RESEARCH ARTICLE

SPDEF drives pancreatic adenocarcinoma progression via transcriptional upregulation of S100A16 and activation of the PI3K/AKT signaling pathway

Hang Jiang 1#, Zhiqian Xue 1#, Liping Zhao 1#, Boyuan Wang 2, Chenfei Wang 3*, Haihan Song 1-4,5*, and Jianjun Sun 1*

Pancreatic adenocarcinoma (PAAD) is a notably aggressive malignancy with limited treatment options and an unfavorable prognosis for patients. The objective of this study was to elucidate the molecular mechanisms by which Sam pointed domain-containing ETS transcription factor (SPDEF) impacts PAAD progression. Analysis of differentially expressed genes (DEGs) and their association with ETS family members, conducted through The Cancer Genome Atlas (TCGA) database, identified SPDEF as a key gene within the molecular framework of PAAD. Kaplan-Meier (KM) survival analysis highlighted the prognostic relevance of SPDEF. In vitro experiments validated the association with cell proliferation and apoptosis, affecting pancreatic cancer cell dynamics. Elevated SPDEF expression was observed in PAAD tumor samples, further establishing its role in this disease. Our in vitro assays revealed that SPDEF regulates mRNA and protein expression levels, significantly affecting cell proliferation. Moreover, an association was established between SPDEF and a reduction in apoptosis, alongside an increase in cell migration and invasion. An in-depth analysis of SPDEF-targeted genes revealed four crucial genes for the advanced prognostic model, among which S100A16 was significantly correlated with SPDEF. Mechanistic analysis showed that SPDEF enhances the transcription of S100A16, which in turn enhances PAAD cell migration, proliferation, and invasion by activating the PI3K/AKT signaling pathway. Our study revealed the critical role of SPDEF in promoting PAAD by upregulating S100A16 transcription and stimulating the PI3K/AKT signaling pathway, thereby deepening the understanding of the molecular evolution of pancreatic cancer and revealing new therapeutic targets in the SPDEF-driven pathways.

Keywords: Pancreatic adenocarcinoma (PAAD), Sam pointed domain-containing ETS transcription factor (SPDEF), S100A16, PI3K/AKT signaling pathway.

Introduction

The global prevalence of pancreatic adenocarcinoma (PAAD) has rapidly increased, highlighting the critical importance of early detection and timely intervention [1, 2]. Due to its insidious nature and the lack of distinctive early symptoms, diagnosis is often delayed, resulting in advanced stages of the disease that are frequently inoperable [3, 4]. Late detection of PAAD complicates treatment and reduces the likelihood of a positive outcome for the patient due to the aggressive progression of the cancer. Furthermore, the inherent resistance of PAAD to several conventional treatments means that even if diagnosed early, the treatment effectiveness may still be limited [5]. The genetic heterogeneity of PAAD suggests that tailored treatment options may be more effective than universal strategies [6, 7]. Advancements in personalized medicine and genomic profiling offer promising avenues in this realm. By recognizing the specific genetic aberrations and molecular mechanisms of individual tumors may enable clinicians to design more precise and effective therapeutic interventions.

The PI3K/AKT signaling pathway is crucial for cellular homeostasis and growth. However, when dysregulated, it can contribute to various diseases, particularly cancer [8, 9]. Its role in PAAD is highlighted by a complex network of signaling cascades, feedback mechanisms, and interactions with other pathways, collectively influencing the progression of cancer cells [10, 11]. Aberrant activation of the PI3K/AKT pathway provides a proliferative edge to pancreatic tumor cells [12]. This signaling pathway promotes unchecked cell proliferation, circumvents apoptotic processes, and stimulates angiogenesis, all of which are crucial for tumor survival [13, 14]. Furthermore, it can promote metastasis, which is a primary factor in PAAD fatalities [15]. Recent studies by Stanciu et al. and Li et al. have enriched our understanding of the intricacies of this pathway in pancreatic cancer. Stanciu et al. [16] emphasized the
role of cytokines, chemokines, and growth factors in activating the PI3K/AKT/mTOR signaling pathway, revealing the external factors that modulate pancreatic tumor intracellular mechanisms. Conversely, Li et al. [17] demonstrated the therapeutic potential of scoparone, a natural compound, in countering pancreatic cancer by targeting this pathway. Therefore, delving into the nuances of the PI3K/AKT signaling pathway, offers insights into pancreatic cancer mechanisms and promises targeted therapeutic avenues.

The Sam pointed domain-containing ETS transcription factor (SPDEF), while implicated in various cellular processes, has not been extensively explored in the context of PAAD. Unveiling the role of novel molecules, such as SPDEF, in the complex molecular landscape of pancreatic cancer may be pivotal for crafting innovative therapeutic avenues. Our investigation focuses on the relationship between SPDEF and S100A16, its purported downstream effector. Elucidation of this regulatory axis may provide insight into novel molecular mechanisms that may influence the aggressiveness of PAAD. S100A16, a member of the S100 protein family known for calcium binding and cellular signaling, may play a nuanced role in tumorigenesis, especially in conjunction with SPDEF [18, 19]. Additionally, the potential interplay between SPDEF, S100A16, and the PI3K/AKT signaling pathway is of considerable interest given the significance of this pathway in various cancers. Such interactions could provide valuable insights into novel avenues of tumor progression and therapeutic resistance. Further investigation into the role of SPDEF may reveal its potential as a prognostic marker, providing valuable insights for predicting disease trajectories and guiding treatment decisions in malignancies like PAAD.

In summary, this research aimed to explore the SPDEF-S100A16-PI3K/AKT axis in the context of PAAD. By elucidating this molecular interaction, the aim is to gain a more complete understanding of the molecular landscape of PAAD and thereby identify potential avenues for targeted therapy, more importantly, hope for patients diagnosed with this formidable malignancy.

**Materials and methods**

**Differential gene expression and survival analysis in PAAD**

The 179 PAAD samples and four normal samples were obtained from The Cancer Genome Atlas (TCGA) database (https://tcga-data.nci.nih.gov/tcga/). By using the “ggplot2” package in R, differential gene expression analysis was done on the TCGA PAAD dataset. Genes were filtered based on fold change (FC) criteria: FC > 2 or FC < 0.5, and P < 0.05 was used as the significance criterion. The distribution of differentially expressed genes (DEGs) was depicted through a volcano plot. The “VennDiagram” package was employed to identify genes that overlapped between DEGs and ETS family genes. The intersection of these gene sets was visualized using Venn diagrams to determine shared candidates for further investigation. The Kaplan-Meier (KM) survival analysis was used to evaluate the prognostic significance of the hub gene (SPDEF) concerning overall survival (OS) and recurrence-free survival (RFS) for patients with pancreatic cancer. Survival curves were plotted using “survival” package in R package, with log-rank test P values. The relationship between SPDEF expression and patient outcomes by comparing high and low-expression groups.

**Expression analysis and staging evaluation of SPDEF in PAAD**

SPDEF expression analysis was performed using the PAAD dataset from the TCGA database. Raw expression data was obtained and processed using R Studio. By using the “ggplot2” package in R, boxplots showing the distribution of SPDEF expression in PAAD samples were visualized and SPDEF expression levels were evaluated. The relationship between SPDEF expression and various pancreatic cancer stages was assessed using the gene expression profiling interactive analysis (GEPIA; http://gepia.cancer-pku.cn/) database. Gene expression data from both the Genotype-Tissue Expression (GTEx) project and TCGA studies were integrated and analyzed by GEPIA. The “Stage Map” function within the GEPIA interface was utilized to investigate SPDEF expression across different tumor stages. Furthermore, the association between SPDEF expression with M (metastasis) and N (lymph node) stages was assessed based on clinical data and medical imaging records. Information on metastases and lymph node involvement was collected from clinical reports, imaging studies, and pathological examinations of patients with PAAD. The multifaceted approach, which involved TCGA, GEPIA, and clinical data, enabled a comprehensive analysis of SPDEF expression patterns and their potential association with the PAAD tumor stage.

**hTFTarget database**

The human transcription factor target gene (hTFTarget, http://bioinfo.life.hust.edu.cn/hTFTarget/) database is specifically designed to predict transcription factor target genes and delineate their regulatory interactions. In this study, the hTFTarget database was utilized to identify genes potentially regulated by SPDEF, aiming to uncover SPDEF target genes. Subsequently, an intersection analysis was conducted between these predicted genes and the upregulated DEGs (Table S1). This approach provided a more comprehensive understanding of the downstream SPDEF-associated regulatory network in the context of PAAD.

**Prognostic analysis of SPDEF-related genes in PAAD**

The hTFTarget database was used to predict SPDEF target genes, which integrates transcription factor-target interactions. A total of thirty genes with significantly altered expression in pancreatic cancer were identified by combining upregulated DEGs and SPDEF-predicted target genes. This subset of genes was subjected to the least absolute shrinkage and selection operator (LASSO) regression analysis to create a predictive model. The optimal lambda value ($\lambda_{min} = 0.0722$) was determined by LASSO regression identifying prognostically significant genes. A prognostic risk model was developed using the expression levels of four significant prognostic genes identified through this analysis process. The risk score formula is as follows:

$$
\text{risk score} = 0.1266 \times \text{MYEOV} + 0.1765 \times \text{ECT2} + 0.0621 \times \text{MMP28} + 0.0291 \times \text{S100A16}
$$
The risk score calculation for pancreatic cancer tumor samples was performed using the TCGA database. Subsequently, samples were dichotomized into high- and low-risk categories based on the median risk score. The performance of the generated risk models was evaluated by survival scatterplots and gene expression heatmaps. The OS was evaluated using KM survival analysis, and the predictive power of the models was evaluated using receiver operating characteristic (ROC) analysis.

Correlation analysis between SPDEF and key prognostic target genes
To explore the potential relationship between SPDEF expression and its key prognostic target genes, a correlation analysis was performed. These four key prognostic genes were derived from risk prognostic models. The correlation between SPDEF and each target gene was calculated using the Spearman correlation coefficient.

JASPAR database
The Just Another Spar Promoter Analysis Resource (JASPAR, http://jaspar.genereg.net/) database provides a comprehensive collection of transcription factor binding profiles and matrices. It offers valuable insights into potential binding motifs that transcription factors may recognize within gene promoter regions. In this study, the JASPAR database was used to predict the putative SPDEF-binding motif within the promoter region of the S100A16 gene. This information was critical for elucidating the direct interaction between SPDEF and the promoter region of S100A16.

Cell culture
In this study, human normal pancreatic ductal cells (HPNE), as well as the PAAD cell lines (BxPC-3, Capan-2, HPAF-II, PANC-1, Mia PaCa-2, and SW1990) were all sourced from the ATCC (Manassas, VA, USA). Cells were cultured in a humidified incubator at 37 °C with 5% CO2 in a suitable media containing 1% penicillin–streptomycin and 10% fetal bovine serum (FBS). Cell viability was evaluated using the CCK-8 assay (Dojindo, Japan), on 96-well plates with a seeding density of 5 × 10^3 cells per well. Subsequently, these cells were loaded into the upper chamber of a six-well Transwell insert. To serve as a chemoattractant, a full medium was added to the lower chamber. After 48 hours of incubation at 37 °C, non-migratory and non-invasive cells remaining in the upper chamber were meticulously removed with a cotton swab.

Protein lysates from PAAD cells were prepared using RIPA lysis buffer (Thermo Fisher Scientific, USA) containing protease and phosphatase inhibitors. The protein concentration was calculated using the BCA Protein Assay Kit from Thermo Fisher Scientific (USA). Equal quantities of protein were separated using SDS-PAGE and transferred to PVDF membranes from Millipore (USA). The membranes were probed with primary antibodies against Akt (1:1000, Cell Signaling Technology), p-Akt (1:1000, Cell Signaling Technology), p-GSK3β (1:1000, Cell Signaling Technology), SPDEF (1:1000, Abcam), S100A16 (1:1000, Abcam), PI3K (1:1000, Abcam), and GAPDH (1:5000, Cell Signaling Technology) as a control. Following incubation with secondary antibodies, bands were visualized using enhanced chemiluminescence (ECL) and documented with a ChemiDoc imaging system.

Cell Counting Kit-8 (CCK-8) assay
Cell viability was evaluated using the CCK-8 assay (Dojindo, Japan), on 96-well plates with a seeding density of 5 × 10^3 PAAD cells per well. Appropriate treatments were administered to each well before adding the CCK-8 reagent. After 0, 24, 48, 72, 96, and 120 h, the absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, USA).

Flow cytometry analysis
PAAD cells were separated using Trypsin-EDTA (Gibco, USA) and washed with phosphate-buffered saline. According to the manufacturer’s recommendations, the cells were stained using fluorescently labeled antibodies specific for SPDEF and S100A16 (Abcam, USA). Data were analyzed using FlowJo software (FlowJo LLC, USA) and flow cytometry was carried out using a flow cytometer (BD Biosciences, USA).

Transwell migration and invasion assay
After 24 hours of transfection, cells were collected and suspended at a density of 5 × 10^4 cells/well per well. Subsequently, these cells were loaded into the upper chamber of a six-well Transwell insert. To serve as a chemoattractant, a full medium was added to the lower chamber. After 48 hours of incubation at 37 °C, non-migratory and non-invasive cells remaining in the upper chamber were meticulously removed with a cotton swab.
The cells adhered to the underside of the membrane were fixed using 4% paraformaldehyde and stained with DAPI to visualize nuclei. The invading or migrating cells were observed and quantified using a fluorescence microscope, and images were captured for subsequent analysis.

**PCR analysis of chromatin immunoprecipitation (ChIP)**

The ChIP assay was performed using the SimpleChIP Plus Enzymatic Chromatin IP Kit from Cell Signaling Technology and an anti-SPDEF antibody. The DNA fragments were enriched and PCR was performed using primers specific for the predicted SPDEF binding motif within the S100A16 promoter region.

**Luciferase activity assay**

A luciferase reporter plasmid with either the wild-type (Wt) or mutant (Mut) S100A16 promoter sequence was co-transfected into PAAD cells with a plasmid encoding SPDEF. The Dual Glo Luciferase Assay System (Promega, Madison, WI, USA) was used following the manufacturer’s instructions to quantify luciferase activity 48 h after co-transfection. Firefly luciferase activity was normalized to the activity of the Renilla luciferase gene.

**Statistical analysis**

The Statistical Analysis System was used for all analyses, and experiments were triple-replicated. Data were represented as mean ± SD. Significance (*P* < 0.05) between treatments was ascertained using analysis of variance and Fischer’s test at the 95% confidence level. Mortality differences across treatments were evaluated using a chi-square test.

**Results**

**Prognostic significance of SPDEF expression in PAAD**

The TCGA database yielded 385 upregulated and 605 downregulated DEGs between PAAD samples and normal samples (Figure 1A). Further analysis identified five overlapping genes among the DEGs and ETS family members (Figure 1B). To evaluate the prognostic significance of SPDEF concerning OS and RFS, KM analysis was conducted. As depicted in Figures 1C and 1D, pancreatic cancer patients with diminished SPDEF expression exhibited significantly enhanced OS (*P* = 0.0024) and RFS (*P* = 0.011). This emphasizes the importance of SPDEF as a prognostic determinant and its critical role in disease trajectory and patient prognosis. Further examination revealed a notable increase in SPDEF levels in PAAD tumor samples, suggesting its oncogenic function (Figure 1E). Analysis of SPDEF expression across PAAD tumor stages identified elevated SPDEF levels in stage 2 tumors (Figure 1F). An in-depth appraisal of both M-stage and N-stage categories corroborated the sustained elevation of SPDEF in PAAD tumor specimens, with its expression independent of M-stage and N-stage distinctions (Figures 1G and 1H). For in vitro evaluations, our selection encompassed HPNE and a spectrum of PAAD cells. Through qRT-PCR and WB analytical methodologies, we ascertained a significant upregulation of SPDEF in pancreatic cancer cells, predominantly within PANC-1 and MIA PaCa-2 lines (Figures 1I and 1J), designating them for further experimental exploration.

**Effects of SPDEF regulation on the phenotype of PAAD cells**

Utilizing qRT-PCR and WB analyses, we studied the effects of SPDEF manipulation on MIA PaCa-2 and PANC-1 cells (Figures 2A–2D). Overexpression of SPDEF significantly increased both mRNA and protein levels, while SPDEF knockdown led to substantial reductions in these levels, with the most pronounced decrease seen in si-SPDEF#1. To understand the functional implications of these alterations, the CCK-8 assay was employed. Cells exhibiting increased SPDEF expression demonstrated enhanced proliferation, as indicated by increased absorbance values. However, cells with reduced SPDEF expression displayed diminished proliferation, evidenced by decreased absorbance values (Figures 2E and 2F). Collectively, our data emphasizes the pivotal role SPDEF plays in influencing the behavior of PAAD cells.

**SPDEF promotes PAAD cell invasion and metastasis and inhibits apoptosis in vitro**

Flow cytometry provides a compelling method to study cellular apoptosis. Our results revealed that enhanced SPDEF expression leads to a decline in apoptosis (Figures 3A–3D). Conversely, cells with downregulated SPDEF exhibited pronounced apoptotic activity. To further investigate the effects of SPDEF on PAAD cell dynamics, we used the Transwell assay to analyze cell migration and invasion capabilities. Cells with higher levels of SPDEF exhibited greater migratory and invasive properties. In contrast, cells inhibited with SPDEF demonstrated significantly diminished capacities in both assays (Figures 3E–3I). Together, these insights underscore the instrumental role of SPDEF in shaping the behavior of PAAD cells.

**SPDEF target gene identification and prognostic value analysis**

The identification of SPDEF target genes is pivotal for deepening our understanding of its function in PAAD. The hTTarget database was employed to identify these potential targets. An analysis intersecting the predicted targets with upregulated DEGs yielded 30 candidate genes. LASSO regression analysis, intersecting the predicted targets with upregulated DEGs yielded 30 candidate genes. LASSO regression analysis, with an optimal *λ* value pinpointed at 0.0722 (Figures 4A and 4B), further distilled this list to four paramount genes: S100A16, MMP28, ECT2, and MYEOV (Figure 4C). Furthermore, KM survival analysis revealed that samples with a higher risk profile have a decreased OS probability (Figure 4D). Additionally, ROC curve analysis indicated that the risk model possessed good prognostic predictive capabilities, with AUC values exceeding 0.7 at the 1-, 3-, and 5-year marks (Figure 4E).

**SPDEF activates transcription of S100A16**

A correlation analysis was conducted involving SPDEF and its four putative targets: S100A16, MMP28, ECT2, and MYEOV (Figure 5A). S100A16 showed the highest correlation with SPDEF, prompting us to further investigate its relationship. Experimental observations revealed that elevating S100A16 levels in PAAD cells led to a surge in SPDEF expression (Figures 5B and 5C). Conversely, reducing S100A16 resulted in decreased SPDEF levels. JASPAR was used to predict potential SPDEF-binding sites within the S100A16 promoter, and several candidates were identified (Figure 5D). To confirm a direct interaction between SPDEF and the S100A16 promoter, ChIP-PCR
was performed. The analysis validated SPDEF enrichment on the S100A16 promoter, with sequences from the binding region being amplified when DNA was precipitated with Flag-tagged SPDEF (Figures 5E and 5F). To further investigate the transcriptional influence of SPDEF on S100A16, we introduced both the Wt and the Mut binding site of the S100A16 promoter into the pGL4.20 vector. After transfection into PAAD cells, luciferase reporter assays showed an increase in S100A16 promoter activity with SPDEF expression. Notably, this amplification was nullified when the SPDEF binding site underwent mutation (Figures 5G and 5H).

**S100A16 mediates SPDEF-induced proliferation, migration, and invasion of PAAD cells**

A series of experiments were conducted to investigate the interaction between SPDEF and S100A16, as well as its consequent effects on the behaviors of PAAD cells. qRT-PCR and WB analyses, as illustrated in Figures 6A–6D, demonstrated a significant decrease in S100A16 expression following its knockdown in PAAD cells. Conversely, the upregulation of S100A16 was associated with its increased expression. Subsequent functional analyses elucidated the implications of these modulations. The CCK-8 assays showed that the proliferation of PAAD cells increased with heightened SPDEF expression following its knockdown, as their migration and invasion capabilities were significantly diminished, falling below those of the control group (Figures 6E and 6F). To further investigate the role of S100A16 in SPDEF-mediated cellular behaviors, we conducted migration and invasion assays. Figures 6G–6L showed a significant increase in the migratory and invasive potential of PAAD cells under SPDEF overexpression. However, a significant finding was observed when SPDEF-overexpressing cells were simultaneously subjected to S100A16 knockdown, as their migration and invasion capabilities were significantly diminished, falling below those of the control group levels.

**Figure 1. Expression and prognostic analysis of SPDEF in pancreatic PAAD.** (A) Analysis of DEGs in PAAD samples and normal control samples in the TCGA database. Red scattered points represent upregulated DEGs, and blue scattered points represent downregulated DEGs; (B) Venn diagram, analysis of overlapping genes between TCGA-DEGs and ETS family members; (C and D) The impact of differential expression of SPDEF on OS prognosis and RFS prognosis in PAAD patients. Blue represents low-expression samples, red represents high-expression samples; (E) Box plot, validation of SPDEF expression in PAAD tumor samples in the GEPIA database; (F–H) Expression of SPDEF in different stage subgroups in the GEPIA database, including M stage and N stage; (I and J) qRT-PCR and western blot detected the expression of S100A16 in control cells and six PAAD cell lines. *P < 0.05; **P < 0.01; ns: Not significant; PAAD: Pancreatic adenocarcinoma; SPDEF: Sam’s pointed domain-containing ETS transcription factor; DEGs: Differentially expressed genes; TCGA: The Cancer Genome Atlas; GEPIA: Gene expression profiling interactive analysis; qRT-PCR: Quantitative real-time polymerase chain reaction; OS: Overall survival; RFS: Recurrence-free survival.
Figure 2. Regulation of PAAD cell phenotype by overexpression and knockdown of SPDEF. (A-D) qRT-PCR and western blot analyses were used to investigate the effect of SPDEF regulation on the phenotype of PAAD cells; (E and F) Functional analysis using the CCK-8 assay to assess cell proliferation in response to SPDEF modulation. *P < 0.05; **P < 0.01. PAAD: Pancreatic adenocarcinoma; SPDEF: Sam pointed domain-containing ETS transcription factor; qRT-PCR: Quantitative real-time polymerase chain reaction; CCK-8: Cell Counting Kit-8.

SPDEF promotes PAAD progression by transcriptionally upregulating S100A16 and activating the PI3K/AKT signaling pathway

To explore the interplay between the PI3K/AKT/p-GSK3β signaling pathway and S100A16 in cellular dynamics, we conducted a comprehensive experiment using PAAD cells (Figures 7A–7F). Initially, we transfected cells with SPDEF overexpression alone, in combination with LY294002 (a PI3K antagonist at 10 μM), or after S100A16 knockdown at 10 μg/mL. Subsequent WB analyses revealed that SPDEF overexpression led to increased levels of p-Akt, and p-GSK3β. Combining SPDEF overexpression with LY294002 resulted in a decline in p-Akt, and p-GSK3β levels, although these levels remained higher than those observed in the control group. Conversely, the combination of SPDEF overexpression and S100A16 silencing led to reduced expressions of p-Akt and p-GSK3β. Significant alterations, as measured by WB, were predominantly observed in p-Akt and p-GSK3β. Collectively, our data highlights the pivotal role that S100A16 plays in orchestrating the influence of SPDEF on the PI3K/AKT signaling pathway, thereby illuminating the complex molecular mechanisms that govern the behavior of PAAD cells.

Discussion

In this study, we delineate the pivotal influence of the SPDEF gene on cancer progression, particularly in the context of the PI3K/AKT signaling pathway. While previous studies have suggested the role of SPDEF in various tumors, its unique role in specific cancer manifestations remains unclear. Thus, the multifaceted functions and mechanisms of SPDEF in PAAD were
SPDEF's role in PAAD progression

Our data highlighted the integral role of SPDEF in the complex progression of PAAD, primarily through the activation of the PI3K/AKT signaling pathway. Although the roles of SPDEF and the PI3K/AKT signaling pathway in cancer are recognized, their specific interaction in PAAD remains incompletely explored. The findings of this study help fill this knowledge gap, providing valuable insights into the molecular mechanisms driving PAAD progression.

Initiating our investigation, we employed a bioinformatics approach to identify SPDEF as a significant regulator within the complex landscape of pancreatic cancer. SPDEF plays a pivotal role in various biological functions [20–22], and while its association with oncogenesis is established, the nuances of its involvement vary across cancer types. For instance, Ye et al. [23] highlighted the dual roles of SPDEF in breast cancer, showcasing its oncogenic and tumor-suppressive capacities. Divergently, in colorectal cancers, Lo et al. [22] described that SPDEF induces cellular quiescence in colorectal cancer by orchestrating the regulation of β-catenin transcriptional targets. These diverse functions underscore the significant impact of SPDEF in cancer biology, marking it as a critical component in developing potential treatments. Our findings indicate a negative correlation between SPDEF expression and disease prognosis, with an observed upregulation of SPDEF in PAAD tumors, notably in stage 2 tumors. This aligns with the sequential expression pattern in the M and N stages, suggesting a potential proto-oncogene role. Moreover, in vitro functional assessment further revealed the extensive role of SPDEF in the
regulation of PAAD cells. Overexpression of SPDEF resulted in altered cell behavior, promoting proliferation, migration, and invasion while inhibiting apoptosis. In conclusion, our study clarifies the multifaceted role of SPDEF in PAAD and offers novel insights into its potential as a diagnostic and therapeutic target.

Through an integrated approach combining bioinformatics analysis, regression techniques, and experimental validation, we precisely identified S100A16 as a target gene of SPDEF, revealing its significant role within the oncogenic landscape. S100A16, part of the expansive S100A family, emerges as a key player in cancer biology, exhibiting diverse functions in tumorigenesis [24, 25]. Intriguingly, Li et al. [26] reported the upregulation of S100A16 and its family members in pancreatic ductal adenocarcinoma (PDAC) tissues compared to normal tissues, a phenomenon inversely related to promoter methylation.

Figure 4. SPDEF target gene identification and prognostic value analysis in PAAD. (A) LASSO coefficient profiles of the 30 candidate genes. The vertical line is drawn at the value chosen by 10-fold cross-validation. (B) Partial-likelihood deviance plot versus log(\(\lambda\)). The dotted vertical lines represent the optimal values using the minimum criteria and the one standard error of the minimum criteria. (C) Display of the four significant genes (S100A16, MMP28, ECT2, and MYEOV) filtered by LASSO regression, along with their risk scores and survival status. The heatmap below shows the z-score of expression for these genes. (D) Kaplan-Meier survival curves for patients grouped by high and low risk. The number of patients at risk in each group is displayed below the survival curve. (E) ROC curve analysis for the prognostic risk model at 1-, 3-, and 5-year survival periods. AUC values for each interval are provided in the legend. PAAD: Pancreatic adenocarcinoma; SPDEF: Sam pointed domain-containing ETS transcription factor; LASSO: Least absolute shrinkage and selection operator; ROC: Receiver operating characteristic.
This increased expression correlates with diminished survival rates in PDAC patients, highlighting the potential of S100A16 as a prognostic marker. Notably, the elevated S100A16 expression in PAAD is intriguingly counteracted by its negative association with immune activity and infiltration, particularly with CD8+ T cells. This sheds light on its dual capacity as a prognostic marker and a therapeutic target for immune interventions. Additionally, Li et al. [24] discovered that S100A16 promotes PDAC metastasis by activating the STAT3 signaling cascade, inducing epithelial-to-mesenchymal transition. This finding suggests that reducing S100A16 levels could improve the effectiveness of drugs such as gemcitabine, making it a potential target in the treatment of PDAC. Another study delineated an overexpression of S100A16 in PDAC, pinpointing its role in advancing the disease through the FGF19-mediated AKT and ERK1/2 signaling pathways, further supporting its potential as a therapeutic target [27]. Our findings designate S100A16 as the primary target gene of SPDEF in PAAD, with SPDEF expression modulating in response to S100A16 levels, indicating a direct link between them. Critically, reduced S100A16 expression mitigated the SPDEF overexpression-induced enhancement of PAAD cell proliferation, underscoring its influence on cancer cell dynamics.

The PI3K/AKT signaling pathway is acknowledged as a pivotal regulatory network in exploring the complex mechanisms underlying pancreatic cancer, playing a central role in numerous biological functions [28–30]. This pathway is critical for maintaining healthy cell function but also plays a crucial role in cancer development [31]. Reports suggest that abnormal activation of the PI3K/AKT signaling pathway leads to unlimited proliferation of pancreatic cancer cells and inhibition of apoptosis, supporting tumor growth and metastasis [32]. Another study found that blocking the PI3K/AKT signaling pathway with certain medications can
prevent pancreatic cancer cells from migrating and invading, highlighting the critical role of this pathway in pancreatic cancer [33]. Building on this foundation, our study delved into the interaction between the PI3K/AKT/p-GSK3β signaling cascade and S100A16 in pancreatic cancer cells. Experimental findings revealed that SPDEF overexpression led to substantial increases in p-Akt and p-GSK3β levels. These expression levels were reduced when combined with LY294002, the antagonist of PI3K, or S100A16 knockdown. These results highlight the significant regulatory role of S100A16 on the PI3K/AKT signaling pathway in modulating the impact of SPDEF. The findings shed new light on the molecular basis of pancreatic cancer.

**Conclusion**

In summary, our investigation highlights the significant role of SPDEF and its target gene, S100A16, in the progression and dynamics of PAAD cells. SPDEF was identified as a crucial factor that significantly affects cell proliferation, migration, and invasion. Notably, its transcriptional upregulation of S100A16 unraveled a significant nexus in shaping PAAD cell behavior. Moreover, the involvement of the PI3K/AKT signaling pathway further elucidates the molecular complexity underpinning the translational effects promoted by SPDEF through S100A16. These findings shed light on potential therapeutic options and highlight the importance of further investigating these molecular dynamics for effective intervention in PAAD.
Figure 7. SPDEF modulates the PI3K/AKT/p-GSK3β signaling cascade in PAAD cells. (A and B) Western blot analysis representing the impact of SPDEF overexpression, either alone or in conjunction with LY294002 treatment, on the expression levels of PI3K, Akt, p-Akt, and p-GSK3β in MIA PaCa-2 and PANC-1 cells. GAPDH was employed as a loading control. (C) Quantification of protein levels in MIA PaCa-2 and PANC-1 cells treated as described in (A and B). (D and E) Western blot analysis showcasing the effect of SPDEF overexpression either individually or following S100A16 knockdown on the expression of PI3K, Akt, p-Akt, and p-GSK3β in MIA PaCa-2 and PANC-1 cells. GAPDH served as a loading control. (F) Quantitative assessment of protein expressions in MIA PaCa-2 and PANC-1 cells treated as mentioned in (D and E). *p < 0.05. PAAD: Pancreatic adenocarcinoma; SPDEF: Sarm pointed domain-containing ETS transcription factor.

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Conflicts of interest: Authors declare no conflicts of interest.

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Data availability: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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### Table S1. List of thirty SPDEF target genes

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