

The Promising Role of TGF- β /SMAD4 in Pancreatic Cancer: The future targeted therapy

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ABSTRACT

The transforming growth factor β (TGF- β) superfamily includes a group of structurally related cytokines that regulate a wide variety of biological processes. The diversity of its action depends on the dynamic tissue microenvironment, and interplays among the factors involving in the signaling pathway. Although its expression is tightly regulated in normal tissue, overexpression of TGF- β has been identified in multiple tumor types, including pancreatic adenocarcinoma. In pancreatic tumorigenesis, TGF- β acts initially as a tumor suppressor through phosphorylation and activation of SMAD4/DPC4 gene. Mutation of SMAD4/DPC4 gene results in carcinogenesis and tumor progression in many cancers like lung, colon, and pancreatic cancer. SMAD4 mutation is now recognized in >50% of pancreatic ductal carcinomas. This review is to evaluate the diagnostic and prognostic value targeting the TGF- β /SMAD4 pathway in pancreatic ductal adenocarcinoma.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the United States, with a median survival rate of fewer than six months and five-year survival rate ranging from 20%–30%¹⁻³. Several etiologic factors have been shown to be associated with PDAC including advanced age, smoking, long-standing chronic pancreatitis, obesity, and diabetes mellitus. Despite advances in both surgical and neoadjuvant treatment, there remains no definitive prognostic marker/molecular target to improve overall survival. The aggressive nature of PDAC is thought to be a result of multiple key genetic aberrations in genes such as KRAS, p16, TP53, and SMAD4. Among these mutations, inactivation of SMAD4 has been found in 55% of pancreatic adenocarcinoma cases and thus is similarly known as DPC4 (deleted in pancreatic cancer)⁴. SMAD4 is a member of a large protein family found on chromosome 18q that serves as the main signal transducer for the TGF- β family. The majority of SMAD4 mutations are due to either homozygous deletion of both alleles or intragenic mutation in one allele with subsequent “second hit” of the second allele.⁵⁻⁷ Thus, the purpose of this review is to elaborate on the role of SMAD4 in pancreatic carcinogenesis and explore its role as both a prognostic and therapeutic marker.

Pancreatic Intraepithelial Neoplasia to Adenocarcinoma

Pancreatic ductal adenocarcinoma follows a malignant progression through a known precursor lesion in most cases, akin to colorectal carcinogenesis. The known precursor lesion is pancreatic intraepithelial neoplasia (PanIN), which proceeds through histological and molecular changes associated with cytological and

architectural atypia to progress to adenocarcinoma. Kirsten Ras sarcoma viral oncogene homolog (KRAS) mutation is an early driver event that leads to constitutive activation of multiple pathways, whereas inactivation of SMAD4 is a late event in carcinogenesis⁸. In the early stage of pancreatic carcinogenesis, down-regulation of SMAD4 results in loss of TGF- β /SMAD4-dependent cell cycle arrest and apoptosis⁹. Although the role of SMAD4 in tumor progression and metastasis is complex and still under investigation, recent studies on genetically engineered mice with combined SMAD4/KRAS activation mutation developed an aggressive form of PDAC with poor prognosis¹⁰.

To highlight the importance of multiple genetic mutations driving PDAC oncogenesis, conditional deletion of SMAD4 was insufficient to induce either PanIN or invasive cancer¹¹⁻¹³. It has been found that SMAD4 loss markedly promotes tumor development initiated by Kras G12D activation and Kras G12D/SMAD4 $-/-$ tumors exhibit both increased proliferation and tumor stromal formation^{11,14}. Furthermore, SMAD4 $-/-$ tumors metastasized more frequently than SMAD4 wildtype tumors¹⁵. Additional studies on in-vivo pancreatic cancer cell lines also found SMAD4 mutation or inactivation is always seen in combination with other gene alterations in K-ras, P53, and/or P16¹⁶.

TGF- β Signaling

In normal physiologic conditions, TGF- β proteins are involved in the regulation of critical cellular functions including tissue differentiation, cell proliferation, migration, apoptosis, immune surveillance, and maintaining tissue homeostasis¹⁷. However, TGF- β is often overexpressed and activated in inflammation, fibrosis and even tumorigenesis. The dual function of tumor suppression and oncogenesis in the tumor microenvironment is through activation of cell signaling pathways. Active TGF- β induces a linear signaling pathway through activation of type II and type I receptor kinase. The ultimate response resulting from SMAD dependent and SMAD independent responses and ligand-induced transcription¹⁸.

SMAD-Dependent pathway

The TGF- β superfamily consists of TGF- β s, bone morphogenetic proteins (BMPs), activins and related proteins¹⁸. These proteins regulate their effects through the induction of signaling pathways. Among these proteins, TGF- β regulates linear signaling pathway either by SMAD-mediated response or SMAD-independent response. Stored form extracellular and inactive TGF- β is a complex form of TGF- β pro-peptide and latent transforming growth factor β binding protein (LTBP)¹⁹. Activation of LTBP releases active TGF- β s and allow it to bind with receptors (TGF- β Rs) to initiate the signaling response. The most important downstream intracellular signaling pathway is

SMAD dependent. TGF- β Rs are type I (TGF- β RI), type II (TGF- β RII), and type III (TGF- β RIII). TGF- β RI and TGF- β RII are transmembrane serine-threonine kinase receptors, consist of extracellular ligand domain, a transmembrane domain, and an intracellular serine-threonine kinase domain. Released mature TGF- β , following binding with the ligand domain form a TGF- β RI/TGF- β RII heteromeric complex, subsequently autophosphorylate TGF- β RII and transphosphorylate TGF- β RI²⁰. Phosphorylation of TGF- β RI leads to activation of its kinase domain, significant step to phosphorylate SMAD proteins; specifically, R-SMAD (receptor-regulated SMAD) in SMAD dependent pathway^{20,7}. Once phosphorylated, R-SMAD associates with the common SMAD/SMAD4, and mediate nuclear translocation of the heterotetrameric complex²¹. The SMAD complex accumulates in the nucleus and directly regulates the expression of different target genes, such as integrin, E-cadherin, collagen, and others^{22,23}. It is thought that this TGF- β /SMAD4 pathway serves as a tumor suppressor in the early stages of PDAC by promoting cell cycle arrest and apoptosis of epithelial cells¹⁸. However, persistent overexpression of TGF- β changes the tumor microenvironment to switch them from a tumor suppressor to an oncogene^{24,25}. This change could be a result of the loss of SMAD4 (30% PDAC with SMAD4 homozygous deletion) or due to inactivation (20% of PDAC)²⁶. Downregulation of SMAD4 in the TGF- β signaling pathway switch over the action of cell cycle arrest and apoptosis in epithelial cells²⁷.

Like TGF- β , BMPs are complex proteins play an important role in early and late embryonic development and maintain tissue homeostasis. Upon activation, BMP induces, interaction with surface receptors; type I and type II, mediates activation of serine/threonine kinase activity and formation of heteromeric receptor complex²⁸. The transduced extracellular signal induces phosphorylation and activation of intracellular signaling of specific proteins including the receptor regulated SMAD proteins (SMAD1, SMAD5, and SMAD8). Activated R-SMAD form complex with Co-SMAD and regulate gene transcriptional response. BMP receptor activation also induces SMAD-independent signaling pathway by activation of intracellular p38 and JNK MAP kinases and small GTPases²⁹.

SMAD-Independent pathway

In SMAD independent or non-canonical pathway TGF- β RI complexes induce signaling through activation of other pathways such as tumor necrosis factor (TNF) receptor-associated factor 4 (TRAF4), TRAF6, TGF- β activated kinase 1 (TAK1 or MAP3K), p38 mitogen-activated protein kinase 1 (p38 MAPK), RHO, phosphoinositide 3-kinase (PI3K), AKT (also known as protein kinase B), extracellular signal-regulated kinase (ERK), JUN N-terminal kinase (JNK) or nuclear factor κ B (NF- κ B). The mode of action and outcome varies according to the activation of the individual pathway.

Among the other non-canonical pathways in TGF- β signaling, the RAS-ERK pathway has been shown to be involved in many different points of carcinogenesis. Primarily ERK functions as a proto-oncogene as it promotes cell proliferation, migration, and tumor metastasis but at some points, it antagonizes tumorigenesis. In breast cancer, ERK inhibits cell apoptosis and in pancreatic cancer, ERK upregulates p21 and thereby facilitates TGF- β mediated cell cycle arrest^{30,31}. Sustained existence of ERK could facilitate cell progression through entering S-phase of cell cycle³². ERK contributes to the TGF- β signaling pathway in different stages; antagonizing the SMAD signaling through phosphorylation of SMAD2 and SMAD3 at SMAD linker region and facilitating the SMAD signaling through phosphorylation of other SMAD3 residues³³⁻³⁶. Principe et al. showed ERK upregulation in pancreatic cancer cells and their study also found ERK-induced cell cycle arrest through TGF- β /SMAD4 mediated p21 upregulation in benign pancreatic cell line³⁴. The author and his group demonstrated the role by showing the reduced level of TGF- β mediated nuclear translocation of SMAD4 and p21 while pharmacologically pretreated with ERK antagonist, in benign and well-differentiated neoplastic pancreatic ductal epithelial cell³⁷. In advanced pancreatic cancer cell phosphorylated ERK has no regulatory effect on TGF- β induced upregulation of p21 and nuclear translocation but it plays a crucial role in TGF- β mediated EMT in all pancreatic cancer cell lines³⁴. Like TGF- β , ERK contributes to the carcinogenesis in various ways and some of its contradictory functions brought it as a topic of further investigation.

In advanced pancreatic cancer overexpression of TGF- β and loss of SMAD4 function is one of the explanations of directing the SMAD-independent pathway. A study has shown that TGF- β could still act as tumorigenesis while SMAD4 RNA was downregulated pancreatic cancer cell lines³⁸. The switching of TGF- β to SMAD independent pathway could also be related to the SMAD4 requirement. Further studies are required for a better understanding of this cross over function of TGF- β in carcinogenesis.

TGF- β /SMAD4, Tumor Suppression to Progression

The cell cycle inhibition or growth inhibitory effect of TGF- β /SMAD4 signaling pathway defines its tumor suppressive role whereas in carcinogenesis overexpression of TGF- β and loss of TGF- β /SMAD4 mediated tumor suppressive action leads to tumor progression¹⁸. The other crucial roles of TGF- β in cancer progression are; immune suppression, angiogenesis, and epithelial-mesenchymal transition (EMT)^{39,40}.

In early neoplastic and benign pancreatic epithelial cells, cell cycle arrest by TGF- β /SMAD4 is the main tumor suppressive function, mediated by upregulation of targets

like; p21^{41,42}. In the cell cycle transition from G1 to S phase is inhibited by cyclin-dependent kinase p21⁴³. Upregulation of p21 is mediated by TGF- β /SMAD4 pathway while non-SMAD also regulate p21 as seen in colon cancer^{44,45}. In a pancreatic cancer patient, high levels of p21 is an indicator of good prognosis as it inhibits cell proliferation as well as acinar to ductal metaplasia⁴⁶. Loss of SMAD4 in PDAC opposes the cell cycle regulation and favors proliferation. The similar result is seen in TGF- β mediated apoptosis effect in the tumor microenvironment. TGF- β regulates expression of different genes through SMAD4 like; GADD45 β r signaling factor domain, the bcl-2 Bim homologous factor, the death-associated protein kinase, and TGF- β -inducible early response gene 1 (TIEG1)⁴⁷. A genetic mutation in TGF- β signaling components or changes in signaling pathways often downregulate apoptosis and cell cycle arrest and leads to cancer progression.

In carcinogenesis, EMT is an important transition that leads to the progression and metastasis of cancer cells³⁹. In benign and cancer cells EMT is mediated by a complex of cellular and molecular changes in transcriptional and translational levels^{48,49}. Depending on the cell type and tissue microenvironment these changes downregulate expression of epithelial junctional proteins and markers and upregulate mesenchymal adhesion and marker proteins⁵⁰. The ultimate transcription factor families that regulate the transition are Snail/Slug, Zeb1/2, and twist family⁵¹. In the TGF- β /SMAD4-dependent pathway, the SMAD3/SMAD4 complex induces transcription of Snail protein and decreases expression of epithelial junction protein E-cadherin and occludin^{52,53}. TGF- β also increases the expression of ZEB transcription factors through SMAD4-dependent pathway and decrease expression of miR-200 family, T β R1 and SMAD2, which reflexly increase ZEB1 and ZEB2 mRNA and protein levels and thus further bolstering the EMT response^{53,54}. In the late stage of tumorigenesis, TGF- β promotes tumor growth by a combined effect of SMAD4-dependent and SMAD-independent effects on EMT^{54,55}. The critical role of TGF- β in cancer dissemination is complex and extensively studied to emphasize that TGF- β drives cancer progression not only by cell growth but also and more importantly by EMT⁴⁰.

In addition to its effect on tumor growth, TGF- β modulates tumor development and progression by changing the tumor microenvironment⁵⁶. TGF- β suppresses immune and inflammatory processes through the inhibition of CD8+ cytotoxic T cells, macrophages, dendritic cells, and NK cells⁵⁶. It has recently been found that sustained activation of pro-inflammatory transcription factor NF- κ B in PDAC is closely involved in driving tumor progression. Intact SMAD4, S100A8, S100A9, and S100A8/A9 share an overall inhibitory effect on NF- κ B, while these molecules do not affect NF- κ B in the presence of SMAD4 homozygous

deletion. Thus, SMAD4 plays a key role in suppressing the inflammatory signaling cascades important in PDAC⁵⁷.

PDAC has a strong effect on the deposition of extracellular matrix (ECM), eliciting a strong desmoplastic reaction. In addition to ECM, endothelial cells, immune cells, and fibroblasts are recruited to the tumor microenvironment and thus contribute to both tumor growth and invasion^{58,59}. TGF- β expression enhances the release of multiple ECM molecules including fibronectin, collagen fibulins, and elastin^{60,61}. Overexpression of TGF- β is a critical mediator of fibrosis in many tumors⁶²⁻⁶⁴. TGF- β also inhibits the degradation of newly synthesized ECM by inhibiting the synthesis of matrix metalloproteinase (MMP) and by inhibiting the expression of genes responsible to produce MMP⁶⁵. T β R β II is also known to be overexpressed in PDAC and correlate with advanced tumor stage, decreased patient survival, and increased expression of genes known to promote angiogenesis and invasion (e.g., plasminogen activator 1 and matrix-metalloproteinase-9)⁶⁶⁻⁶⁸. Additionally, high levels of SMAD2 have been documented in PDAC⁶⁸, leading to a more potent response to TGF- β signals.

The correlation of TGF- β overexpression and cancer progression is established in several malignancies^{69,70}. An elevated level of TGF- β is found in both tissue and plasma of PDAC patients and is correlated with the presence of metastases in CRC, PDAC, as well as prostate and breast cancers⁶⁹. Additionally, cancer cells, in general, have been shown to secrete higher amounts of TGF- β than their normal cell counterparts. Conversely, reduced levels of circulating TGF- β isoforms in patient serum was found to be associated with prolonged survival⁷¹.

SMAD4 gene inactivation is associated with poor prognosis in clinically diagnosed PDAC cases, due to an increased incidence of metastatic disease. Studies by Blackford et al. have shown that SMAD4 mutation was more prevalent in unresectable PDAC cases with a higher metastatic burden, whereas SMAD4 wildtype cases were more amenable to chemotherapy and surgical resection⁷⁰. Furthermore, clinical studies have proved that SMAD4 protein expression, acts as an important prognostic marker. In a series of over 200 PDAC cases, median survival was shown to be 19.2-20.5 months for intact SMAD4 expression compared to 13.7-14.7 months for loss of SMAD4 expression, respectively⁶⁷. Several meta-analyses have also proven the association of SMAD4 loss with poor prognosis^{15,67,70}.

SMAD4 inactivating mutations occur in approximately 20% of all pancreatic cancer. They are typically within the MH1 (DNA binding) or MH2 (transcriptional activation) domains of the protein. Additional mutations include deletion of the entire chromosome with a point, frameshift, nonsense, and missense mutations reported. Functional

studies have shown missense mutations within the MH2 domain result in the loss of protein stability and disruption of the dimerization ability of the SMADS⁷². Furthermore, a study by Xu et al. (2000) found that although SMAD4 proteins with MH1 domain point mutations are translated at similar rates as wild-type proteins, they have a shorter half-life and are more rapidly degraded by a ubiquitin-mediated pathway⁷².

SMAD4 as a Prognostic Marker

The diagnosis of pancreatic adenocarcinoma frequently occurs at an advanced stage, leading to inoperability and a median survival rate of 4-8 months following diagnosis. When the disease is limited to the pancreas, surgical resection can improve the 5-year survival rate to 15-20%. The surgical and pathological prognostic factors for PDAC are tumor location (localized in the pancreas), margin status, vascular invasion, tumor size, and TNM staging. Recent studies have proven that tumor expression of TGF- β /SMAD4 plays a pivotal role in cancer prognosis and survival of the patient. More than 50% of PDAC show SMAD4 alteration with concomitant mutation of INK4A/ARF tumor suppressor gene and a Kras oncogene⁵. A SMAD4 related survival study of 25 PDAC patients (8 patients with gene alteration) show SMAD4 gene mutated cases survival period was shorter than SMAD4 nonmutated cases (median survival 5 and 10 months, respectively, $P=0.001$)⁷³. In a different study of surgically resected pancreatic adenocarcinoma patients, survival was shown significantly longer (unadjusted median survival, 19.2 months) with tumors expressed SMAD4 protein as compared with SMAD4 protein expression (14.7 months, $P = 0.03$). This SMAD4 survival benefit was not related to other prognostic factors including tumor size, margin status, lymph node status, pathological stage, blood loss, and use of adjuvant chemoradiotherapy¹⁰. The median survival differences of about 5 months explain the clinical significance of SMAD4 expression PDAC. A meta-analysis of 4247 patients in 20 published articles concluded that the immunohistochemical loss of SMAD4 predicted poor overall survival in both Asian and Caucasian patients with pancreatic cancer, but did not correlate with tumor size, differentiation, or lymph node metastasis⁶⁸. In another meta-analysis of 1762 patients from 14 studies found that loss of SMAD4 correlated significantly with poor overall survival. The multivariate analysis showed that the loss of SMAD4 predicted poor prognosis in patients with less advanced disease (likely Stage I to Stage II pancreatic cancer)¹⁵.

A recent development in gene sequencing technology allows us to know the detail of cancer genome and to identify genetic markers for prognosis of pancreatic cancer. Blackford et al studied a series of 24 adenocarcinoma patients by doing protein-coding gene sequencing. The result showed a mutation of 39 genes in more than one of these cases. In their next study, they showed somatic

mutation correlated with the survival of the patients. They examined all the members of the TGF- β pathway (SMAD3, SMAD4, TGF β R1, and TGF β R2) individually and together. The survival analysis of mutated and non-mutated SMAD4 gene showed a significant difference. Cases with the intact SMAD4 gene had a median survival rate longer than SMAD4 mutated gene (14.2 [95% C.I., 12.5 – 20.5 and 11.5 [95% C.I., 8.5 – 16.0] month respectively)⁷⁰. The recent extensive molecular study is going on to definitively define the sub-groups of pancreatic cancer who could be benefited by targeted therapy.

Current Targeted Therapy for the TGF- β /SMAD Pathway

Role of TGF- β signaling as a potent tumor suppressor in most of the solid tumors focus on developing the therapeutic target. In advanced cancers, there is a selective loss of TGF- β induce growth inhibitory function and induction of other activities that lead to growth, invasion, and metastasis of cancer cells^{56,69}. Current studies differentiate the tumor suppressive and promoting the role of TGF- β at the cellular level to potentiate the anti-tumor effect and at the same time suppress the pro-oncogenic effect. TGF- β /SMAD dependent signaling pathway is thought to be the tumor suppressive whereas the other TGF- β /SMAD independent pathway is considered as pro-oncogenic. The dual functions of TGF- β bring a challenge to identify the selective pathway of the tumor for the appropriate therapeutic intervention. There are several proposed therapeutic approaches to target the TGF- β pathway. According to Massage et al, three best approaches to inhibit TGF- β signaling pathways are 1) translational level inhibition, 2) ligand-receptor level inhibition and 3) inhibition of receptor-mediated signaling⁶⁹. The aim of each of these targeted therapies is to inhibit tumor-promoting function and maintain the tumor suppressive function of TGF- β . One of the other conventional approaches is to target TGF- β induced epithelial-mesenchymal transition (EMT). Advancement of large molecule inhibitors and small molecule inhibitors are designed to inhibit the generation of TGF- β and downstream signaling pathway.

AP-12009, one of the antisense oligonucleotides (ASO) act directly against the mRNA of TGF- β 2. An ongoing study has shown a survival benefit of pancreatic adenocarcinoma and melanoma using AP-12009, supporting the promising role of AP-12009 on aggressive tumors overexpress TGF- β 2⁷⁴. The other ASO under study and preclinical training are AP-11014 and AP-15012²⁸. Monoclonal antibodies are the choice of targeted therapy for many cancers. In the TGF- β signaling pathway, these antibodies target the ligand-receptor binding and prevent subsequent ligand signaling²⁸. Some of these monoclonal antibodies are in under advance the clinical investigation, including Lerdelimumab (CAT-152, Trabio TM), metelimumab (CAT-

192) antibody against TGF- β 2, TGF- β 1. In tumor immune microenvironment, TGF- β exerts its immunosuppressive function through antagonizing Interleukin (IL)-15 mediated natural killer (NK) cell proliferation. The suppression of the cytotoxic function of NK and T cells, through SMAD dependent pathway, is another key role in tumor immune evasion^{75,76}. Inhibition of TGF- β mediated signaling has shown a reversal of immune evasion function by the restoration of immune activity against the tumor cells⁷⁷. Targeting TGF- β mediated immunosuppression in the tumor microenvironment is a new therapeutic target but the effects of the TGF- β inhibitor on human immune system needs to be further evaluated.

The dual role of TGF- β in oncogenesis makes it a potential target for cancer therapy. Extensive studies have been done to evaluate its nature of interactions with TGF- β -receptors and subsequent signaling pathway (SMAD dependent and SMAD independent). Preclinical and clinical studies are going on targeting at different levels of interactions are showing promising results.

Conclusion

PDAC is a devastating malignancy highlighted by the early metastatic spread and advanced stage at diagnosis. Most of the cases are not amenable to surgical resection. Several studies have shown a strong association of TGF- β with cancer prognosis, metastasis, and survival. Additionally, the role of SMAD-dependent signal transduction pathway is important to explain the association of TGF- β with PDAC carcinogenesis. TGF- β targeted therapy is now established for several human cancers and several preclinical and clinical data have shown that TGF- β blockade could be effective in the treatment of PDAC. Checkpoint inhibitors introduce a new therapeutic guideline in treating cancer. Monotherapy with checkpoint inhibitors could not sufficiently treat an advanced pancreatic cancer patient. There is increasing evidence of treating several cancers with targeting TGF- β signaling suggesting the combination of check-point inhibitors with TGF- β signaling inhibition could increase the survival. Thusly, the precision targeting of the TGF- β /SMAD4 signaling pathway could be critical in the treatment of PDAC.

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Author Contributions

S.A. and R.U. formulated the original hypothesis, designed the study and wrote the manuscript. C.S and M.Z.D wrote the manuscript.

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