CLINICAL IMPLICATIONS OF PANCREATIC CANCERS IN IMMUNE ASSOCIATED THERAPIES

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Abstract: Pancreatic ductal adenocarcinoma (PDAC) is regarded as a devastating disease with poor prognosis. Late detection, aggressive pathogenesis, immune deserting of tumor and surgical restrictions are major challenges that limit therapeutic interventions. In this review, we discuss stratification, immune biomarkers, their analytical assessments, and clinical implications in therapeutic fortes according to patient suitabilites. On those lines, we also highlight the significant drawbacks in clinical interventions that could lead to potential research scopes such as increasing drug efficacy, extending patient survival and quality of life. Additionally, we have also discussed the genomic and immunological attributes of pancreatic cancer for better understanding and development of potential treatment approaches, focusing mainly on immunotherapies.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is known as one among the lethal malignancies with 10% of 5 year survival rate with increasing incidences and detrimental mortality rate over the next two decades. The aggressive form of cancer is worsened due to lack of effective therapies and preventive strategies [1].

Genetically, the activation of oncogenes and inactivation of tumor suppressors leads to pancreatic cancers. The genetic alterations mostly observed in the precursor lesions (PanINs& IPMNs) are hierarchical [2]. PDAC is projected as the 7th leading cause of cancer associated deaths. The patients, mostly 40 years and above, rarely children or young adult; seldom exhibit any symptoms until it reaches lethal stage. Thus, the diagnosis and therapeutic interventions are

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Dedication: **Dr. P.D. Gupta has been a significant influence in my life for catalyzing my interest in science and clinical research since childhood. My father's collaboration with him has always been very interesting. His research contributions to life sciences have been impeccable. I dedicate this manuscript to him with due respect and humility**

tricky, hence is highly pursued for clinical relevance. The incidents are predominant in males but are not necessarily gender biased. About 10-15% PDAC have hereditary bias, with no causative gene known, whereas 7% will probably have an inherited germline mutation such as BRCA which could be a plausible detection method [3]. Endocrine tumours are rare while exocrinetumours arepredominant,likePDACs and pancreatic neuroendocrine tumours (NETs). PDACs are sub-classiûed into Squamous, Adenosquamous,Colloidand Adenocarcinomas. The tumour stages of PDACs are stage I conûned to pancreas, stage II inûltrates bileductsnegativeinlymph nodes,stageIII,Positivelymphnodes,StageIV-A,MetastasesandStageIV-B,inûltrates distant organs.

Subclasses and Stratification: PDAC are classified based on morphologic, molecular, and immunologic features. Researchers identified 5 subtypes by using gene expression data with consensus clustering [4]. Nine histological subtypes, suggested by World Health Organization (WHO) [5]. Multiple molecular subtypes are based on formalin fixed and paraffin embedded(FFPE) tissue [6]. Immune gene signatures of patient stratification suggested immune subtypes (ISs), IS1, IS2 and IS3 in which, IS3 has higher immune infiltration [7]. Based on neo-antigens, immunological and stromal features, the subtypes are categorized as hot, cold, mutational cold, and mutational active [8]. Based on expression profile, expression of CD8 and PD-L1, PD-L1+/CD8 high, PD-L1+/CD8 low, PD-L1-/CD8 high, and PD-L1-/CD8 low is shown [9]. Functional immunology-based subtypes are - immune-escape, immune-rich and exhaust. The subtyping signatures are described [10].

Biomarkers and immunotherapy based biomarkers: Pancreatic pre-neoplastic lesions, accumulate genetic alterations, leading to the development of malignancy. Non-specific symptoms lack sensitive or specific biomarkers for early diagnosis. Measurement of biomarkers requires invasive resected biopsy/needle biopsy analysis by immunohistochemistry (IHC) and molecular biology assays. Non-invasive biomarkers derived from blood, urine, saliva, and stool are also considered in the evaluation. CA19-9, the traditional serological biomarker is important in prognostic and diagnostics but lacks sensitivity and speciûcity for effective diagnosis. It has been combined with additional biomarkers, carcinoembryonic antigen(CEA) and CA125 which showed desired improvement [11].

[11]. There are limited publications suggesting presence of tumor- infiltrating lymphocytes examined on H &E sections on PDAC. The key biomarkers for diagnostic/prediction/prognosis and genomic alterations and their associated pathways are described [12,13]. The signiûcance of liquid biopsy and the use of diagnostic panels facilitated the availability of more biomarkers [14]. The improved analytical technologies in genetic proflingand immunological land scapes of disease progression are a boon. Data from blood derived cell free DNA(cfDNA), extracellular exosomes and circulating tumor cells (CTCs) are examples of liquid biopsy outcomes [15].The pancreatic cancers subtypesare defined [16]. Micro RNAs (miRNA) are non-coding RNAs that regulate gene expression by degrading target mRNA. Six miRNAs (miR-452, miR-105, miR-127, miR-518a-2, miR-187, and miR-30a-3p) were identified in nodal disease using blood samples. In urine samples, levels of miR-143, miR-223, and miR-30e are elevated than the controls whereas miR-143, miR-223 and miR-204 are at higher levels in control than diseased stages II-IV patients. Promotor methylation in ADAMTS1 and BNC1 was relevant with PDAC in a cohort of 123 patients. Micro satellites are short sequences of six base pairs, present in repetitive patterns in genomic DNA and instability of micro satellite, mediated by mismatch repair proteins (MMRs) were observed in PDACs [17].

KRAS mutations are signiûcant in PDAC (90-95%) and its genomic landscape, dominated by Kirsten rData from blood derived cell free DNA (cfDNA), extracellular exosomes ,and circulating tumor cells (CTCs) TP53 (75%), CDKN2A (44%), SMA4 (22%) and CDKN2B (21%) [18]. KRAS is essential in regulating cell proliferation and angiogenesis. Certain studies suggested that molecular detection of KRAS mutation in histological samples is a sensitive detection method for PDAC. Detection within resection margins imply tumor cell persistence whereas venous margins imply tumor spread. So far there are no effective, targeted diagnostic approaches against most tumours with Ras mutations [19].

Checkpoint inhibitors, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or the axis of programmed cell- death protein1(PD-1) and its corresponding ligand PD-L1 are significant immune markers. However, PDAC lacks effective T cell filtration and hence inflammatory signalling required for PD-L1 expression is not fulfilled. The oncogenic signalling

to activate PD-L1 is poorly understood [20]. Recent approaches to biology of TME, enables the identification of angiogenic immunomarkers such as CD31, CD105 and desmoplasia-related biomarkers such as alpha smoothmuscle actin (±-SMA) and collagen I. CD3 (T-lymphocytes), CD4 (T-helper lymphocytes), CD8 (cytotoxic-lymphocytes), CD68 (macrophage marker) and CD206 (M2 macrophage marker, related immune cells infiltration are identified [21].

Besides, gene mutational markers, others like CXC chemokine, their ligand CXCL12, cytokines like IL-2,IFN,IL-7,IL-15 and IL-21, classical CTLA-4, PD-1, PDL1, LAG3, TIM3, CSF1R, CD47, IL6, IL6R, VEGF, and VEGFR like antagonistic antigens [22] and focal adhesion kinase (FAK) and connective tissue growth factor (CTGF) like proteins, identifying liquid biopsy based CAFs as a whole and components of CAFs can be consider as biomarkers. Mesothelin, co-expressed with CA-125 and telomer based hTERT and all the above mentioned biomarkers are targeted and immunotherapy clinical trials were made in PDAC. Some of theclinical strategies practiced aregiven [15]

Analytical methods to assess biomarkers: Advancement of imaging modalities with high spatial are temporal resolution helpful for clinicians to understand and deal with pancreatic cancers. Imaging techniques such as positron emission tomography is reviewed [23]. Classical biomarker, CA19-9 is the most important carbohydrate antigen, a solid phase enzyme linked immunosorbent assay based on the sandwich principle is used to measure concentration [24]. The micro-titer wells coated with a monoclonal antibody binds to antigenic site of the CA 19-9 molecule and incubated with anti-CA19-9 antibody conjugated with horse-radish peroxidase/ fuorescence tags, followed by substrate addition and colour/fuorescencewas measured. Commercial kits for CA19-9, CA125 are available with multipaneled kits [25] developed an automated multi-marker ELISA kit using 3 biomarkers (leucine-rich alpha-2-glycoprotein [LRG1], transthyretin [TTR], and CA 19-9) that were previously discovered and proposed a diagnostic model for PDAC based on this kit for clinical usage. Meta-analysis[26] implied sensitivity of KRAS mutation testing in endoscopic pancreatic mucus varies and is not a potential diagnostic marker yet which could frame a potential research question. They furtheridentified the high specificity of KRAS+ enabled differentiating PDAC from healthy controls.

Immunohistochemistry/ Immunofuorescence of the resected/biopsy tissue are routinely used to diagnose tumours, determine their grade, and identify the cell type of a metastasis. Multi-panel kits are used to identify cytokines, however are deemed insufficient [27]. Dewaxed tissue sections are hydrated, blocked the non-specific sites, and used for different staining including immunostaining and hemotoxylin and eosin. Epitope retrieval by enzymatic or heat-induced activation is commonly performed [28]. The Cancer Genome Atlas (TCGA) and other international consortia are dedicated to performing comprehensive genomic and epigenetic analyses of selected tumor types. Major contributions from IHC or tissue arrays from FFPE tissue revealed immune cell infiltration, immune cytolysis activity, activation of the interferon pathway, tumor mutational burden, and copy number alterations [29]. FFPE retrieved tissue and plasma samples of the patient are useful in identifying expression profile by real time polymerase chain reaction(RT-PCR) and next generation sequencing (NGS). In NGS library preparations and in gene expression studies, role of qRT-PCR is important [30].

Liquid biopsy techniques are being developed as non-invasive techniques for diagnostic, prognostic, predictive, and detecting tumor recurrence. Mutant KRAS of ctDNA by Ion-Torrent was recently reported. ctDNA was amplified by PCR using KRAS exon 2 speciûc primers, supplemented with Ion-Torrent adapters P1 and A, to allow binding to the ion sphere Particles. Additionally, 20–30 diûerent for ward primers, each with a different barcode, were used for amplification for multiple samples in a single reaction. Amplicons were subjected to Qiagen purification kit and sequenced. Isolation and enrichment of liquid biopsy components should be devoid of contamination. ctDNA, ddPCR and NGS are preferred analytical methods. Purification of exosomes by ultracentrifugation, gradient centrifugation, chromatography, gel-filtration, and affinity-based or antibody-coated magnetic beads are recommended. Omics may prove a potential backbone to predict mutant models and targets for therapeutic interventions. Associated proteins of stroma, cancer associated fibroblasts, exosomes, glycosylated/other post translational proteins, serological proteome analysis (SERPA) and biomarkers for monitoring treatment response are all subjected to proteomics/metabolomics based technologies. Recently it was reported transcriptomic profiles from both PDAC microenvironment and epithelial cells to develop a Master

Regulators (MR)-Gradient model that allows significant interpretations on transcriptional networks and metabolomics pathways that underlies PDAC heterogeneity [31]. The robust principal component analyses worked in combinations of methylome, and metabolome data generated from patient xenografts and experimental measures of metabolites, western blot and immunofluorescence microscopy and established potential PDAC prognostic approaches, subjected to epigenetic modulations.

Immunotherapies associated with PDAC : Till date, both chemo and immunotherapeutic regimens have limited efficacy in treating PDAC. Densely packed stroma and inaccessible tumor microenvironment (TME) in fibrotic and hypoxic state is referred as immunologically 'cold' tumor, partly responsible for therapy resistance [22]. Additionally, significant genetic heterogeneity and resistance to cytotoxic chemotherapy fuels the disease progression causing a setback in immune evasion of PDAC. Low mutational and immunosuppressive landscapes are hallmark of PDAC, resulting in limited neoantigens and anti-tumor T cell infiltration (TIL). Further, the cancerous cells release pro-tumorigenic chemokines, cytokines, transforming growth factor beta (TGF-β), macrophage colony-stimulating factor (M-CSF) and vascular endothelial growth factor (VEGF) enables the tumor from immune surveillance to immune tolerance to immuno-suppressive. Rationale behind immunotherapies is to increase the number and activity of TIL to evoke anti-tumor T-cell responses. Two conceptual models have emerged: (1) restoring elements of the cancer immunity to stimulate productive T-cell immuno-surveillance and (2) redirecting the immune reaction to enhance the efficacy of cytotoxic therapies.

Immunotherapies are categorized into immuno-modulators (i.e., immune checkpoint inhibitors, cytokines, and adjuvants), immune stimulatory agonists, bispecific antibodies, oncolytic viruses, adoptive cell therapies (i.e., T cells and NK cells) and cancer vaccines. So far, targeted therapies have not established significant prospects. KRAS inhibitors, novel anti stromal therapies, small molecule multikinase inhibitors etc could be potential scope for the future. To overcome stromal thickness and the barriers mentioned above, tumor debulking through chemo/radio therapies may be needed. Immunotherapeutic strategies in PDAC are elucidated along with immunotargets of PDAC [32]. Neoadjuvant chemotherapy (NAC) is predominantly administered to PDAC patients at advanced stages. There are
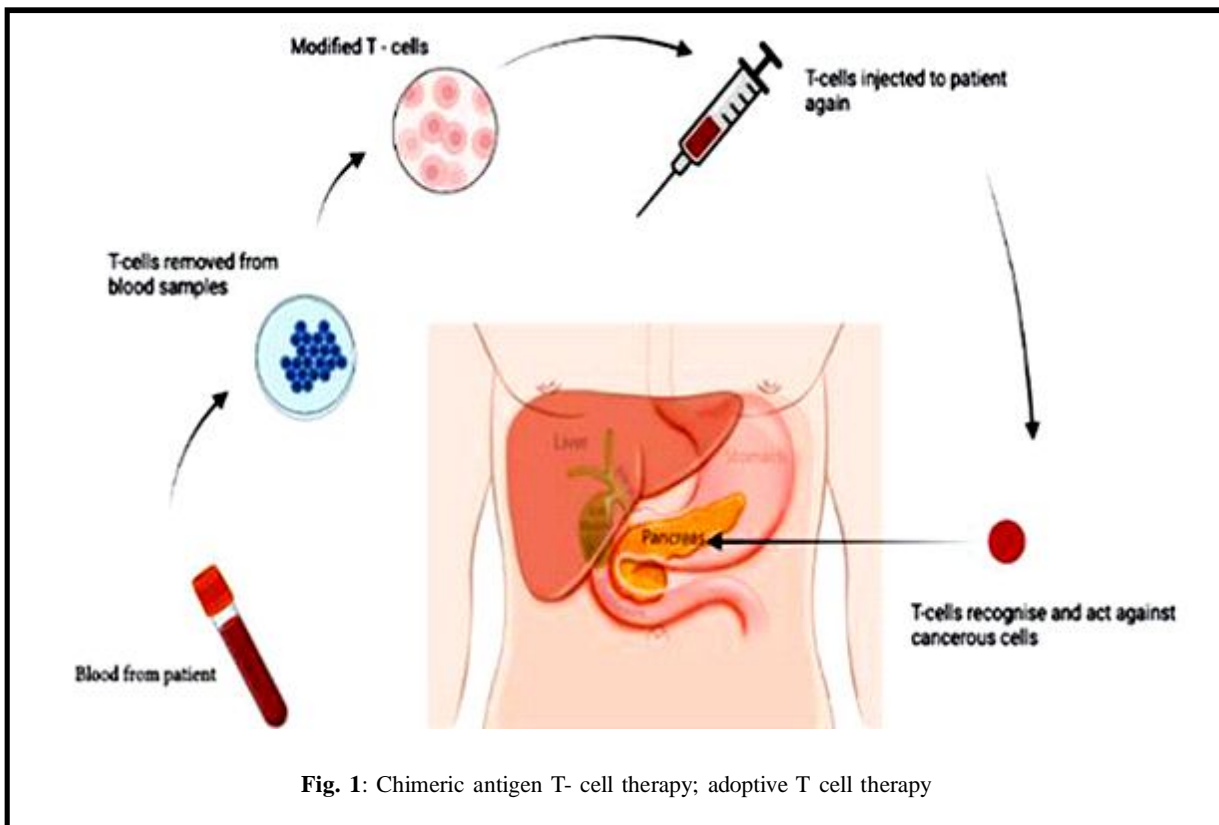

Fig. 1: Chimeric antigen T- cell therapy; adoptive T cell therapy

studies indicating response and survival to an extent. There are no established biomarkers to predict NAC responses, however. One potential PDAC immunomarker is Syndecan-1 (SDC1), significant in tumorigenesis generally but specifically in pancreatic cancer. Serum SDC1 levels established effective prognosis in PDAC cohorts with respect to healthy controls [33].

Immune checkpoint inhibitors (ICI): Combining chemotherapy with ICI (CTLA-4,PD-L1) failed to show progress whereas upregulation of the CTLA-4- and PD1-encoding genes in immuno-subtype of PDAC is reported. Immunotherapy surely instigates an anti-tumor effect but aren't potent enough. For example, increased number and activity of tumor infiltrating lymphocytes (TIL) positively correlates with improved survival and high number of regulatory FOXP3+ T-cells (Treg), which suppresses the activity of effector T-cells, correlates with poor survival in PDAC suggesting that this tumor is sensitive to immunotherapy.

Cancer Vaccines : GVAX is the most extensively tested vaccines in PDAC manifestations. Constituted vaccine provides a representative source of PDAC antigens and these vaccines, convert non-immunogenic to immunogenic TME, which is ideal setup for other therapies. Antigens derived from mutations in driver genes are optimal targets and thus, targeting KRAS mutant protein with a single- or poly-peptide vaccine. The application of bispecific T-cell engagers (BiTEs) may be a suitable option too but immunosuppressive environment of PDAC with low T cells activity may need some in depth knowledge.

CD40 agonists : Pancreatic tumours show high expression of CD40, a cell membrane receptor of the tumor necrosis factor family that modulates immune response [34]. The basic idea is to enhance antigen presentation by activating dendritic cells. CD40 agonist in low immunogenic land scape of PDAC, may be helpful in T cell activation [35,36]

Oncolytic viral (OV) therapy & Adoptive T cell therapy (ACT): These viruses are replication-competent viruses, which replicate within the host, and preferentially target and lyse tumor cells, thereby, inducing immunity. Oncolytic viruses are modified to express checkpoint inhibitory antibodies or immune stimulatory molecules, directing their expression in

tumor tissues. However, clinical efficacy is limited. Despite difficulties in its tumor-site delivery, the chimeric antigen receptor (CAR) T cells have gained clinical approval. CAR T cell are genetically engineered T cells, modified to express chimeric antigen receptors (CARs). The effectiveness of CAR T cell therapies in PDAC is limited as it lacks ideal receptor targets (Fig. 1) [22].

Conclusion and Prospects: Classical diagnostic methods of biomarkers involved more sample quantity either from resected tumor or from formalin fixed paraffin embedded tissues. The buzz around precision medicine demands diagnosis based on biomarkers on personalized stratification, especially in more teratogenous diseases like PDAC. PDAC tumor development is a kind of wound in pancreas and so, the wound-healing biomarkers can be checked in early stages of PDAC. Matrix metallo-proteinases (MMPs) involved in wound healing [37] and its role in inflammation and cancer also is reported. Indeed, MMP-8 plays prognostic roles in cancers including PDAC [38]. By using omics technologies, they can be screened from the blood sample almost like a clinical blood profile. Gene expression profile also became handy using RT-PCR and transcriptome analysis. Using enrichment methods such as immuno-precipitation, magnetic beads and affinity-based techniques can be adopted wherever possible. Unfortunately, clinical trial implications of these agents in PDAC have not been affirmative in vitro. There is currently no effective treatment or cure for PDAC. The lack of possibilities for an early detection is a major setback. The efficacy of surgical and medical approaches is ambiguous whereas the scope for alternative approaches such as immunotherapies appears promising, although achieving efficiency is very tricky. Thus, it is very important to improve our understanding of underlying mechanisms of PDAC in poor therapeutic responses, to enhance drug efficacy and improve quality of life.

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