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The up-regulation of SPTAN1 expression in Pancreatic adenocarcinoma is associated with tumor immune invasion and poor clinical prognosis

Wei Guo[1†], LingYu Hu[2†], ZhaoFeng Gao[2], XiaoRong Liu[2], XiaoDan Yang[2*] and XiaoGuang Wang[2*]

Abstract

Background Pancreatic adenocarcinoma (PAAD) is a common malignancy with a very low survival rate. More and more studies have shown that SPTAN1 may be involved in the development and progression of a variety of tumors, including rectal cancer, Pancreatic adenocarcinoma, etc., and may affect their prognosis.

Methods Bioinformatics technology was used to analyze the relationship between SPTAN1 expression in PAAD and immune cell infiltration, immune regulatory factors and chemokines, and cell experiments were used to verify the relationship between SPTAN1 knock down and migration, invasion, apoptosis and cycle changes of PAAD cell lines. In addition, immunohistochemical staining of SPTAN1 was performed by tissue microarray (TMA) to study the relationship between high expression of SPTAN1 and clinicopathological features and overall survival rate.

Results The expression of SPTAN1 is significantly correlated with immune cell infiltration, immunomodulators, chemokines and their receptors. In addition, it was found that the knock-down of SPTAN1 inhibited the migration and invasion ability of PAAD cell lines, promoted the apoptosis of cell lines, and also affected the changes of cell cycle. Immunohistochemical staining using tissue microarray (TMA) showed that the high expression of SPTAN1 was associated with M stage ($P=0.004$) and CA199 ($P=0.012$), and the overall survival rate of the high expression group was significantly lower than that of the low expression group ($P=0.043$).

Conclusion Our results suggest that up-regulation of SPTAN1 is related to cell migration, invasion, apoptosis and cycle changes, and is associated with tumor immune invasion and poor prognosis of PAAD.

Keywords Pancreatic adenocarcinoma, Nonerythroid spectrin αII, Prognosis-related biomarker, Clinicopathoogical characteristics

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Introduction

Pancreatic adenocarcinoma is a deadly disease with a high mortality rate due to its early difficult diagnosis and easy metastasis, ranking fourth among the causes of cancer death, and its overall survival rate is only 5–10% five years after diagnosis [1, 2]. In the next 30 years, Pancreatic adenocarcinoma is expected to become the second leading cause of cancer death in the United States [3] and the third leading cause of cancer-related death in Europe [4]. The main risk factors for Pancreatic adenocarcinoma are smoking, high-fat diet, diabetes, coffee, alcohol consumption, some occupational exposure and genetic factors. But all are the result of a synergistic effect of multiple factors [5]. Radical surgery is the only effective treatment at present, but more than 80% of patients are already in the advanced stage when diagnosed, and the surgical treatment effect is not good. Even if radical surgery is performed for early Pancreatic adenocarcinoma patients, supplemented by chemoradiotherapy and targeted therapy, most patients still have local recurrence and metastasis after surgery, and the postoperative 5-year survival rate is still less than 10% [6]. Therefore, it is necessary to explore new and more effective therapeutic targets and identify new prognostic markers to increase the early diagnosis rate of Pancreatic adenocarcinoma and improve the survival and prognosis of patients.

Nonerythroid spectrin αII (SPTAN1) is a filamentous cytoskeleton protein that plays an important role in stabilizing the plasma membrane and organelles, ensuring important cellular properties, including polarity and cell stability. In addition, it is involved in cell adhesion, intercellular contact, and apoptosis [7]. Studies have found that the change of expression of SPTAN1 in tumors is due to its multiple functions and its role in adhesion and migration. SPTAN1 can affect the growth and progression of tumors in both positive and negative directions according to its specific regulatory effects, and can be used as a potential marker protein for tumor formation, tumor aggressiveness and evaluation of treatment efficiency [8]. In addition, studies have found that SPTAN1 is involved in the development of various cancers, such as colorectal cancer, gastric cancer, lung cancer, and bladder cancer, by affecting the progression process in tumor cells [9–12]. These results suggest that SPTAN1 may serve as a novel prognostic marker for patients with multiple malignancies.

Therefore, in this study, we evaluated the correlation between SPTAN1 expression and the prognosis of PAAD and explored the role of SPTAN1 in the progression of PAAD. The expression, prognosis, tumor immune microenvironment and regulatory mechanism of SPTAN1 in PAAD were analyzed. To explore the prognostic value of SPTAN1 gene in patients with PAAD. In this study, we investigated the effect of SPTAN1 on cell cycle, scratch, migration and invasion of Pancreatic adenocarcinoma cells. The expression level of SPTAN1 in tumor samples from PAAD patients was studied and the correlation between SPTAN1 expression and clinical outcomes of PAAD patients was determined.

Materials and methods

Bioinformatics analysis

Data acquisition

The Cancer Genome Atlas (TCGA) mainly collects clinical data, genomic variation, mRNA expression and other data of various human cancers (including tumor subtypes), which is an important data source for cancer researchers. The expression of SPTAN1 gene was obtained using the Cancer Genome Atlas (TCGA) and matched with the adjacent tissues. Table 1 shows the types and cases of cancer in the TCGA dataset. We then used the GEPIA2 (http://gepia.cancer-pku.cn//) data site (based on the TPM data type in the TCGA database) to analyze SPTAN1 expression in 33 cancers. In addition to UVM without para-tumor samples, all other cancer types contained tumor tissue and para-tumor samples [13].

GEPIA2 database analysis

Gene Expression Profiling Interactive Analysis (GEPIA) (http://GEPIA.cancer-pku.cn//) tool was used to analyze target genes, compare their expression levels in different tumors with matched adjacent tissues, and identify the prognostic value of this gene in predicting PAAD outcomes. Subsequently, the expression of SPTAN1 in tumor tissue samples and para-cancer samples with statistical significance ($P<0.05$) was further analyzed with box diagram on GEPIA2 website.

TIMER2 database analysis

By TIMER2 database (http://timer.cistrome.org/) Immune Association components. Utilizing R package immunedeconv, the R guarantee integrates 6 state-of-the-art algorithms: TIMER, xCell, MCP-counter, CIBERSORT, EPIC, and quanTIseq [14]. To estimate the correlation between SPTAN1 and various immune infiltrations.

TISDB database analysis

TISIDB (http://cis.hku.hk/TISIDB/index.php), a variety of types of data in database integration the tumor immune resources [15]. We used this database to analyze the relationship between SPTAN1 and 45 immunostimulants, 24 immunosuppressants, 41 chemokines, and 18 receptors in PAAD.

Table 1 Cancer types evaluated from the TCGA dataset

Types of cancer	TCGA dataset	No. of cancer tissues	No. of normal tissues
Adrenocortical carcinoma	TCGA-ACC	77	128
Bladder Urothelial Carcinoma	TCGA-BLCA	404	28
Breast invasive carcinoma	TCGA-BRCA	1085	291
Cervical squamous cell carcinoma and endocervical adenocarcinoma	TCGA-CESC	306	13
Cholangio carcinoma	TCGA-CHOL	36	9
Colon adenocarcinoma	TCGA-COAD	275	349
Lymphoid Neoplasm Diffuse Large B-cell Lymphoma	TCGA-DLBC	47	337
Esophageal carcinoma	TCGA-ESCA	182	286
Glioblastoma multiforme	TCGA-GBM	163	207
Head and Neck squamous cell carcinoma	TCGA-HNSC	519	44
Kidney Chromophobe	TCGA-KICH	66	53
Kidney renal clear cell carcinoma	TCGA-KIRC	523	100
Kidney renal papillary cell carcinoma	TCGA-KIRP	286	60
Acute Myeloid Leukemia	TCGA-LAML	173	70
Brain Lower Grade Glioma	TCGA-LGG	518	207
Liver hepatocellular carcinoma	TCGA-LIHC	369	160
Lung adenocarcinoma	TCGA-LUAD	483	347
Lung squamous cell carcinoma	TCGA-LUSC	486	338
Mesothelioma	TCGA-MESO	87	0
Ovarian serous cystadenocarcinoma	TCGA-OV	426	88
Pancreatic adenocarcinoma	TCGA-PAAD	179	171
Pheochromocytoma and Paraganglioma	TCGA-PCPG	182	3
Prostate adenocarcinoma	TCGA-PRAD	492	152
Rectum adenocarcinoma	TCGA-PEAD	92	318
Sarcoma	TCGA-SARC	262	2
Skin Cutaneous Melanoma	TCGA-SKCM	461	558
Stomach adenocarcinoma	TCGA-STAD	408	211
Testicular Germ Cell Tumors	TCGA-TGCT	137	165
Thyroid carcinoma	TCGA-THCA	512	337
Thymoma	TCGA-THYM	118	339
Uterine Corpus Endometrial Carcinoma	TCGA-UCEC	174	91
Uterine Carcinosarcoma	TCGA-UCS	57	78

Cell experiment and clinical sample verification

Verification experiments on PAAD cells

Cell source and culture

The human PAAD cell line (BXPC3, CFPAC-1) was purchased from the Center for Typical Culture Preservation (Manassas, Virginia, USA). DMEM high glucose cell culture medium, penicillin–streptomycin mixture and fetal bovine serum were purchased from BI Limited. Human PAAD cell lines (BXPC3, CFPAC-1) were prepared with DMEM high-glucose complete medium containing 10% fetal bovine serum, and 1% double antibody was added to the complete medium. All cells were cultured at 37℃ in a constant temperature incubator containing 5%CO2 by volume, and passed every 3 days at a ratio of 1 : 2 or 1 : 3.

RNA Transfection

Small interference SPTAN1 targeting SPTAN1 was purchased from Genepharma Biotechnology (Shanghai, China), the transfection procedure was followed by Lipofectamine 2000 instructions, and SPTAN1 was used to transfect PAAD cells. The SPTAN1 sequences are as follows: SPTAN1-1:sense: GCUGGAAGAUUCCUAUCG A(dT)(dT); Antisense: UCGAUAGGAAUCUUCCAG C(dT)(dT); SPTAN1-2:sense: GGUGCAGUACUUACG AGAA(dT)(dT); Antisense: UUCUCGUAAGUACUG CACC(dT)(dT); SPTAN1-3:sense: GCAGAAGUUCAG

CGCUUUA(dT)(dT); Antisense: UAAAGCGCUGAA CUUCUGC(dT)(dT).

Flow cytometry

The effect of SPTAN1 on the cell cycle of PAAD was detected, the cell suspension was prepared and thoroughly mixed, and transferred into a 3.5 mL special flow tube. The flow cytometry of German Partec CyFlow@ space was used for detection. The samples stained by PI standard dyeing process were excited by 488 nm light source. To test the cell cycle, Results Using Muticycle function in FCsExpress4 software to fit, complete cell cycle maps were obtained, including G1 phase, G2/M phase and S phase cell ratio, respectively.

The effect of SPTAN1 on apoptosis of PAAD cells was detected. The transfected cells were collected, centrifuged, and counted. After centrifugation, cells were prepared into 1×10^5 suspensions using 200ul Binding Buffer, filtered and transferred to a sterile flow tube. 2ul FITC Annexin V Annexin V was added into the flow tube, gently mixed, then 2ulPI was added, and the samples were uniformly mixed and stained at room temperature (25℃) for 15 min without light. The samples were examined by flow cytometry or fluorescence microscopy within 1 h.

Wound healing assay

The effect of SPTAN1 knockdown on PAAD cell migration was studied using wound healing assay. First, the cells of each group were inoculated into a 6-well plate according to 3×105 cells per well, and transfected when the cell density reached 50%. After fluid exchange, the cell fragments were cleaned with PBS with a 200μL gun tip, then the serum-free medium was added, photographed under the microscope, and then placed in an incubator for further culture. The cell migration state was observed under microscope and photographed 24 h after transfection. The ratio of residual wound area to initial area was calculated and quantified by ImagePro Plus V 6.0 (Media Cybernetics, Bethesda, Maryland, USA).

Transwell invasion and migration assay

The working solution of Matrigel matrix glue with a mass concentration of about 200 μg/mL was prepared in advance, and was added to the upper chamber of the cell used in the invasion experiment at the rate of 100 μL/ well (not in the migration experiment), and incubated in the incubator at 37℃ for 30 min, waiting for the matrix glue to set. Transfected cells were inoculated into the upper compartment of Transwell at 5×104 / well. Serum-free medium was used in the upper compartment, and 650μL DMEM medium containing 20%FBS was added to the lower compartment. The transfected cells were cultured in an incubator for 24 h. The next day, the upper chamber was removed, the culture medium was sucked, the normal saline was washed three times, the cotton swab was gently wiped, and then the normal saline was washed for 5 ~ 10 min with 0.1% crystal violet. The field of vision was randomly selected under the microscope to take photos, and the number of cell migration in each group was calculated using ImageJ software, and the average number of membrane penetrating cells was calculated. The experiment was repeated three times.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the human PAAD cell line (BXPC3, CFPAC-1) using TRIzol reagent (Invitrogen No. 12183–555). mRNA is reversed-transcribed into cDNA using the reverse transcription reagent PrimeScript™ RT Master Mix(Perfect Real Time) (TAKARA item No. RR036A). According to the instructions, the qRT-PCR system was used for testing, and the reagent TB Green Premix Ex Taq II (Tli RNaseH Plus) (TAKARA No.: RR820A) was used for qRT-PCR analysis. The primers were synthesized by Shanghai Bioengineering Company, and the primers sequence was as follows: SPTAN1 follow: 5′- TACGAGAATGTGAGGACGTGA -3′ and SPTAN1 reverse: 5′- CATGAGCAGCCATATCTG TTTG -3′; Human GAPDH follow: 5′-AGAAGGCTG GGGCTCATTTG-3′ and Human GAPDH reverse: 5′-AGGGGCCATCCACAGTCTTC -3′;

Verification experiments on PAAD clinical samples

The PAAD Tissue Microarray (TMA) was obtained from the National Human Genetic Resources Sharing Service Platform (No. 2005DKA21300). The experimental procedure was approved by the Ethics Committee (No.: YB M-05–02) of Shanghai Core Biotech, which authorized the collection of tissue samples from Pancreatic adenocarcinoma patients. TMA included 90 Pancreatic adenocarcinoma tumor tissues, which also included 80 matched para-cancerous tissues. Informed consent forms were signed by all patients, who underwent pancreatectomy between January 2008 and August 2015. The clinicopathological information included age (year), sex, pathological grade, T stage, N stage, TNM stage, nerve invasion, vascular invasion, CA199, CA125, AFP, Ki67 and CEA. Among them, No data was no follow-up information.

Immunohistochemical staining evaluation

Immunohistochemical kit (EnVision™ FLEX+, Mouse, High pH, (Link) Brand:Dako K8002) immunohistochemical staining of PAAD TMA, in order to evaluate the expression of SPTAN1 in the sample tissues, was performed by two pathologists with more than 10 years of experience using the Aperio scanner (Aperio XT, Leica

Microsystems GmbH, magnification, × 200) Immunohistochemical staining was scored. The cell membrane/cytoplasm staining intensity and SPTAN1 staining positive rate of cancer tissue and matched adjacent tissue (epithelium) were interpreted, and the cell membrane and cytoplasm staining were scored respectively. The staining intensity score was 0 points (negative), 1 points (+), 2 points (++), 3 points (+++). Staining positive score: 0 points (negative), 1 points (1–25%), 2 points (26%-50%), 3 points (51–75%), 4 points (76%-100%). Cancer and adjacent cancer were scored by the product of staining intensity score and staining positive rate score. < 8 was divided into low antibody expression group, > =8 was divided into high antibody expression group.

Statistical analysis

Bioinformatics data analysis is done automatically using online databases (The two independent samples were tested by t-test instead of Wilcoxon rank sum test for non-parametric tests). The expression of SPTAN1 in PPAD specimens and matched paracancer tissues was analyzed using Fisher precision test and Chi-square test, and the relationship between SPTAN1 expression and clinicopathological features was evaluated. All data were represented by (± s), the comparison between two independent samples was conducted by independent sample t test, and the comparison between two or more subgroups was conducted by one-way analysis of variance. Log-rank p-value < 0.05 or p-value < 0.05 were considered statistically significant.

Results

Bioinformatics analysis

Expression of SPTAN1 in different databases

Firstly, in order to study the expression of SPTAN1 in different cancers and its clinical prognostic value, we conducted relevant bioinformatics analysis. The results showed that compared with normal tissues, SPTAN1 was highly expressed in a variety of human tumors, including diffuse large B-cell lymphoma (DLBC), acute myeloid leukemia (LAML), Pancreatic adenocarcinoma (PAAD), thymoma (THYM), and gastric adenocarcinoma (STAD) ($P < 0.05$, Fig. 1A). In the GEPIA database, compared with normal tissues, the expression of SPTAN1 protein in PAAD, CHOL, DLBC, LAML, STAD and THYM was significantly increased, while the expression of SPtan1 protein in LUAD, LUSC, GBM and TGCT was decreased (Fig. 1B).

High expression of SPTAN1 is associated with poor prognosis in patients with different cancers

We analyzed data from 178 patients with PAAD in the TCGA dataset. These patients were divided into low SPTAN1 expression group ($n = 89$) and high SPTAN1 expression group ($n = 89$). Low expression of SPTAN1 was significantly associated with improved survival ($P = 0.033$). Data from 364 PAAD patients in the TCGA dataset were also analyzed. The patients were divided into low SPTAN1 expression group ($n = 182$) and high SPTAN1 expression group ($n = 182$). Low expression of SPTAN1 was significantly associated with improved survival ($P = 0.0013$) (Fig. 1C).

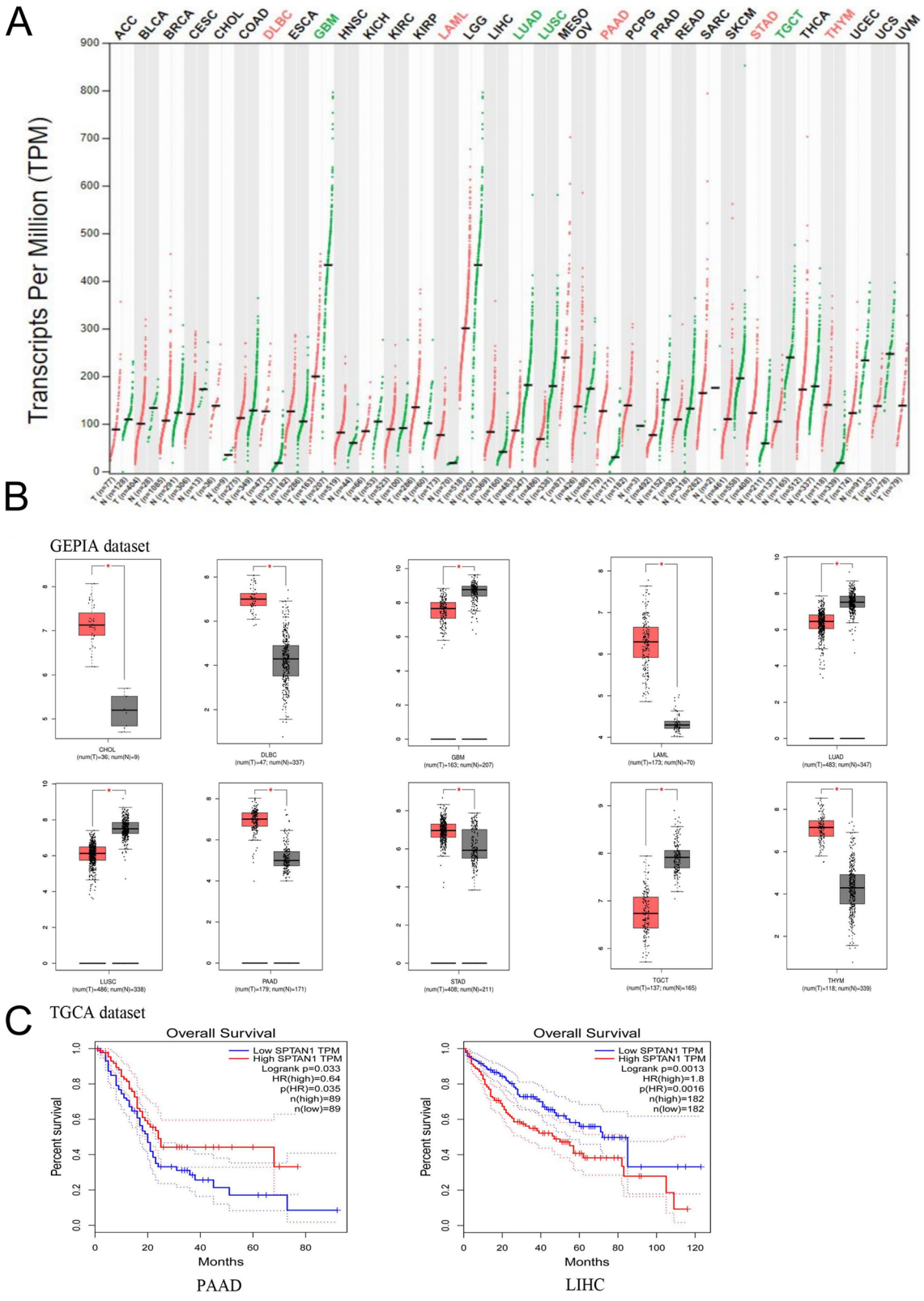
Correlation between immune infiltration and SPTAN1 expression in PAAD

Studies have found that immune infiltration plays a key role in the occurrence and development of tumors [16]. In PAAD, there was no significant change in the infiltration of immune cells at different SPTAN1 copy numbers (Fig. 2A).

TIMER database was used to evaluate the correlation between SPTAN1 expression and the degree of immune cell infiltration in PAAD, and a significant positive correlation was found between SPTAN1 expression and immune cells CD4 + T cells ($P = 1.29 \times 10^{-6}$) (Fig. 2B). In addition, associations between SPTAN1 expression and immune cell biomarkers were evaluated in patients with PAAD. They found a significant positive correlation between SPTAN1 and immune cell biomarkers in PAAD. Including CCR7, CD163, CD19, CD1C, CD4, CD79A, CD8A, CD8B, CEACAM8, HLA-DRA, IRF5, ITGAM, ITGAX, MS4A4A, NOS2, PTGS2, and VSIG4 (Fig. 2C).

(See figure on next page.)

Fig. 1 SPTAN1 expression profiles in cancer and normal tissues from the Cancer Genome Atlas. Note: **A** SPTAN1 was found in the tumor dataset (T; Red dots) and matching para-cancerous tissue (N; Green dots) expression in the data set. Each single point represents SPTAN1 expression in a single sample. Comparisons between tumors and normal tissues were performed using the GEPIA tool, and significantly elevated expression was identified by high log2FC values and percentage values greater than the threshold. The cancer type shown in red has significantly higher SPTAN1 expression than the corresponding normal tissue. **B** through GEPIA SPTAN1 in PAAD protein were measured data set CHOL, DLBC, LAML, STAD, THYM, LUAD, LUSC, expression of GBM and TGCT. **C** with Kaplan Meier plotter plot of different SPTAN1 in cancer survival. HR: hazard ratio *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$



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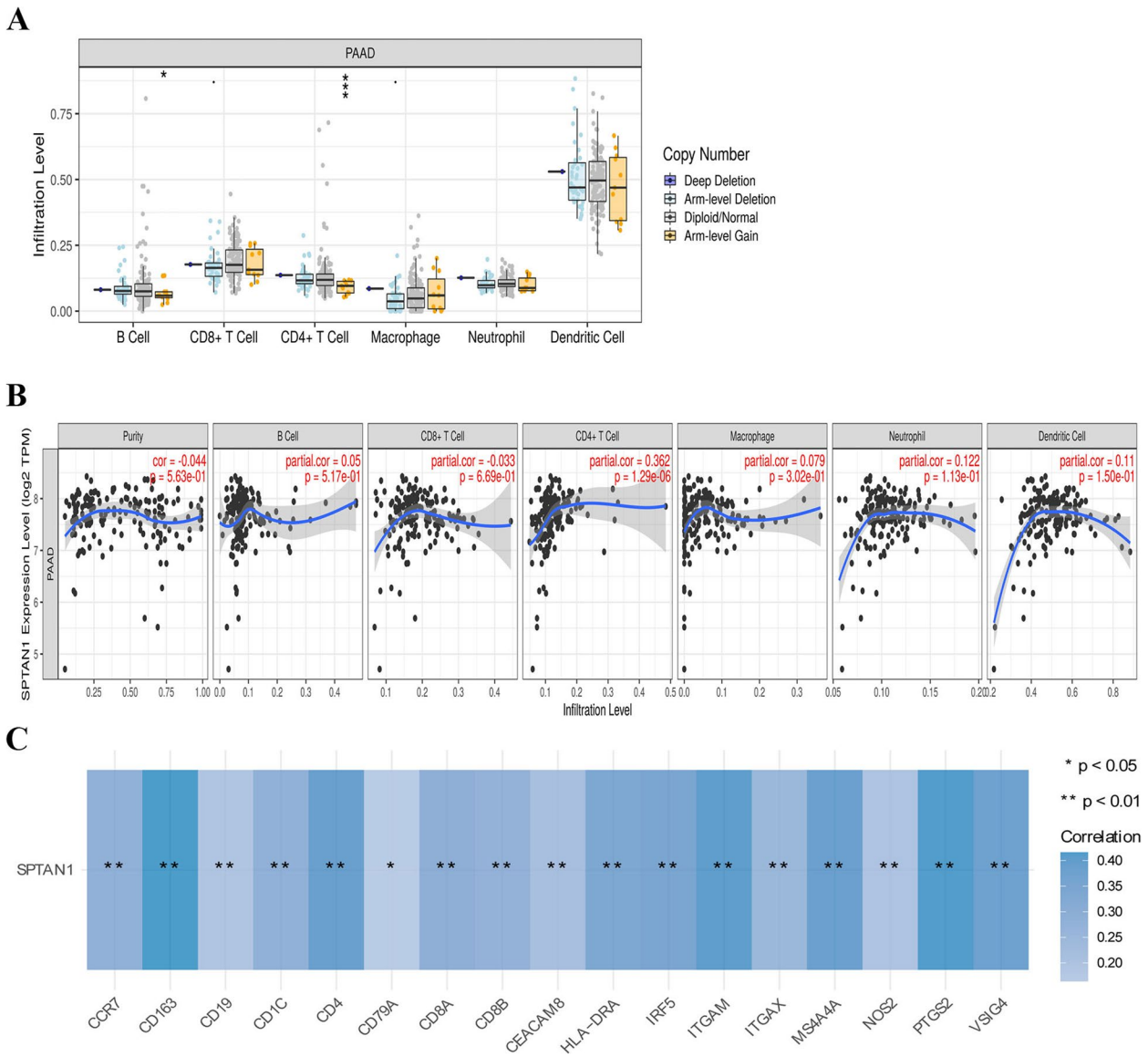

Fig. 2 Correlation between immune infiltration and SPTAN1 expression in PAAD. Note: **A** Infiltration levels of various immune cells in PAAD with different copy numbers of SPTAN1. **B** Correlation between SPTAN1 expression levels in PAAD and infiltration levels of B cells, CD8 + T cells, CD4 + T cells, macrophages, neutrophils, or dendritic cells. **C** Correlation analysis of SPTAN1 and PAAD immune cell biomarkers. *, $P < 0.05$; **, $P < 0.01$

Relationship between SPTAN1 expression and immunomodulators in PAAD

Studies have found that immunomodulators are involved in influencing various functions of the immune system [17]. SPTAN1 was significantly associated with a number of immunosuppressants, including ADORA2A, TGFB1, TGFBR1, CD160, and LAG3 ($P < 0.05$) (Fig. 3A). In addition, the expression of SPTAN1 is closely related to the following immune-stimulating factors: CD276, TMEM173, TNFRSF4, ICOSLG, TNFRSF25, TNFSF15, KLRK1, and NT5E ($P < 0.05$) (Fig. 3B) suggested that SPTAN1 might be involved in regulating the escape process of tumor immunity.

Relationship between SPTAN1 expression and chemokines in PAAD

Studies have found that chemokines play an important role in the initiation and stabilization of the immune system, and are involved in almost all destructive or protective immune and inflammatory response processes [18]. The expression of SPTAN1 was significantly correlated with chemokines CCL4, CXCL16 and CCL7 ($P < 0.05$) (Fig. 4A). In addition, we further investigated

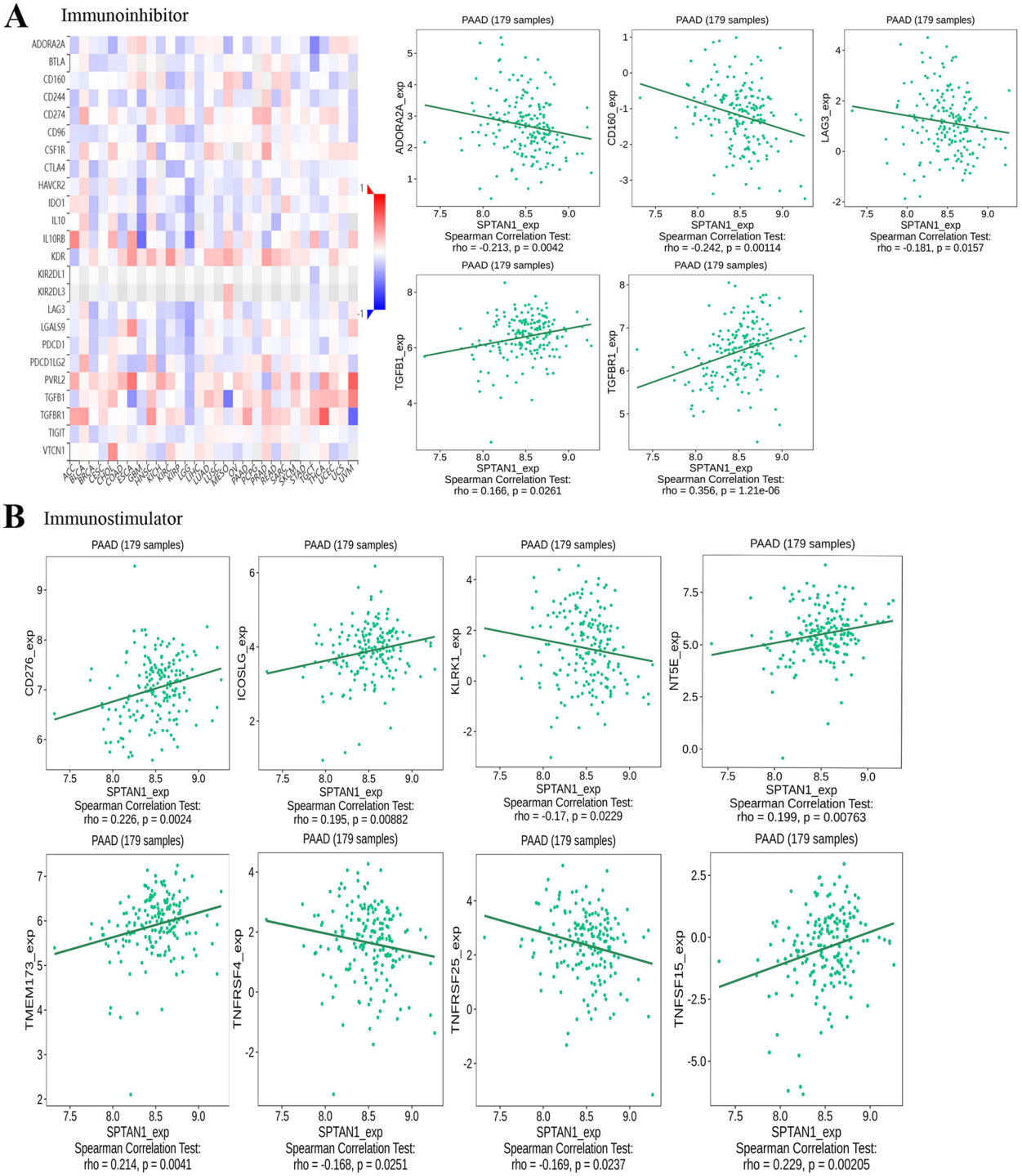

Fig. 3 TISDB database showed the correlation between SPTAN1 expression and immunomodulators in Pancreatic adenocarcinoma. Note: **A** immunosuppressants and **B** immunostimulants

the correlation between SPTAN1 and chemokine receptor expression. The results showed that the expression of SPTAN1 was significantly correlated with CCR8 and CCR9 ($P<0.05$) (Fig. 4B). These results further indicate that SPTAN1 is involved in the immunomodulatory process of PAAD.

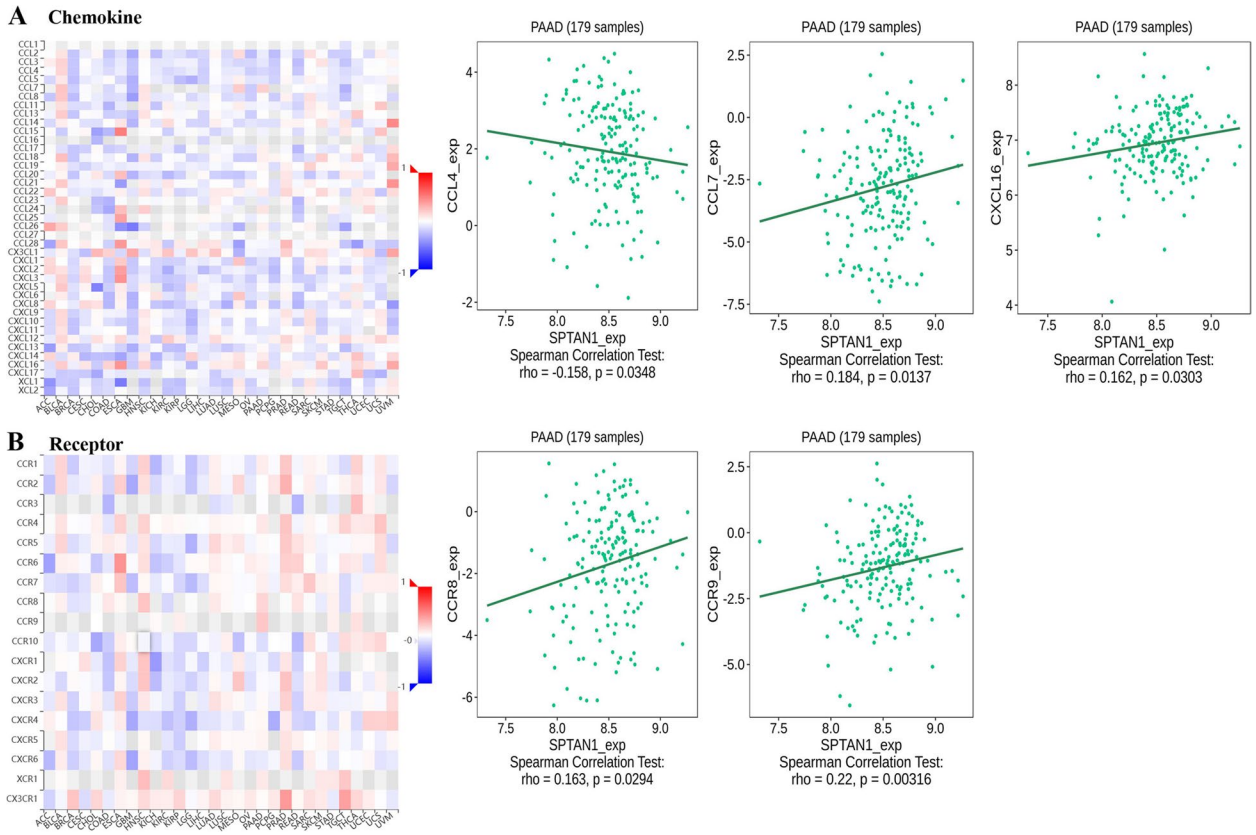

Fig. 4 In the TISDB database, the correlation between the expression of SPTAN1 and chemokines and receptors in Pancreatic adenocarcinoma was shown. Note: **A** chemokines, **B** chemokine receptors

Pancreatic adenocarcinoma cells were verified in vitro

Quantitative PCR results showed that SPTAN1 had the highest expression level in BXPC-3, HPDE6-C7 and CFPAC-1 (*, $P<0.05$) (Fig. 5A). Therefore, we selected cell lines BXPC-3 and CFPAC-1 for related experimental studies. In addition, after si-SPTAN1 transfection, compared with siCtrl group, the mRNA expression of SPTAN1 gene at the target sites of si-Sptan1-1, si-SPTAN1-2 and si-SPTAN1-3 groups was significantly inhibited (*, $P<0.05$), and the knockdown efficiency reached 71%,48% and 13% (Fig. 5B).

SPTAN1 promotes the migration of PAAD cells

To investigate whether SPTAN1 promotes PAAD migration, we used SPTAN1 to knock down SPTAN1 expression in BXPC-3 and CFPAC-1 cells. After transfection, SPTAN1 KD1 and SPTAN1 KD2 significantly inhibited PAAD cell migration after 24 h compared to the KD-NC group. However, there was no significant difference in migration between the SPTAN1 KD1 and SPTAN1 KD2 groups. Thus, these results suggest that SPTAN1 promotes PAAD cell migration in vitro (Fig. 5C).

Effect of SPTAN1 knock-down on migration and invasion of PAAD cells in vitro

Transwell assay was used to investigate the effect of SPTAN1 knock-down on the migration and invasion ability of BXPC-3 and CFPAC-1 cells. Compared with the KD-NC group, cell migration and invasion were significantly reduced in SPTAN1KD1 and SPTAN1KD2 groups, while there was no difference between SPTAN1KD1 and SPTAN1KD2 groups. These results demonstrated that SPTAN1 knock-down significantly inhibited the migration and invasion of Pancreatic adenocarcinoma cells in vitro (Fig. 5D, E).

Effect of knocking down SPTAN1 on apoptosis and cell cycle of PAAD in vitro

Flow cytometry was used to detect the effect of knock-down SPTAN1 on apoptosis of BXPC-3 and CFPAC-1 cells. Compared with the KD-NC group, apoptosis was significantly increased in SPTAN1KD1 and SPTAN1 KD2 groups. There was no significant difference in apoptosis between SPTAN1 KD1 and SPTAN1KD2 groups. These results suggested that SPTAN1 knock-down promoted apoptosis of PAAD cells in vitro (Fig. 6A, B). In

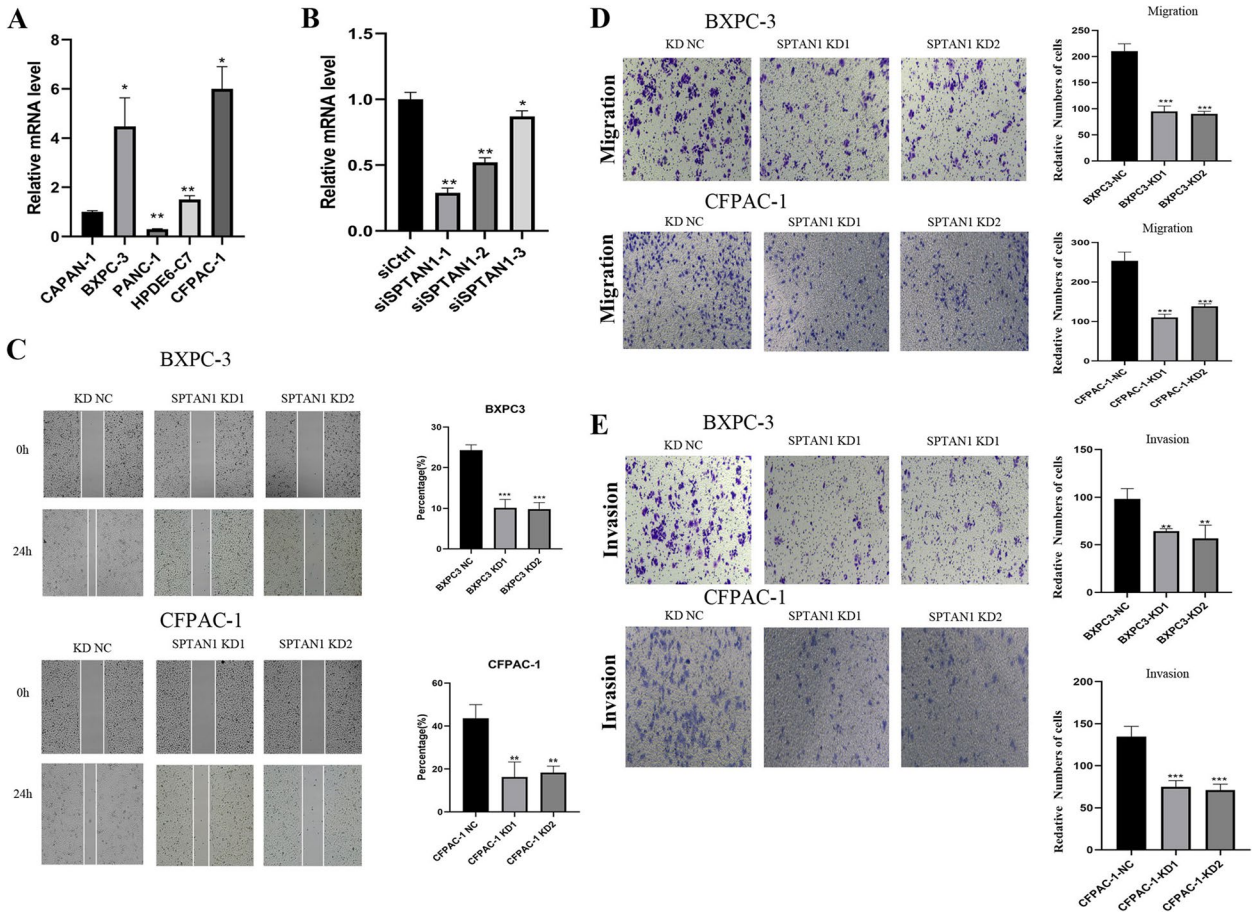

Fig. 5 Effect of SPTAN1 knock-down on migration and invasion of PAAD cell lines. Note: **A** The expression of SPTAN1 was the highest in BXPC-3 and CFPAC-1. **B** The mRNA expression level of SPTAN1 gene at SPTAN1-1 and SPTAN1-2 groups was significantly inhibited. **C** PAAD cell migration was significantly reduced after SPTAN1 knock-down. Wound healing measurements were performed at 24 h in BXPC-3 and CFPAC-1 cells, using the gap width at 0 h for each group as a reference. **D**, **E** Compared with the migration and invasion of KD-NC, the migration and invasion ability of cells in SPTAN1KD1 and SPTAN1KD2 groups were significantly decreased, while there was no significant difference between SPTAN1KD1 and SPTAN1KD2 groups. (*, $P<0.05$; **, $P<0.01$; ***,$P<0.001$)

addition, the effect of SPTAN1 knockdown on the cell cycle of BXPC3 and CFPAC-1 was examined. Compared with the KD-NC group, knockdown of SPTAN1 significantly increased the number of G1/G0 phase cells in SPTAN1KD1 and SPTAN1KD2 groups, and decreased the number of G2 phase cells, while there was no significant difference in cell cycle between SPTAN1KD1 and SPTAN1KD2 groups (Fig. 6C, D).

Expression of SPTAN1 in PAAD clinical samples

We collected 86 PAAD clinical samples and matched paracancer tissue samples in the hospital. Then, we used immunohistochemical staining to detect the expression level of SPTAN1 in PAAD clinical samples and matched paracancer tissue samples (Fig. 7A). Micrograph of SPTAN1 immunohistochemical staining in tissue samples from PAAD clinical patients.

Correlation between the expression level of SPTAN1 and clinicopathological features of PAAD patients

The expression level of SPTAN1 was correlated with M stage ($P=0.004$) and CA199 stage ($P=0.012$). However, the expression of SPTAN1 was not significantly correlated with other clinicopathological features, such as CEA, CA125, Ki67, etc. (Table 2).

Relationship between SPTAN1 expression and overall survival rate

Kaplan–Meier analysis was used for univariate survival analysis. The results showed that the overall survival rate of high SPTAN1 expression group was significantly lower than that of low SPTAN1 expression group ($P=0.043$; Fig. 8). Therefore, it suggests that there is a significant correlation between high expression of SPTAN1 and poor prognosis of PAAD, and further suggests that

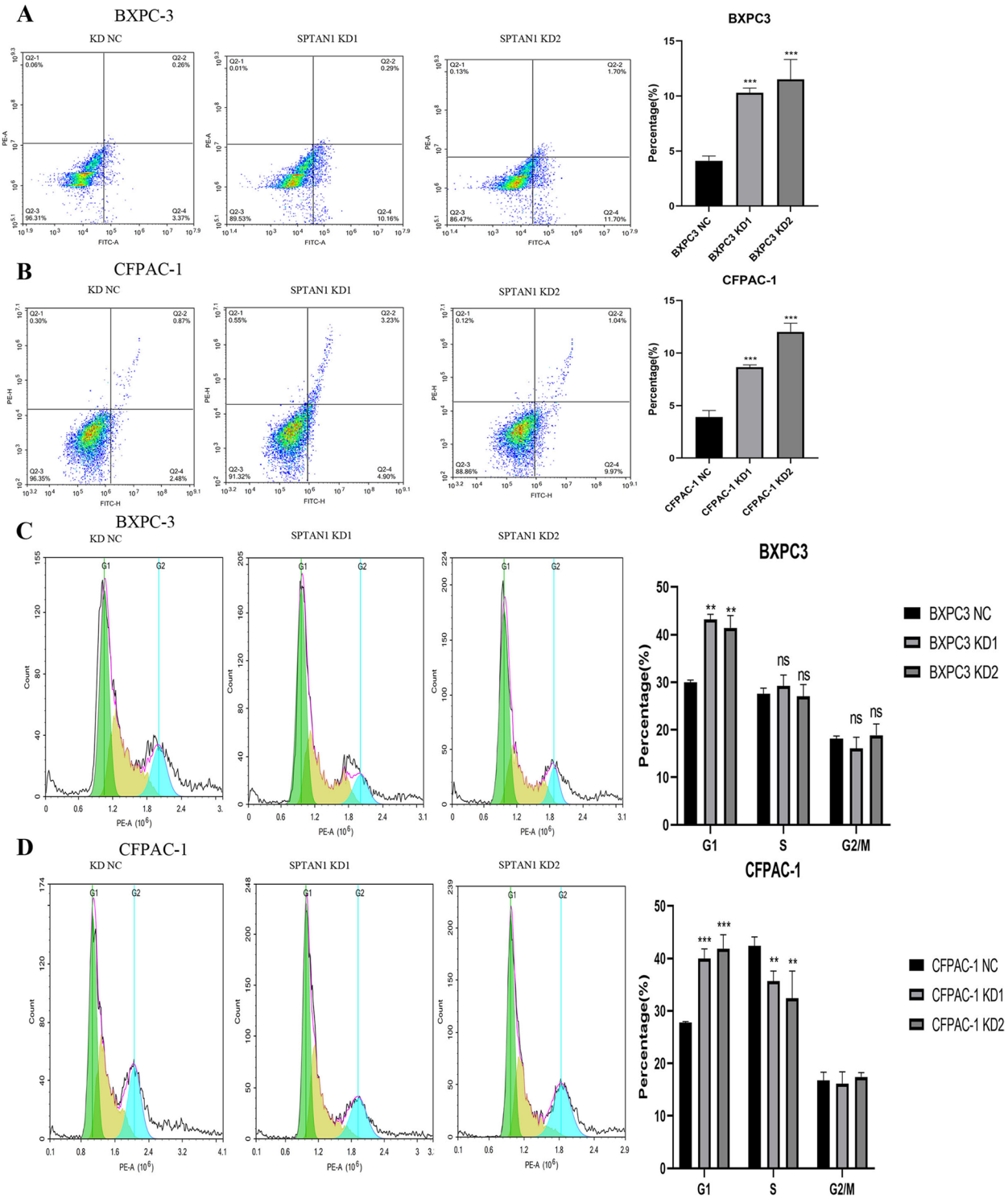

Fig. 6 Effect of SPTAN1 knock-down on apoptosis and cycle of PAAD cell line. Note: **A**-**B** In BXPC-3 and CFPAC-1 cells, compared with the KD-NC group, the apoptosis of SPTAN1KD1 and SPTAN1KD2 cells was significantly increased, but there was no significant difference between SPTAN1KD1 and SPTAN1KD2 cells. **C**-**D** When detecting the cell cycle, the number of cells in G1/G0 phase increased in SPTAN1KD1 and SPTAN1KD2 group and decreased in G2 phase in BXPC-3 and CFPAC-1 cells compared with KD-NC group. There was no significant difference in cell cycle between SPTAN1KD1 and SPTAN1KD2 groups (**, $P < 0.01$; ***, $P < 0.001$)

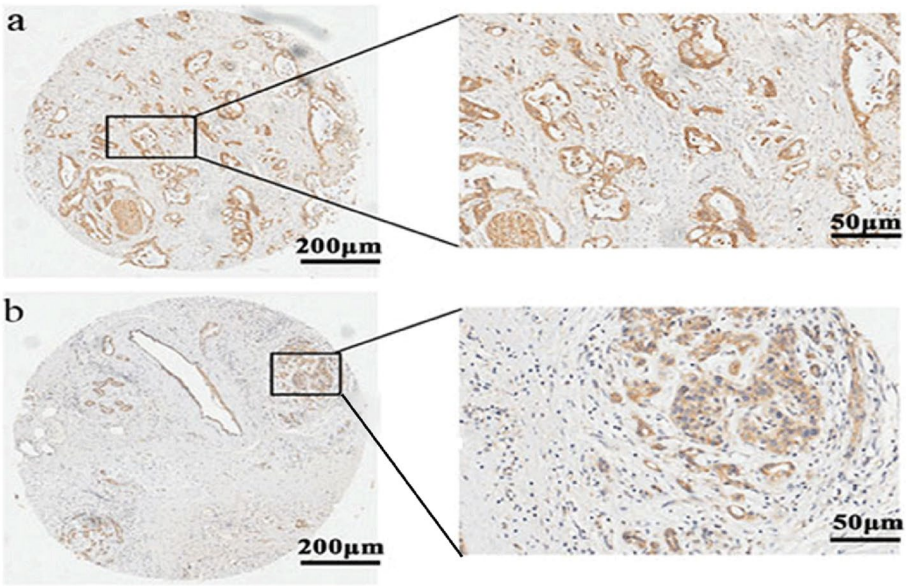

Fig. 7 Micrograph of SPTAN1 immunohistochemical staining in tissue samples from PAAD clinical patients. Note: **A** SPTAN1 positive staining. **B** SPTAN1 negative staining

SPTAN1 may be an independent prognostic factor for the prognosis of PAAD patients.

Discussion

Pancreatic adenocarcinoma is the most malignant digestive system tumor, and its incidence is on the rise, which is one of the main causes of cancer-related death [13]. The prognosis of Pancreatic adenocarcinoma is very poor, the five-year survival rate is less than 5%, and surgery is the only treatment method, and the difficulty in early diagnosis leads to a low surgical rate. Therefore, intravenous chemotherapy, immunotherapy and other comprehensive treatments are particularly important, but the overall efficacy is very limited at present [19]. In recent years, with the continuous update of minimally invasive techniques and surgical AIDS, the quality and safety of Pancreatic adenocarcinoma surgery have been greatly improved. In addition, the application of genomic detection in metastatic Pancreatic adenocarcinoma is also increasing, and the analysis from its biological perspective can enable clinicians to better discover potential therapeutic targets. Similarly, targeted drugs such as PARP inhibitors and immune checkpoint inhibitors have also appeared one after another and achieved good results [20].

SPTAN1 is a cytoskeletal scaffold protein. When cross-linked with filamentous actin, SPTAN1 is involved in various cellular processes, including cell adhesion and DNA repair. Studies have shown that SPtan1 is related to the migration and metastasis of tumor cells [21]. Christopher et al. reported that SPTAN1 was significantly correlated with the development and progression of rectal cancer. Through in vitro cell experiments, it was found that cells knocking down SPTAN1 were not sensitive to chemotherapy [7]. In this study, we used TCGA, GTEx and other data sets to conduct relevant bioinformatics analysis to analyze the expression of SPTAN1 in pancancer cells. It was found that the expression of SPTAN1 in PAAD was significantly increased at both mRNA and protein levels compared with normal samples, and SPTAN1 was also associated with the prognosis of PAAD. The results were verified in vitro cell experiments and clinical tissue samples from PAAD patients, and our results all support the cancer-promoting effect of SPTAN1 in PAAD.

In recent years, immunotherapy has made great achievements in solid malignant tumors, but Pancreatic adenocarcinoma is considered to be a non-immunogenic tumor, most tumor cells can escape the recognition of host immune cells, and regulate immune response and immune tolerance to evade immune surveillance and promote disease progression. Our study showed that SPTAN1 expression was significantly positively correlated with immune cell CD4+T cells and with most immune cell biomarkers in PAAD, suggesting that SPTAN1 may be involved in the immune regulation of PAAD.

Immune stimulants and immunosuppressants regulate the degree of immune response, and these receptors and ligands are called immune checkpoints [22]. Studies have found that immunotherapy using checkpoint inhibitors can significantly change the treatment of Pancreatic

Table 2 Relationship between SPTAN1 expression and clinicopathological characteristics in PAAD patients ($n=86$)

Clinicopathoogical characteristics	variables	SPTAN1 expression		total	χ2	*p* value
		low	high			
Age (year)	< = 55	25	4	29	2.195	0.138
	> 55	41	16	57		
Age (year)	< = 60	33	11	44	0.154	0.695
	> 60	33	9	42		
Sex	Female	24	11	35	2.209	0.137
	Male	42	9	51		
Grade	I	9	1	10	1.535	0.464
	II	53	17	70		
	III	4	2	6		
T stage	I	5	0	5	3.383	0.184
	II	30	8	38		
	III-IV	30	12	42		
	No data			1		
N stage	N0	27	5	32	2.078	0.354
	N1	29	12	41		
	N2	9	2	11		
	No data			2		
M stage	M0	54	10	64	8.162	**0.004***
	M1	12	10	22		
	No data			0		
TNM stage	I	21	3	24	4.774	0.092
	II	23	5	28		
	III-IV	22	12	34		
	No data			0		
Vasculature violations	NO	38	11	49	0.177	0.674
	YES	25	9	34		
	No data			3		
Invasion of nerve	NO	22	6	28	0.164	0.685
	YES	41	14	55		
	No data			3		
CEA	negative	44	12	56	0.416	0.519
	Positive	18	7	25		
	No data			5		
CA125	negative	29	14	43	0.68	0.41
	Positive	17	5	22		
	No data			21		
CA199	negative	9	8	17	6.278	0.012*
	Positive	51	11	62		
	No data			7		
Ki67	I	8	3	11	0.062	0.97
	II	13	6	19		
	III-IV	14	6	20		
	No data			36		

*Statistically significant ($p<0.05$)

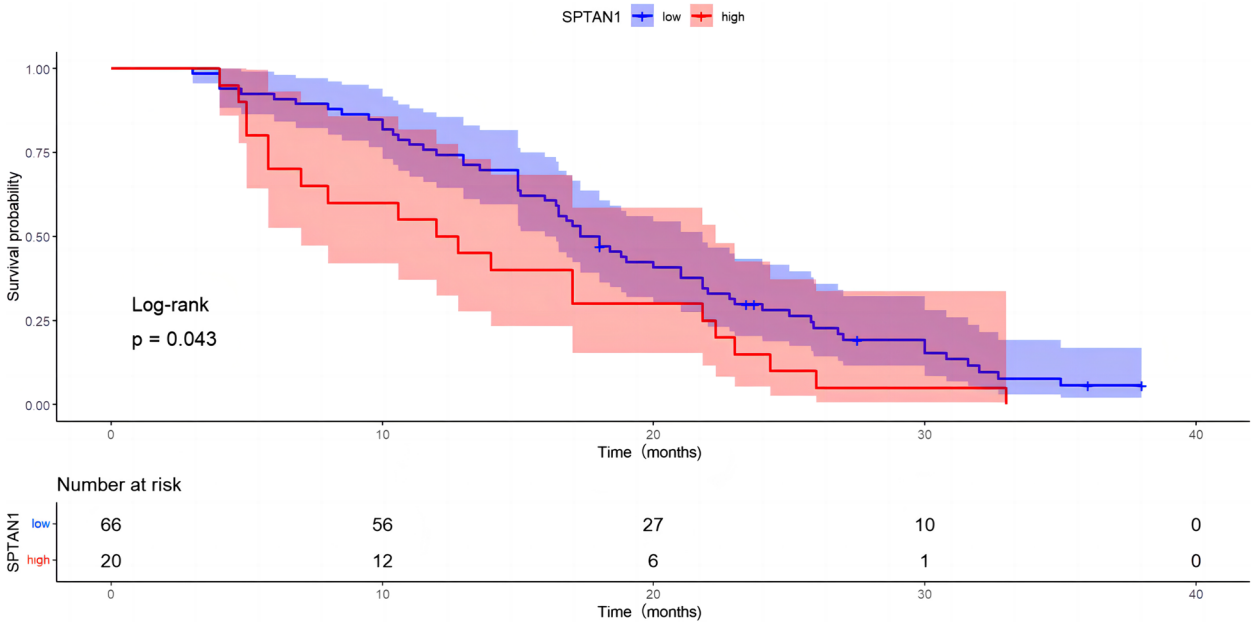

Fig. 8 Kaplan–Meier analysis of PAAD patients. Effect of SPTAN1 protein expression on overall survival. Note:Immunohistochemical staining of PAAD tissue microarray was used to determine the expression level of SPTAN1. SPTAN1, non-red genealogical protein αii; PAAD: Pancreatic adenocarcinoma

adenocarcinoma [23]. We found that SPTAN1 was significantly associated with immunosuppressants and immunostimulants in PAAD. In addition, the expression of SPTAN1 is significantly associated with many chemokines and chemokine receptors, suggesting that SPTAN1 may not only be involved in immune regulation in Pancreatic adenocarcinoma, but that these molecules may also be potential immunotherapeutic targets for SPTAN1 in PAAD. Therefore, how to improve the immune microenvironment of Pancreatic adenocarcinoma, transform non-immunogenic tumors into immunogenic tumors, and improve immune non-response and tolerance is also a new choice for Pancreatic adenocarcinoma treatment.

In addition, the above related results were verified in vitro cell experiments and clinical tissue samples from PAAD patients, and our findings all support the cancer-promoting effect of SPTAN1 in PAAD. The expression of SPTAN1 in PAAD samples was significantly higher than that in the matched para-cancerous tissues from patient samples. In vitro cell experiments, the knock-down of SPTAN1 inhibited the migration and invasion ability of PAAD cell lines, promoted the apoptosis of the cell lines, and also affected the changes of the cell cycle. In addition, immunohistochemical staining of clinical tissue samples showed that high expression of SPTAN1 was significantly correlated with M stage and CA199. Survival analysis showed that the overall survival rate of PAAD patients with high SPTAN1 expression was significantly lower than that of patients with low SPTAN1 expression. These results all suggest that the expression level of SPTAN1 in PAAD patients may be important in predicting the prognosis of PAAD patients.

In addition, studies have shown that SPTAN1 is positively correlated with the proliferation, migration and invasion of gastric, bladder and lung cancers, but the specific mechanism of SPTAN1 is still under continuous study [11, 12]. In this study, the expression and prognosis of SPTAN1 in PAAD were evaluated by bioinformatics analysis, as well as the relationship between SPTAN1 expression and PAAD cell line migration, invasion, apoptosis, and cell cycle, all of which were verified in vitro cell experiments. Finally, we performed immunohistochemical analysis of SPTAN1 expression in clinical PAAD samples to explore whether SPTAN1 can be an independent prognostic factor for PAAD.

There are still major limitations to the current study, for one thing, most databases are still being updated and expanded, which could affect the results. Second, when exploring the relationship between SPTAN1 expression levels and clinicopathological features, incomplete clinicopathological data collection may confound the results. In addition, these results still need to be validated in large-scale clinical trials at home and abroad.

Conclusions

In conclusion, our findings suggest that SPTAN1 expression in PAAD is significantly increased and may be an independent predictor of poor prognosis in PAAD. The expression of SPTAN1 is positively correlated with immune cell infiltration, immunomodulators, chemokines and their receptors, which further indicates that SPTAN1 plays a carcinogenic role by regulating immune process. After further continuous research, SPTAN1 may be used for early diagnosis of PAAD patients and become a prognostic marker, thereby improving the survival rate of PAAD patients.

Abbreviations

PAAD	Pancreatic adenocarcinoma
SPTAN1	Nonerythroid spectrin αII
TCGA	The CancerGenome Atlas
GTEx	The Genotype-Tissue Expression
GEPIA	Gene Expression Profiling Interactive Analysis
qRT-PCR	Quantitative real-time PCR

Authors' contributions

W. Guo designed this study and drafted the manuscript. L. Hu collected samples. Z. Gao and X. Liu performed the experiments and analyzed the results. X. Yang and X. Wang provided the conceptualization and methodology. All authors contributed to the manuscript and approved the submitted version.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

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