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REVIEW

Molecular therapeutics in pancreas cancer

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Abstract

The emergence of the "precision-medicine" paradigm in oncology has ushered in tremendous improvements in patient outcomes in a wide variety of malignancies. However, pancreas ductal adenocarcinoma (PDAC) has remained an obstinate challenge to the oncology community and continues to be associated with a dismal prognosis with 5-year survival rates consistently less than 5%. Cytotoxic chemotherapy with gemcitabine-based regimens has been the cornerstone of treatment in PDAC especially because most patients present with inoperable disease. But in recent years remarkable basic science research has improved our understanding of the molecular and genetic basis of PDAC. Whole genomic analysis has exemplified the genetic heterogeneity of pancreas cancer and has led to ingenious efforts to target oncogenes and their downstream signaling cascades. Novel stromal depletion strategies have been devised based on our enhanced recognition of the complex architecture of the tumor stroma and the various mechanisms in the tumor microenvironment that sustain tumorigenesis. Immunotherapy using vaccines and immune checkpoint inhibitors has also risen to the forefront of therapeutic strategies against PDAC. Furthermore, adoptive T cell transfer and strategies to target epigenetic regulators are being explored with enthusiasm. This review will focus on the recent advances in molecularly targeted therapies in PDAC and offer future perspectives to tackle this lethal disease.

Key words: Pancreas neoplasm; Vaccines; Targeted therapy; Immunotherapy; Kirsten rat sarcoma oncogene

Core tip: The treatment of pancreas ductal adenocarcinoma is in an exciting phase due to a tremendous surge in knowledge regarding the molecular mechanisms that underlie pancreas cancer that has fueled interest in devising novel strategies to target signal transduction factors downstream to kirsten rat sarcoma oncogene, desmoplastic tumor stroma and cancer stem cells. Furthermore, immunotherapy by utilizing vaccines and immune checkpoint inhibitors is gaining momentum. Alluring results from studies evaluating molecularly targeted therapies have not only proven the feasibility of this approach but are also indicative of a paradigm shift in management of pancreatic cancer in the near future.

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INTRODUCTION

Over the past few decades, pancreas ductal adenocarcinoma (PDAC) has claimed notoriety by proving to be one of the most recalcitrant solid-organ malignancies. As a telltale sign of its lethality, PDAC accounts for less than 3% of new cancers diagnosed annually in developed nations and in the United States, yet it is the fourth leading cause of cancer related mortality[1]. Ominously, PDAC is also poised to surpass breast, prostate and colon cancers to become the second leading cancer related cause of death by 2030[2]. Owing to the late stage at presentation, most patients with PDAC are not candidates for surgical resection. Even patients with early-stage disease who undergo surgical resection and adjuvant therapy eventually relapse and succumb to it[3,4].

Patients with advanced disease have a dismal prognosis with 5-year survival rates of less than 5%[5]. Following the initial success of gemcitabine in the metastatic setting[6], oncologists have traditionally relied upon cytotoxic chemotherapy to tackle locally advanced and metastatic disease but with limited success. After nearly two decades of research to identify optimal regimens for metastatic PDAC, the PRODIGE 4/ACCORD 11 and MPACT trials have proven the efficacy of combination chemotherapy with meaningful increase in overall survival (OS) although accompanied by the risk of increased toxicity[7,8]. The median survival for patients with metastatic disease still remains less than 1 year[7,8].

MOLECULAR THERAPEUTICS IN PDAC

The dawn of the era of precision-medicine in oncology has led to tremendous gains in understanding various molecular mechanisms of PDAC oncogenesis, but translating this knowledge to the bedside with targeted therapy has been a daunting task. The complex biology of PDAC has posed a formidable challenge against successful targeted interventions (summarized in Table 1). However, in recent years several innovative approaches have achieved early success to pave the way for impactful molecular therapeutic strategies.

GENETIC HETEROGENEITY OF PDAC

Similar to the adenoma-carcinoma sequence in colon cancer, the development of PDAC represents the culmination of progressive increments in dysplasia in precursor lesions collectively termed pancreatic intraepithelial neoplasia (PanIN)[9]. Molecular profiling studies in genetically engineered mouse models (GEMM) have demonstrated that histological progression of PanINs from low to high-grade occurs in tandem with successive accumulation of gene mutations such as activation of the *KRAS* oncogene, inactivation of the tumor suppressor cyclin dependent kinase-N2A (*CDKN2A*) gene and the eventual inactivation of TP53 and deleted in pancreatic cancer 4 (*DPC4*/*SMAD4*) genes[10]. All of the same genetic alterations also occur in established PDAC but at a higher frequency (Table 2). Patients with familial forms of PDAC also harbor germ-line mutations in BRCA2 and partner and localizer of BRCA2 (*PALB2*) genes[11,12]. In a sentinel genomic analysis of 24 pancreatic tumors, Jones *et al*[13] classified the genetic alterations in PDAC into a core set of 12 cellular signaling pathways that encompass an incredibly high 63 gene mutations within an individual tumor. A recent study of 109 micro-dissected pancreatic tumors by whole-exome sequencing corroborated the high mutational burden and also identified other novel genetic mutations that confer adverse prognosis such as MYC amplification[14]. Abnormalities in Wnt and Hedgehog signaling, chromatin remodeling and DNA repair mechanisms occur at a high frequency in PDAC[14,15]. In addition to remarkable variations in genetic abnormalities in individual tumors, the realization that PDAC genes function through a relatively small number of pathways confers a level of genetic heterogeneity that makes molecular targeting exceptionally difficult.

Targeting KRAS and downstream signal transduction

The four human *RAS* genes encode for small guanosine triphosphatases (GTPases) and under normal circumstances cycle between an active GTP-bound and an inactive guanosine diphosphate (GDP) bound state[16]. Upwards of 95% of PDACs possess activating mutations of the *KRAS* gene, most commonly at the G12 residue[17]. Mutant *KRAS* remains persistently active in the GTP-bound state and results in uninterrupted downstream signal transduction of growth signals such as rapidly activated fibrosarcoma homolog B (BRAF), mitogen activated protein kinase (MAPK) and phosphatidyl inositol-3 kinase (PI3K)/mammalian target of rapamycin (mTOR)[16].

Despite intensive efforts, direct pharmacologic inhibition of *KRAS* has been unsuccessful because of the high binding affinity of the oncoprotein to GTP and inability to identify an easily accessible active site within *KRAS* that is susceptible to competitive allosteric inhibition[18]. To overcome these difficulties, alternate approaches have been attempted but with limited success. Van Cutsem *et al*[19] attempted inhibition of farnesylation, a crucial step in post-translational modification of *KRAS* proteins that is essential for membrane anchorage of RAS, using the farnesyl-transferase inhibitor tipifarnib in combination with gemcitabine. But no improvement in OS was observed when compared to gemcitabine plus placebo in patients with advanced PDAC. Likewise, other strategies such as dislodging *KRAS* from the plasma membrane and preventing interactions with *KRAS* activating proteins have been effective in pre-clinical models but are yet to be translated to the clinical setting[20,21].

Substantial efforts have also been devoted to inhibition

Table 1 Barriers to effective molecularly targeted therapy in pancreatic ductal adenocarcinoma

PDAC biology	Barrier
Genetic heterogeneity	Inability to directly inhibit *KRAS*
	Convergence of signal transduction pathways downstream from KRAS with feedback inhibitory loops
Overexpression of EGFR, IGF-1R	Escape from growth factor dependence in later stages of tumorigenesis
Desmoplastic stroma	Hypoxic tumor milieu impairs effective drug delivery
Overexpression of angiogenic factors	Secretion of angiostatic factors in tumor microenvironment
PDAC stem cells	Difficult to eradicate subpopulation of cells capable of self-renewal
	Resistance to chemotherapy, radiation
Low immunogenicity	Evasion of host immunity
	Abundance of immunosuppressive cells in tumor milieu

PDAC: Pancreatic ductal adenocarcinoma; KRAS: Kirsten rat sarcoma oncogene; EGFR: Epidermal growth factor receptor; IGF-1R: Insulin like growth factor-1 receptor.

of downstream signal transduction, especially the PI3K and MAPK (RAF/Mek/ERK) pathways, as they are more amenable to pharmacological inhibition[22-24]. Disconcertingly, this strategy has proven unsuccessful because inhibition of the MEK pathway resulted in feedback activation of the PI3K pathway mediated by the epidermal growth factor receptor (EGFR)[25]. To counter this Ko *et al*[26] investigated the effect of dual inhibition of EGFR and MEK with erlotinib and selumetinib respectively. In this phase Ⅱ non-randomized trial of 41 patients with chemotherapy refractory PDAC, 26% had stable disease for 12 wk or more and 38% of patients had a greater than 50% decline in CA 19-9 levels[26]. Though this combination approach showed promise, it needs to be validated in larger studies.

Targeting BRCA2 and PALB2

The main role of the tumor suppressor genes *BRCA2* and *PALB2* is to repair double stranded DNA breaks by homologous recombination. In patients with germ-line mutations of these genes, DNA repair occurs by alternate means, predominantly by base-excision repair mediated by the enzyme poly(ADP-ribose) polymerase (PARP). Inhibition of PARP in patients with BRCA mutations renders tumor cells incapable of repairing DNA damage and cell death ensues. This concept is known as "synthetic lethality" and represents a great example of targeted therapy in PDAC[27].

In a preclinical study, the PARP inhibitor 3-amino-benzamide in combination with gemcitabine showed strong anti-tumor activity by inducing apoptosis in PDAC cell lines[28]. Remarkably, neoadjuvant iniparib plus gemcitabine induced a complete pathological response in a patient with recurrent PDAC harboring the BRCA2 mutation[29]. In a phase Ⅰ study of olaparib plus gemcitabine in patients with advanced solid tumors that also included 15 patients with PDAC, no differences in efficacy endpoints were noted with the combination[30]. However, these patients were genetically unselected and were unable to receive full-dose gemcitabine due to myelotoxicity attributed to olaparib. The role of *PARP* inhibitors in PDAC might thus be limited to patients with *BRCA2* mutations. The combination of PARP inhibitors with cytotoxic chemotherapy is also being investigated (ClinicalTrials.gov identifiers: NCT01585805 and NCT01296763, Table 3).

OVEREXPRESSION OF GROWTH-FACTOR RECEPTORS ON TUMOR CELLS

PDAC cells overexpress the EGFR and its ligand transforming growth factor-α (TGF-α)[31]. In transgenic mouse models, EGFR signaling mediated by TGF-α has been shown to be essential for the onset of ductal metaplasia, a precursor lesion that progresses to PanIN and eventually to PDAC[32]. EGFR signaling is perceived to be a vital cog in mediating the oncogenic effects of *KRAS* as evidenced in mouse models wherein genetic or pharmacological inhibition of EGFR signaling eliminates tumorigenesis[33,34]. The exact mechanism by which EGFR overexpression contributes to the development of PDAC is unclear but could be related to induction of the Notch pathway[35]. EGFR overexpression is also associated with increased propensity for liver metastasis and poor prognosis[36]. Paradoxically, despite compelling pre-clinical evidence, the EGFR inhibitor erlotinib showed only a marginal clinical benefit for patients with advanced PDAC by prolonging OS by a mere 2 wk[37]. It is hypothesized that EGFR signaling might be essential earlier in tumorigenesis while advanced PDAC cells escape EGFR dependence[38].

Targeting insulin-like growth factor-1 receptor

PDAC cells also overexpress insulin-like growth factor-1 receptor (IGF-1R)[39]. IGF-1R signaling promotes tumorigenesis by activating PI3K, MAPK, AKT and Rac pathways[40]. This results in uncontrolled cellular proliferation, survival and metastasis. Based on exciting and positive results from preclinical[41] and early-phase trials[42,43]. ganitumab, a fully humanized monoclonal antibody against IGF-1R combined with gemcitabine was investigated in a phase Ⅲ, double-blind, placebo-controlled randomized controlled trial (RCT) as first-line therapy for patients with metastatic PDAC[44]. Though the ganitumab-gemcitabine combination was safe, the study was terminated early based on results of a pre-planned futility analysis which revealed no improvement in the primary objective of OS, and the reason for lack of efficacy is yet unclear[44]. Inhibition of IGF-1R using small interfering RNA (siRNA) had an anti-proliferative effect on HPAC and Panc-1 pancreatic cancer cell lines invoking the possibility of a novel target for

Table 2 Frequency and consequences of common genetic mutations in pancreatic ductal adenocarcinoma

Mutation category	Frequency in PDAC	Effects of mutation	Consequence
Gain of function			
KRAS	> 95%	Continuous transduction of downstream growth signals (BRAF/MAPK, PI3K/mTOR)	Enhanced cell growth and survival
Loss of function			
CDKN2A	95%	Disruption of RB1 by CDK4	Uncontrolled cellular proliferation
TP53	75%-85%	Impaired DNA damage repair, loss of cell cycle checkpoint activation	Chromosomal instability, aneuploidy
DPC4/SMAD4	50%	Loss of inhibition of TGF-β	Loss of cell growth inhibition
BRCA2	6%-17%	Impaired DNA damage repair by homologous recombination, loss of cell-cycle checkpoint activation	Genomic instability
PALB2	1%-3%	Impaired BRCA2 function	Genomic instability

KRAS: Kirsten rat sarcoma oncogene; BRAF: Rapidly activated fibrosarcoma homolog B; MAPK: Mitogen activated protein kinase; PI3K: Phosphatidyl inositol-3 kinase; mTOR: Mammalian target of rapamycin; CDK: Cyclin dependent kinase; DPC4: Deleted in pancreatic cancer 4; TGF-β: Transforming growth factor-β; BRCA2: Breast cancer 2; PALB2: Partner and localizer of BRCA.

Table 3 Summary of selected ongoing clinical trials evaluating molecular therapies in pancreatic ductal adenocarcinoma (according to www.clinicalTrials.gov, accessed July 2015)

Category	Clinical trial number	PDA setting	Medications studied	Phase	Status	Estimated completion
Tumor suppressor genes						
	NCT01585805	Locally advanced/ metastatic	Gem and Cisplatin ± Veliparib *vs* Veliparib alone	II	Recruiting	07/2017
	NCT01296763	Advanced	Irinotecan + Cisplatin + Mitomycin C ± Olaparib	I / II	Ongoing, not recruiting	01/2014
Recombinant hyaluronidase						
	NCT01959139	Metastatic	FOLFIRINOX ± PEGPH20	I / II	Recruiting	12/2017
	NCT01839487	Metastatic	Gem + Nab-paclitaxel *vs* Gem + Nab-paclitaxel + PEGPH20	II	Recruiting	04/2016
Vaccine therapy						
	NCT02004262	Metastatic	Cy + GVAX + CRS-207 *vs* Chemotherapy *vs* CRS-207	II	Recruiting	12/2016
	NCT01072981	Adjuvant	Chemotherapy *vs* Chemo-radiotherapy ± Algenpantucel-L	III	Ongoing, not recruiting	06/2016
	NCT01836432	Neoadjuvant	FOLFIRINOX ± Algenpantucel-L	III	Recruiting	09/2015
Immune checkpoint	NCT02472977	Metastatic	Ulocuplumab (CXCR4) and nivolumab (PD1)	I B	Recruiting	7/2017
CAR-T cell therapy						
	NCT01897415	Metastatic	Autologous redirected RNA mesothelin specific CAR-T cells	I	Not recruiting	01/2015
	NCT01583686	Metastatic	CAR-T cell receptor	I / II	Recruiting	12/2018
Micro-RNA-21 targeted therapy						
	NCT01274455	Locally advanced	Gem + Plasmid DNA CYL-02	I	Not recruiting	12/2013
Signal transduction inhibitors						
Janus kinase targeted	NCT02119663	Locally advanced/ metastatic	Capecitabine + Ruxolitinib *vs* Capecitabine + Placebo	III	Recruiting	06/2017
	NCT02117479	Locally advanced/ metastatic	Capecitabine + Ruxolitinib *vs* Capecitabine + Placebo	III	Recruiting	12/2015
Wnt targeted	NCT02050178	Metastatic	OMP-54F28 + Gem-Nab-paclitaxel	I	Recruiting	12/2016
	NCT01764477	Metastatic	PRI-724 + Gem	I	Recruiting	03/2016
Notch inhibitor	NCT01647828	Locally advanced/ metastatic	OMP-59R5 + Gem-Nab-paclitaxel	I / II	Recruiting	01/2016
TGF-β inhibitor	NCT01373164	Locally advanced/ metastatic	LY2157299 + Gem	I / II	Not recruiting	11/2015

PDA: Pancreas ductal adenocarcinoma; Gem: Gemcitabine; FOLFIRINOX: 5-fluorouracil, leucovorin, irinotecan, oxaliplatin; CAR-T: Chimeric antigen receptor T cell; TGF-β: Transforming growth factor-β; Cy: Cyclophosphamide.

future clinical studies[45].

DESMOPLASTIC STROMA

The stroma of PDAC is characterized by an intense fibrotic reaction termed "desmoplasia"[46]. This is attributed to collagen, laminin, fibronectin, hyaluronan and various other components of the extracellular matrix (ECM) secreted by activated pancreatic myofibroblasts (stellate cells) in response to stimuli from TGF-β, platelet derived growth factor (PDGF) and fibroblast growth factors (FGF) produced by the tumor microenvironment (TME)[46]. The accumulation of ECM components renders the tumor milieu rigid, and the ensuing increase in extracellular fluid pressure results in collapse of blood vessels in the tumor stroma. The resultant hypoxic peri-tumoral milieu is thus a significant impediment to the effective delivery of chemotherapy to the tumor[47]. Furthermore, matrix metalloproteinases (MMP) produced in the ECM damages the structural integrity of the ECM to self-perpetuate tumor invasion and metastasis[48]. The desmoplastic stroma in PDAC represents an ever-changing compartment that not only functions as a mechanical barrier to drug delivery, but also favors tumorigenesis and invasion.

STROMAL TARGETING STRATEGIES

Pegylated recombinant hyaluronidase

Hyaluronan is a visco-elastic glycosaminoglycan found in abundance in normal tissues, notably in joint cartilage[49]. It is also found in the stroma of PDAC, where it contributes to significantly elevated interstitial fluid pressure (IFP) and vascular collapse[47]. In the KPC mouse model, enzymatic depletion of hyaluronan with pegylated recombinant hyaluronidase (PEGPH20, Halozyme, San Diego, CA) rapidly normalized the IFP and hence restored normal vascular caliber[50]. Importantly, co-administration of PEGPH20 and gemcitabine resulted in an 83% increase in survival and a dramatic decrease in metastatic burden in mice, owing to the enhanced delivery of gemcitabine to the tumor[50]. Based on the encouraging results from a phase Ⅰb trial that combined PEGPH20 with gemcitabine[51], this strategy is now being investigated in phase Ⅱ trials in combination with conventional chemotherapy regimens for metastatic PDAC (ClinicalTrials.gov identifiers: NCT01959139 and NCT01839487, Table 3). Initial reports demonstrate that patients with high hyaluronan expressing tumors have greater clinical benefit[52].

Nanoparticle albumin-bound (nab)-paclitaxel with gemcitabine

Though cytotoxic agents are not considered to be within the realm of targeted therapy, nab-paclitaxel might be an exception. In a small yet novel study, the combination of nab-paclitaxel and gemcitabine was administered to 16 patients in a neo-adjuvant fashion[53]. The effects on tumor stroma were determined by endoscopic ultrasound (EUS) elastography and examination of surgically resected tumor specimens. Not only was there a significant decrease in tumor stiffness on EUS elastography, but also a decrease in cancer associated fibroblasts (CAF) and significant disruption of the intense collagen architecture[53]. Similarly, stromal disruption was also noted in a patient-derived xenograft mouse model treated with the same combination[54]. In this study, genetically engineered mice bearing tumors received nab-paclitaxel, gemcitabine or the combination of the two. The intra-tumoral concentration of gemcitabine was nearly 3-fold higher in mice treated with nab-paclitaxel plus gemcitabine than in those receiving gemcitabine alone. The exact mechanism of action of nab-paclitaxel in depleting tumor stroma has not been elucidated, but could be mediated by secreted protein acidic and rich in cysteine (SPARC) - a matrix glycoprotein and marker of activated fibroblasts[55] proposed to be a crucial driver of PDAC invasiveness[54,56,57].

Targeting myofibroblasts/stellate cells

Though the anti-inflammatory properties of $1,25(OH)_2D_3$ have been well established[58], the finding that activated myofibroblasts (also known as stellate cells) overexpress the vitamin D receptor (VDR) was an unexpected finding[59]. The VDR plays an important role in the transcriptional regulation of activated myofibroblasts by converting them back to their quiescent state[59]. This is substantiated by a preclinical study in mouse models in which calcipotriol, a VDR agonist resulted in stromal depletion, facilitated intra-tumoral delivery of gemcitabine and caused reduction in tumor volume[59]. Unlike other therapies that focus on stromal ablation, reprogramming the stroma using vitamin D analogs might be a useful adjunct to PDAC therapy.

Hedgehog pathway inhibition

The hedgehog (Hh) signaling cascade activates the Gli family of receptors when the sonic Hh ligands bind to its receptor Patched1 that in-turn relieves the repression on Smoothened1 (Smo)[60]. This paracrine signaling is vital for the proliferation of the desmoplastic stroma in PDAC[60]. IPI-926 is a powerful inhibitor of Smo, which when administered in combination with gemcitabine to KPC mice resulted in increased mean vessel density in the stroma and increased intra-tumoral concentration of gemcitabine[61]. However, when the combination of gemcitabine with IPI-926 resulted in worse progression free survival (PFS) and OS compared to gemcitabine plus placebo in a phase Ⅱ trial that had to be terminated early[62]. More recent studies have shown a possible protective effect of the stroma, which when depleted resulted in a more aggressive and hypervascular phenotype[63,64]. The incongruity in outcomes between pre-clinical and clinical trials is a fine example to exemplify the complexity of targeting the TME in PDAC.

ANGIOGENESIS

Tumor cells can activate quiescent endothelial cells through

an "angiogenic switch" which causes overexpression of pro-angiogenic factors, chiefly vascular endothelial growth factor (VEGF)[65]. VEGF and its two high-affinity tyrosine kinase receptors namely flk-1/KDR and flt-1 are overexpressed in PDAC and associated with disease progression[66]. VEGF enhances MAPK phosphorylation in pancreatic cancer cell lines, and PD9805 an inhibitor of MAPK inhibits the proliferative effects of VEGF[67]. In contrast to pancreatic cancer cell lines, actual pancreatic tumors have a much lower microvessel density compared to normal pancreatic tissue[68]. Unsurprisingly, anti-angiogenic therapy directed against circulating VEGF using bevacizumab in combination either in combination with gemcitabine alone or with gemcitabine and erlotinib has been unsuccessful[69,70]. Likewise, VEGF receptor targeted agents such as axitinib and aflibercept have not improved outcomes either[71,72]. As explained previously, the desmoplastic stroma contributes significantly to altered vasculature. Additionally, the abundance of angiostatic factors such as angiostatin and endostatin that are secreted in the TME also explains the discordance between VEGF overexpression and lack of clinical benefit with VEGF inhibition[46,73].

TUMOR STEM CELLS

Cancer stem cells (CSC) constitute a very small proportion of pancreatic tumors (< 1%), but have the potential for unlimited proliferation[74]. They were identified in PDAC using a xenograft model of immunocompromised mice and proven to have a 100-fold higher tumorigenic potential compared to non-tumorigenic cancer cells[74]. A distinct population of $CD133^+$ PDAC stem cells also predicts propensity to metastasis[75]. Moreover, cancer stem cells are extremely resistant to chemotherapy and radiation[76,77], attributed to the overexpression of the early developmental sonic hedgehog (SHH) pathway[78]. The self-renewing nature of CSCs poses a significant challenge in molecular therapeutics of PDAC.

Targeting CSCs in PDAC

Data emerging from preclinical studies have demonstrated that it is indeed possible to target and eliminate CSCs. Salinomycin, an antibiotic with a greater than 100-fold efficacy against CSCs compared to paclitaxel, inhibited the growth of $CD133^+$ pancreatic CSCs and the effects were synergistic with gemcitabine, which curbed the growth of non-CSC cells[79]. Pancreatic CSCs also overexpress epithelial cell adhesion molecule (EpCAM) and this feature has been the focus of immunotherapy directed against CSCs[80]. MT110 is a bi-specific T cell engaging antibody (BiTE) that simultaneously targets EpCAM on CSCs and T cell-CD3 complexes on T cells to effectively eliminate the highly tumorigenic CSCs both *in vivo* and *in vitro* in a mouse model of PDAC[80]. Natural agents such as isoflavones, 3,3'-diindolylmethane (DIM) and curcumin analogues have also garnered attention because of their inhibitory effects on CSCs through cell-signaling molecules and microRNAs (miRNA)[81]. Pancreatic CSCs also overexpress Nodal and Activin belonging to the TGF-β superfamily and pharmacological inhibition or knockdown of their receptor activin-like 4 and 7 (Alk 4/7) reversed gemcitabine resistance in an orthotopic mouse model and dramatically reduced their tumorigenicity[82]. In addition to newer agents, the anti-neoplastic effects of the timeworn drug metformin are also attributed to its activity against pancreatic CSCs[83]. Results from these preclinical studies await clinical translation.

IMMUNE BIOLOGY OF PDAC

The immune system serves as an innate defense against tumorigenesis and metastasis. To counteract immune-surveillance, tumors develop adaptive mechanisms and PDAC is adept at immune evasion because of its inherently low immunogenicity[84,85]. The lack of anti-tumor effector T lymphocytes in preclinical mouse models of PDAC compared to a very high proportion of immunosuppressive cells such as regulatory T cells (Tregs), tumor-associated macrophages and myeloid derived suppressor cells tips the balance in favor of tumorigenesis[85]. Tumor and stromal cells also secrete several inflammatory mediators, notably TGF-β and interleukin 10 (IL-10) which down-regulate T cell and antigen presenting cell (APC) proliferation in the PDAC microenvironment[86,87]. Despite the purported low immunogenicity of PDAC, the presence of $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells (CTL) in resected pancreatic tumors was associated with longer OS, suggestive of a definite immune response against PDAC[88]. Though it has been a challenging endeavor to devise effective strategies to harness the host's immune system against PDAC, results of recent vaccine trials and immune checkpoint inhibitors in PDAC have been quite encouraging.

VACCINE THERAPY

Immune mediated anti-tumor response occurs in two steps; first, tumor associated antigens (TAA) are presented by APC, notably dendritic cells to effector/CTL, which in turn recognize antigenic epitopes bound to major histocompatibility (MHC) molecules. Next, concomitant binding of co-stimulatory molecules such as B7-1 on APCs and CD28 on T cells results in T cell activation. However, tumor cells lack the additional co-stimulatory molecules and immune evasion ensues[89]. Vaccine-based therapies are designed to circumvent immune evasion by delivering TAAs to APCs and stimulate a robust cell-mediated immune response to attack and eliminate tumor cells.

Initial vaccine designs for PDAC utilized peptide antigens such as mucin-1 (MUC1), carcinoembryonic antigen and protein products of *KRAS* oncogene that are capable of binding exact MHC molecules[89]. Because peptide vaccines contain only single antigenic epitopes, it leads to immune tolerance with minimal and transient efficacy[90]. The expansion of proteonomics and gene expression based assays has led to the identification of

several TAAs that are selectively expressed by pancreatic cancer cells and has widened the scope for development of whole-cell vaccines that utilize these antigens to trigger tumor-specific immunity. Mesothelin is one such example of a TAA that is overexpressed in nearly all PDACs (but not in normal cells) and is implicated in cell adhesion and metastases[91,92]. Mesothelin-specific $CD8^+$ T cell responses have been associated with improved OS following vaccine therapy[93].

Granulocyte-macrophage colony-stimulating factor vaccines

GVAX: GVAX is a whole-cell irradiated allogeneic vaccine that is composed of tumor cells from two pancreatic cell lines (Panc 10.05 and Panc 6.03) that have been genetically modified using a plasmid vector encoding for the granulocyte-macrophage colony-stimulating factor (*GM-CSF*) gene[94]. When injected transdermally, high GM-CSF secretion at the vaccine site causes mobilization and differentiation of APCs, a feature that patients with PDAC typically lack. APCs subsequently migrate to regional lymph nodes and activate $CD4^+$ and $CD8^+$ T cells to mount an effective anti-tumor response[95].

Initial trials demonstrated the safety and tolerability of GVAX when administered in the adjuvant setting followed by conventional chemoradiation. Delayed-type hypersensitivity (DTH) reactions and induction of mesothelin-specific $CD8^+$ cells correlated with prolonged disease free survival in the phase Ⅰ and phase Ⅱ trials respectively[94,96]. Based on the favorable results in the adjuvant setting, GVAX was studied in the metastatic setting in patients who had progressive disease after gemcitabine[97]. In this open-label phase Ⅱ study, the combination of GVAX with immune-modulating dose of cyclophosphamide (Cy) was compared to GVAX alone. The rationale for adding Cy was to enhance treatment related immune response by inhibiting immunosuppressive Tregs. Although OS was better in the combination arm compared to GVAX alone, the results were not statistically significant (median OS - 4.7 mo *vs* 2.3 mo). $CD8^+$ T cell responses to mesothelin were enhanced in the combination arm and associated with a trend towards prolonged PFS[97]. Whether metronomic Cy plus GVAX will counter immune tolerance mediated by Tregs is being evaluated in a randomized clinical trial (NCT00727441, Table 3).

GVAX-prime and CRS-207-boost

CRS-207 is a live-attenuated strain of Listeria monocytogenes, genetically engineered to secrete mesothelin into the cytosol of APCs. In addition to activating effector T cells by delivering TAAs directly to the APCs, the cytokine mediated inflammatory response that is triggered by CRS-207 also serves to recruit more APCs[95]. The synergy between GVAX and CRS-207 was demonstrated in a phase Ⅰ trial[98] which led to a multi-center, randomized phase Ⅱ trial among 90 patients exclusively with PDAC[99]. Patients with previously treated metastatic PDAC were randomized 2:1 to either 2 doses of GVAX immune priming followed by 4 doses of CRS-207 as a boost (arm A) or 6 doses of GVAX alone (arm B). All patients received Cy to inhibit Tregs. After a median duration of follow-up of 6.6 mo, the OS was 6.1 mo in arm A compared to 3.9 mo in arm B (HR for death, 0.59; 95%CI: 0.36 to 0.91, P = 0.02). Toxicity with the combination was minimal and included transient fevers, fatigue, lymphopenia and elevated liver enzymes. As with previous studies, detection of enhanced mesothelin specific $CD8^+$ T cell responses was associated with longer OS regardless of treatment arm[99]. A larger phase Ⅱb trial is currently underway to compare the combination of GVAX plus CRS-207 to CRS-207 alone or chemotherapy alone in the metastatic setting (NCT02004262, Table 3).

Algenpantucel-L

Algenpantucel-L (NewLink Genetics Corporation, Ames, IA) is an allogeneic vaccine that contains two PDAC cell lines (HAPa-1 and HAPa-2) that have been genetically engineered to express α(1,3)-galactosyl epitopes (α-Gal)[100]. Though human cells lack the α-Gal epitopes, the gut flora stimulates antibodies against it. These antibodies are the primary mediators of hyperacute rejection characterized by rapid organ destruction through complement activation within minutes of organ transplantation[100]. When these antibodies are coupled with tumor cells such as in algenpantucel-L, it promotes opsonization and phagocytosis of tumor cells by APCs and results in T cell activation. In a phase Ⅱ study of 70 patients with resected PDAC, algenpantucel-L was added to either gemcitabine or 5-fluorouracil based chemoradiotherapy[100]. After a median follow-up of 21 mo, the DFS and OS at 1 year were 62% and 86% respectively. Notably, the OS in this trial was better than the reported 81% in the sentinel RTOG-9704 trial using the same chemoradiotherapy regimen. Patients who received a higher dose of 300 million cells/dose fared better than those who received 100 million cells/dose with regard to both DFS (81% *vs* 51%) and OS (81% *vs* 68%) at 12 mo respectively, suggesting a strong dose-response effect. Apart from mild adverse events such as injection site pain and induration the vaccine was well tolerated. Phase Ⅲ trials evaluating algenpantucel-L in the adjuvant (NCT01072981) and neoadjuvant setting (NCT01836432) are ongoing (Table 3).

Immune checkpoint inhibitors

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is expressed on the surface of activated T cells and down-regulates immune activation by competitively inhibiting the binding of CD28 to B7-1 and turning off the intracellular signaling cascade of B7-1[101]. Monotherapy with ipilimumab (Yervoy, Bristol-Myers Squibb Company), an anti-CTLA-4 mAb, was ineffective in the treatment of locally advanced or metastatic PDAC[102]. However, the combination of GVAX with ipilimumab showed striking clinical and immunological synergy in previously treated patients with advanced PDAC[103]. Compared to single-agent ipilimumab, patients

in the combination therapy arm had better OS at 1 year (27% *vs* 7%) although the study was not powered to detect differences in OS. Significantly however, combination therapy was not associated with increased adverse events, despite the higher dose of ipilimumab (10 mg/kg) used in this study. Increase in peak mesothelin-specific T cells and enhancement of the T cell repertoire was associated with longer OS[103]. Most responders in this study required at least 12 wk of therapy, thus underscoring the need for selecting patients with early stage disease in future trials to evaluate delayed responses often seen with immunotherapy.

Programmed cell death ligand-1 (PD-L1) and its receptor PD-1 are expressed on the cell surface of tumor cells as well as activated T cells. This receptor-ligand interaction down-regulates $CD4^+$ and $CD8^+$ T cells and is a natural immune checkpoint to prevent excessive immune mediated tissue damage[104]. PD-L1 expression is up regulated in PDAC cells and results in a blunted T cell response against the tumor[104]. Blocking the interaction between PD-1 and PD-L1 successfully augmented anti-tumor immune responses *in vitro* and formed the basis for investigating the efficacy of BMS-936559, an anti-PD-L1 mAb in various solid tumors[105]. Durable responses were noted in patients with melanoma, non-small cell lung cancer and renal-cell carcinoma but disappointingly no responses were seen in 14 patients with PDAC[105]. The resistance to PD-L1 inhibition in PDAC is due to the high expression of fibroblast activation protein (FAP) by carcinoma-associated fibroblasts (CAF) in the tumor stroma[106]. These FAP^+ CAFs produce chemokine (C-X-C motif) ligand 12 (CXCL12) that binds with chemokine receptor 4 (CXCR4) to prevent T cells from infiltrating the tumor and causing local immunosuppression. Inhibition of CXCR4 by AMD3100 in a GEMM of PDAC caused cancer regression synergistically with PD-L1 inhibition[106]. Therefore it might be feasible to overcome tumoral immunosuppression through a combined approach. A clinical trial to test this hypothesis has recently opened to enrollment as a phase Ⅰ study of ulocuplumab (anti-CXCR4) and nivolumab (anti-PD1) (NCT02472977, Table 3). Since these immune-modulating agents are not cancer T cell specific and can cause activation of other quiescent T cell populations, autoimmune toxicities occur frequently[107]. Hence future studies will also need to focus on effective management of these toxicities.

CD40 agonist therapy

CD40 belongs to the tumor necrosis factor receptor superfamily and is expressed by multiple APCs including dendritic cells, B cells and macrophages[108]. Activated CD40 plays a crucial role in the priming and activation of tumor-specific T cells, but also mediates T cell independent antitumor immunity by activating macrophages[108]. CP-870, 893 is a CD40 agonistic mAb that potentiates anti-tumor immunity by these aforementioned mechanisms. In the initial pre-clinical study CP-870, 893 when administered in combination with gemcitabine in the KPC mouse model caused rapid regression of tumors mediated by T cell-independent macrophage infiltration[109]. Notably, depletion of tumor stroma was also noted and attributed to the effect of stromal infiltrating macrophages. In a phase Ⅰ trial conducted subsequently, 22 patients with previously untreated advanced PDAC were administered CP-870, 893 with gemcitabine[110]. The radiological response rate (19% *vs* 9.4%) and median OS (8.6 mo *vs* 6.8 mo) were better than expected with single agent gemcitabine. The addition of gemcitabine is postulated to cause antigenic release akin to that of a vaccine with co-stimulation of APCs by CD40 agonist therapy[111]. Apart from transient cytokine release syndrome and depletion of B cells, none of the auto-immune toxicities seen with the immune check-point inhibitors were noted[110].

FUTURE STRATEGIES

Adoptive T cell transfer

Stemming from the successes in hematological malignancies notably acute lymphoblastic leukemia[112], adoptive T cell transfer is an exciting new paradigm that holds tremendous promise in PDAC. This therapeutic strategy involves *ex vivo* genetic engineering of T cells collected from patients to produce chimeric antigen receptors (CAR) capable of recognizing mesothelin expressed on PDAC cells[113,114]. Infusion of CAR-T cells back to the patient results in immediate recognition of tumor cells and obviates antigen processing and HLA expression. In preclinical studies, CAR-T cells exhibited potent anti-tumor activity[115]. Beatty *et al*[116] have also reported a marked decline in ascitic fluid malignant cell burden in a patient with metastatic PDAC, in addition to transient decline in [^{18}F] fluorodeoxyglucose uptake on positron emission tomography (PET) scan after infusion of CAR-T cells. CAR-T cell therapy is a subject of active research in PDAC and studies are ongoing (ClinicalTrials.gov identifiers: NCT01897415 and NCT01583686, Table 3).

Targeting epigenetic regulators

Epigenetics is the study of changes in gene expression by mechanisms other than changes in the DNA code. Histone modification by acetylation or methylation, DNA methylation and miRNA expression are the main mechanisms of epigenetic regulation[117]. Histone acetylation by histone acetyltransferase promotes transcriptional activity but histone deacetylases (HDAC) repress transcription of tumor suppressor genes and are overexpressed in PDAC[118]. HDAC inhibitors serve to abrogate the transcriptional repression and impair tumorigenesis by playing a crucial role in differentiation, cell-cycle inhibition and apoptosis in tumor cells[117]. Multiple HDAC inhibitors such as hydroxamic acid derivatives (vorinostat), cyclic peptides (romidepsin), short-chain fatty acids (valproic acid) and benzamides have been studied, but results in PDAC have been disappointing[119,120]. However, the recognition that miRNAs play an important role in PDAC has resulted in increased attention towards exploiting them as potential

therapeutic targets[121]. Acting at the post-transcriptional level, these non-coding RNAs play a crucial role in apoptosis, differentiation and proliferation. Aberrant overexpression of multiple miRNAs, particularly miRNA-21 has been demonstrated in PDAC and its inhibition by Lentiviral vectors has shown promising antitumor effects in preclinical studies[121,122]. MiRNA targeted therapy especially in combination with chemotherapy is in its early stages and expected to gain momentum in the future (ClinicalTrials.gov identifier: NCT01274455, Table 3).

Targeting signal transduction

As described previously, targeting signaling pathways downstream from *KRAS* has been unsuccessful so far. However, there is renewed interest in targeting the effects of Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway after its importance in PDAC and associated cachexia became apparent[123,124]. The addition of the JAK inhibitor ruxolitinib to capecitabine in patients with refractory metastatic PDAC in a phase Ⅱ trial showed OS benefit for a subgroup of patients with elevated levels of C-reactive protein[125] and has formed the rationale for phase Ⅲ trials evaluating ruxolitinib in metastatic PDAC (ClinicalTrials.gov identifiers: NCT02119663 and NCT02117479, Table 3). Global genomic analysis data also revealed alterations in genes in the Wnt/Notch and TGF-β signaling pathways in all PDACs[13]. Ongoing clinical trials to evaluate the efficacy of specific inhibitors of these pathways are currently underway (ClinicalTrials.gov identifiers: NCT02050178, NCT01764477: Wnt inhibitors, NCT01647828: mAb against Notch, NCT01373164: Oral anti TGF-β receptor type 1, Table 3).

CONCLUSION

As evinced in this review, with improved understanding of the biology, genetic basis and molecular mechanisms that initiate and propagate PDAC carcinogenesis, the focus has shifted from identifying effective cytotoxic chemotherapy regimens to molecularly targeted therapies. These efforts have been further burnished by significant strides in the field of onco-immunology that now allows for cautious optimism that effective therapeutic options for PDAC are finally within reach.

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