumor samples need further characterization to establish potential functions in tumorigenesis. Taking a converse route, Perez-Mancera et. al., carried out a functional genomic screen for KRAS interactors using transposon-mediated insertional mutagenesis in a mouse model of pancreatic cancer and observed a loss of deubiquitinase Usp9x in over 50% of the murine pancreatic tumors(28). Although no analogous mutations in this gene were observed in human pancreatic cancer tissues, lower USP9X expression level was correlated with poor survival after surgery, and inversely associated with metastatic burden in advanced disease. Thus, USP9X has been proposed to signify an important tumor suppressor gene with prognostic and therapeutic relevance in PDACs.

Thus overall, application of high throughput genomics to pancreatic cancers have yielded several novel leads in a short span of time that are likely to be vigorously followed up in the coming years. With increasing access to low cost, high throughput sequencing and with better understanding of the implications for these genetic alterations in diagnostic and therapeutic management, it is expected that some of these findings may inform personalized therapeutic options in the future.

The increasing footprint of tumor microenvironment in pancreatic cancer

The desmoplastic stroma enveloping PDAC is comprised of cancer-associated fibroblasts (CAFs), infiltrating immune cells, blood vessels, and extracellular matrix, together constituting an elaborate microenvironment that is favorable for tumor growth and may also act as an effective physical barrier to chemotherapeutics. Several studies have explored the role of individual components of stroma in sustaining pancreatic cancer, and provide multiple potential therapeutic avenues. In one such study, Olive et. al. associated poor vasculature of the stroma with the activity of fibroblasts. Using an inhibitor of the hedgehog pathway reduced the proliferation of cancer associated fibroblasts, increased stromal vasculature, leading to improved permeability of the chemotherapeutic agent gemcitabine and reduced tumor burden (29). In another study, the authors targeted the hyaluronic acid (HA) matrix in the stroma that presents a physical barrier to drug delivery, and report that enzymatic ablation of stromal HA expands the stromal microvasculature, improving efficacy of gemcitabine treatment, resulting in a near doubling of overall survival using a genetically engineered mouse model of pancreatic cancer (30).

The fact that oncogenic KRAS (G12D) readily induces PanIN lesions in experimental mouse models but progression to PDAC requires local inflammation indicates the requirement for a permissive environmental milieu. Several cytokine and immune modulators of tumor-stroma interaction have been described recently that help us better understand this dynamic milieu as well as point to potential therapeutic avenues (31). The stroma infiltrating immune cells secrete IL-6 that activates STAT3 and triggers progression of PanINs to PDAC (32). Another study highlights the role of the chemokine CXCR2 in mediating the induction of CTGF by fibroblasts that promotes the progression of PanINs to PDAC (33). Notably, CXCR2 inhibitors were found to inhibit tumor progression by disrupting tumor-stromal interactions and improved survival in a mouse model of PDAC (34). PDAC stroma containing high levels of intratumor T helper type 2 cell infiltrate (Th2 (GATA-3+)) was associated with poor survival and was shown to result from PDAC activated CAFs secreting thymic stromal lymphopoietin (TSLP) that induced myeloid DCs to secrete Th2-attracting chemokines (35). On the other hand, Beatty et. al. reported tumor regression in some patients with surgically incurable PDAC by using an immune-reactivating agonist CD40 antibody in combination with gemcitabine chemotherapy (36).

Follow up studies in mouse models of PDAC revealed that tumor regression by CD40 was through recruitment of macrophages that infiltrated and killed the tumor cells as well as depleted the tumor stroma, independent of activated T cells or gemcitabine (36). Further, tumor progression triggered by inflammation has been hypothesized to result from abrogation of the oncogene driven senescence characteristic of low-grade mPanINs. Thus, it was proposed that anti-inflammatory drugs can potentially block mPanIN expansion, reducing the risk of developing PDAC (37).

While tumor microenvironment research largely pertains to the components of stroma and the tumor stroma interaction, the oxidative state of tumor cells constitutes a local environmental cue as well. One study exploring ROS metabolism in the context of oncogene expression in murine cells found that ROS are actively suppressed in K-RAS (G12D) and B-RAF (V619E) expressing cells and in human pancreatic cancer through activation of the NRF2 mediated antioxidant program (38). Interestingly, abrogation of the NRF2 pathway impaired K-RAS (G12D)-induced proliferation and tumorigenesis in vivo, thus presenting yet another potential avenue for therapeutic intervention (38). Overall, these recent studies suggest that each component of the tumor-stroma ecosystem contributes to pancreatic tumorigenesis and sustenance, and in fact present multiple layers of vulnerabilities that may need to be integrated for successful therapeutic strategies.

The driving force of pancreatic cancer stem cells

The precise identity of the cell of origin for pancreatic ductal adenocarcinoma (PDAC) continues to be debated. The hypothesis that a subset of cells that renews the adult pancreas such as stem cells, progenitor cells, facultative stem cells or trans-differentiated bone marrow cells might also serve as cancer stem cells (CSCs) has merit, but needs to be demonstrated (39). In that light the report showing a role for TGF- β superfamily members Nodal and Activin that are known regulators of embryonic stem cell fate in pancreatic cancer stem cells is of significant interest. Nodal and Activin were found to be overexpressed in pancreatic CSCs, and their knockdown abrogated self-renewal capacity and in vivo tumorigenicity (40). Impressively, the combination of a stroma-targeting hedgehog pathway inhibitor and inhibitor of Nodal/Activin in mouse tumors provided long-term, progression-free survival, suggesting a route to pancreatic CSC therapy (41).

In another independent study the potential CSC function of a known pancreatic stem cell marker c-MET was analyzed (42). In this study, pancreatic cancer cells from low passage primary human pancreatic cancer xenografts were sorted on the basis of c-MET expression and were found to form tumorspheres and tumor xenografts in NOD-SCID mice more readily as compared with c-MET negative populations. The combination of c-MET and CD44 cell surface expression identified the most highly tumorigenic population studied, when compared to other CSC markers, such as ALDH and CD133. Significantly, c-MET inhibition through shRNAs or use of the c-MET inhibitor XL184, inhibited tumor sphere formation as well as reduced tumor growth in vivo and knocked down the CSC population (42). In addition, the c-MET inhibitor XL-184 was able to completely block the development of metastasis using an intra-cardiac injection model, highlighting the potential utility of targeting c-MET in the neoadjuvant or adjuvant setting, where patients who undergo surgical resection are at high risk for metastatic recurrence.

Evidence of metastasis as an early event in pancreatic tumorigenesis

Two recent publications independently examining early events in pancreatic tumorigenesis—one using mouse model of PDAC (43) and the other involving computational modeling of human pancreatic cancer (44), have arrived at a provocative common conclusion that metastasis is likely an early event during tumorigenesis (45), not the last step in a series of

sequential acquisitions of mutations (46, 47). Rhim et. al. utilized a YFP labeled, in vivo lineage tracking system to follow the fate of pancreatic epithelial cells expressing mutant KRAS and TP53 in mouse models of PDAC (43). In presence of inflammation induced by cerulein, PanIN lesions progressed to PDAC, with pancreatic cells displaying surface markers of EMT and CSCs found in the circulation of mice harboring PDAC, as well as interestingly, in mice with only PanIN lesions. This suggests that metastatic cells may bud off from the primary tumor/preneoplastic lesions much sooner than previously believed.

Pancreatic cancer cells and autophagy

Autophagy describes the phenomenon of systematic degradation of cell organelles and macromolecules through recruitment of lysosomes. Autophagy has been associated with oncogenic K-RAS-induced malignant cell transformation in cell culture models (48), and recently, pancreatic cancer cells were shown to display elevated levels of autophagy (49). Inhibition of autophagy led to growth suppression of pancreatic cancer cells in vitro, and treatment with the common anti-malarial drug chloroquine, that is also a potent inhibitor of autophagy, led to tumor regression and prolonged survival in pancreatic cancer xenografts and genetic mouse models (49). Another study with experimental mouse models has reported an autophagy-dependent anticancer immune response following treatment with chemotherapeutic agents (50). These are exciting new leads that may provide additional avenues for pancreatic cancer therapy.

Improved mouse models of pancreatic cancer

Genetically engineered mouse models (GEMMs) of PDAC harboring combinations of KRAS, TP53 (and p16INK4/SMAD4) mutations have enabled precise analyses of PDAC biology, tumor development and progression, and more recently, the role of inflammation and the tumor microenvironment (51). Mouse models of PDAC are also being used to develop new therapeutic and diagnostic approaches (52). While, transgenic mice harboring mutations in KRAS and TP53 provide a powerful model of pancreatic cancer, aberrant expression of the transgenes commences in embryonic pancreas, which does not mimic human pancreatic cancer development, where somatic mutations affect the adult pancreas. A recent publication describing transgenic mice with tetracycline inducible expression of the oncogenic Kras^{G12D} in combination with active/inactive alleles of the tumor suppressor gene p53 provides a very useful addition to the existing PDAC models (53). Using reversible induction experiments, mutant KRAS was found to be required for both the initiation and maintenance of pancreatic cancer in mice. Another study using inducible KRAS has elaborated the role of anabolic glucose metabolism in the maintenance of pancreatic cancer that could potentially help delineate a molecular link between diabetes and pancreatic cancer (54). The inducible model of PDAC may emerge as the preferred model to tease out the contributions of individual mutations in pancreatic cancer.

Even as mouse models are useful, these are no substitute for analysis of human tumors. Hidalgo's group established the experimental protocol for engraftment of primary human PDACs in nude mice followed by generation of low passage xenografts and primary tumor cell lines for therapeutic testing (55). Primary human tumor xenografts provide a powerful tool for genomic/proteomic analysis of individual tumors, as well as it enabling pre-clinical testing of novel drugs and targets in vivo to explore personalized therapeutic options. In a recent report, they systematically evaluated the factors affecting engraftment and the predictability of clinical outcome based on experiments with low-passage xenografts (56). We expect to see this experimental approach being adopted by more researchers, particularly in conjunction with clinical sequencing, to test the efficacy of personalized therapeutic targets(57).

Advances in imaging of early pancreatic cancer lesions

Highly sensitive and specific early detection of pancreatic cancer is needed to extend the available therapeutic time window, but a major limitation is availability of biomarkers for early detection of the disease. Recently two independent studies using mouse models of PDAC have demonstrated the potential of non-invasive detection of pancreatic cancer cells by coupling serine protease cathepsin activity in preinvasive mPanIN lesions and PDAC as compared to benign or inflamed pancreatic tissue with fluorescent substrate probes (58, 59). Eser et. al. identified the serine protease cathepsin to be specifically overexpressed in preinvasive mouse PanIN lesions and PDAC as compared to benign or inflamed pancreatic tissue. They combined a cathepsin-activated near-infrared probe with confocal fluorescence laser microscopy to detect and grade murine PanIN lesions in real time in vivo (59). Elsewhere, in vivo imaging in mice using Plec-1 targeting peptides as a contrast agent for single photon emission computed tomography could distinguish primary and metastatic PDACs from benign tissues (60, 61). Follow up studies testing these leads in a human clinical trial setting are in order.

Conclusions

Pancreatic cancer research is gathering steam with several conceptual breakthroughs made in the field of mutational profiles of tumors, tumor-stroma interactions, cancer stem cells, epithelial to mesenchymal transition, and metastasis using high throughput sequencing applications, robust transgenic mouse models as well as primary tumor xenografts and cell lines (Figure 1). We envision further maturation of the field in the near future will lead to the much needed therapeutic breakthroughs in this 'jewel in the crown of the 'emperor of all maladies'.

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We apologize to all the authors whose work could not be cited due to space limitation.

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Key points

1. The pancreatic ductal adenocarcinoma (PDAC) niche in the pancreas is comprised cancer cells, cancer cells undergoing epithelial to mesenchymal transition (EMT), cancer stem cells (CSCs), and cancer associated stroma.
2. Most PDACs are characterized by the presence of hallmark mutations in KRAS, p16INK4/CDKN2A, TP53, and SMAD4.
3. High throughput sequencing has led to the discovery of novel recurrent mutations in PDACs as well as rarer subtypes of benign/neoplastic lesions of pancreas.
4. Genetically engineered mice with inducible KRAS mutation provide a powerful tool to model pancreatic tumorigenesis.
5. Future therapeutic strategies need to target tumor, stromal, and stem cells, engaging tumor immune response and autophagy.

Figure 1.
Pancreatic cancer in mouse and man: emerging concepts in pancreatic cancer biology.