Integrative analysis of TNFRSF6B as a potential therapeutic target for pancreatic cancer

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Background: Pancreatic cancer is one of the most lethal malignant tumors worldwide with poor outcomes. Previous studies have shown that *tumor necrosis factor receptor superfamily member 6b* (*TNFRSF6B*) plays an important role in cancer progression and immunosuppression. However, the mechanisms by which *TNFRSF6B* influence pancreatic cancer, and the regulatory networks involved remain to be further studied.

Methods: This study analyzed the mRNA information and clinical data of patients from The Cancer Genome Atlas (TCGA) and the ONCOMINE databases. The gene co-expression data regarding *TNFRSF6B* was obtained from the c-BioPortal and used to explore the functional network of *TNFRSF6B* in pancreatic cancer, as well as its function in tumor immunity. Short hairpin (sh) RNA knock-down experiments were performed to examine the functional roles of *TNFRSF6B* in pancreatic cancer cell lines.

Results: The expression of *TNFRSF6B* was elevated in pancreatic cancer tissues compared to normal pancreatic tissues, and its high expression was associated with poor prognosis of patients with pancreatic cancer. *TNFRSF6B* was found to be widely involved in cell cycle processes, apoptosis, apoptosis signaling pathways, immune responses, and responses to interferon. Knock-down of *TNFRSF6B* expression inhibited pancreatic cancer cell proliferation and invasion *in vitro*. Moreover, *carcinoembryonic antigen-related cell adhesion molecule 1* (*CEACAM1*) was found to be co-expressed with *TNFRSF6B*, and there was a positive correlation between these molecules in pancreatic cancer cells.

Conclusions: This report suggested that *TNFRSF6B* has a critical role in the progression and metastasis of pancreatic cancer. These findings provide novel insights into the role of *TNFRSF6B* in the functional network of pancreatic cancer, and suggest that *TNFRSF6B* may be a potential therapeutic target.

Keywords: *TNFRSF6B*; pancreatic cancer; functional networks; tumor immunity

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Introduction

Pancreatic cancer is one of the most common gastrointestinal malignant tumors in the world. It accounts for 5% of cancer-related deaths and is recognized as the

fourth leading cause of cancer-related mortality (1). This is largely due to late diagnosis, low resection rates, and high drug resistance to chemotherapy (2). Diagnosis of the disease is exceedingly difficult due to a lack of

early symptoms and the malignancy can spread rapidly to surrounding organs. At diagnosis, more than 70% of patients present with advance stage disease or distant metastases, making surgical treatment difficult. Invasion and metastasis are the most important characteristics of pancreatic cancer, and thus, there is an urgent need to understand the molecular mechanisms involved in these processes.

In recent years, secretory proteins have increasingly gained prominence in the fields of tumor invasion, metastasis, and drug resistance (3,4). Pancreatic cancer cells can produce a large number of secretory proteins, which promote extracellular matrix (ECM) degradation and tumor metastasis (5). These tumor-specific proteins play an important role in many cellular functions, including signal transduction and interaction with plasma membrane (6,7). *TNFRSF6B* is a soluble secretory protein which lacks the transmembrane structure in its amino acid sequence (8). The overexpression of *TNFRSF6B* protein may block apoptosis and immune monitoring by competitively binding with first apoptosis signal ligand (FasL) (9), tumor necrosis factor-like cytokine 1A (TL1A) (10), or lymphotoxin analogue (LIGHT, also known as TNFSF14) (11). Therefore, *TNFRSF6B* is also known as *decoy receptor 3 (DcR3)*. Previous literature demonstrated that *TNFRSF6B* is highly expressed in human tumors such as lung cancer (12,13), gastric cancer (14), pancreatic cancer (15,16), and colon cancer (17,18), and it has been reported that overexpression of DcR3 is correlated with shortened total survival time of cancer patients (19). Our previous study found that *TNFRSF6B*-mediated signal transduction pathways play an important role in regulating proliferation, apoptosis, migration, and invasion of pancreatic cancer cells (20). *TNFRSF6B* is the key node in the regulatory network for the occurrence and development of pancreatic cancer, however, its regulatory relationship is complex, and the precise mechanisms are still unclear.

This report investigated *TNFRSF6B* expression in pancreatic cancer using databases including The Cancer Genome Atlas (TCGA), the Gene Expression Profiling Interactive Analysis (GEPIA), and ONCOMINE. Through multidimensional analyses, the functional networks and potential relationships of *TNFRSF6B* in pancreatic cancer, and its role in tumor immunity were evaluated. This study provides novel insights into potential therapeutic targets and strategies for the treatment of pancreatic cancer. We present the following article in accordance with the MDAR checklist (available at [https://](https://dx.doi.org/10.21037/jgo-21-303)

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Methods

Acquisition of patients and data

All mRNA information and clinical data of patients were obtained from the University of California, Santa Cruz (UCSC) website (<https://xena.ucsc.edu/>). The cBioPortal website (<https://www.cbioportal.org/>) was search to obtain the co-expression gene data related to *TNFRSF6B*. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Since all data were extracted from publicly available databases, approval by the ethics committee and patient consent were waived.

TCGA database analysis

To provide comprehensive analysis of cancer genome profiles, the TCGA applies high-throughput technologies based on microarrays and next-generation sequencing methods. The research network structure includes many centers utilizing different platforms to provide global information related to cancer genomics.

ONCOMINE database analysis

The levels of *TNFRSF6B* gene expression in various types of cancers were identified in the ONCOMINE database (<https://www.oncomine.org/resource/login.html>) (21). The threshold applied was a P value of 1E-4, fold change of 2, and gene ranking of 10%.

GEPIA database analysis

The Gene Expression Profiling Interactive Analysis (GEPIA) database (<http://gepia.cancer-pku.cn/>) is an interactive database that includes 9,736 tumors and 8,587 normal samples from the TCGA and the Genotype-Tissue Expression (GTEx) projects (22). GEPIA was used to generate survival curves based on gene expression with the log-rank test.

TIMER database analysis

TIMER (<https://cistrome.shinyapps.io/timer/>) is a comprehensive resource for systematic analysis of immune infiltrates across diverse cancer types from the TCGA,

which includes 10,897 samples across 32 cancer types (23).

The correlation between *TNFRSF6B* expression and the abundance of immune infiltrates, including macrophages, neutrophils, dendritic cells, B cells, CD4⁺ T cells, and CD8⁺ T cells was analyzed.

GeneMANIA analysis

GeneMANIA (<http://www.genemania.org>) is a flexible, user-friendly web interface for constructing protein-protein interaction (PPI) networks, generating hypotheses related to gene function, analyzing gene lists, and prioritizing genes for functional assays (24). The website can set the source of the edge of the network, and it features several bioinformatics methods including physical interaction, gene co-expression, gene co-location, gene enrichment analysis, and website prediction. GeneMANIA was used to visualize the gene networks.

Construction of the co-expression network

The co-expression gene data of *TNFRSF6B* was downloaded from c-BioPortal and used to identify genes with Spearman's Correlation greater than 0.4 and less than -0.4. A total of 1,143 genes were extracted from 168 patients with complete clinical data. To further analyze the relationship between these co-expression genes, the R packet WGCNA (weighted gene co-expression network analysis) was used to construct a co-expression network. The "pickSoftThreshold" function was used to select the best soft threshold, and finally the soft threshold was determined to be 11. The minimum number of genes in the module was defined as 30.

Identification of the hub module and functional annotation

The correlation between ME (module eigengenes) and the clinical information of each module was analyzed. The modules closely related to prognosis were selected. The relationship between different modules and age, gender, T stage, N stage, TNM stage, overall survival (OS) time, disease-specific survival (DSS) time, progression-free survival (PFI) time, and disease-free interval (DFI) was studied. At the same time, an in-depth analysis of the genes in the key modules was performed. CLUEGO was used for KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) enrichment analysis of genes in key modules to explore possible pathways and molecular mechanisms. GeneMANIA (<https://genemania.org/>) was

used to construct the PPI network of 10 hub genes and *TNFRSF6B*.

Immune cells and immune checkpoint genes

Single-sample gene set enrichment analysis (SsGESA) was performed to estimate the status of immune cells in patients with pancreatic cancer. The Tumor Immune Estimation Resource (TIMER) website (<http://timer.cistrome.org/>) was used to evaluate the infiltration of six different kinds of immune cells in patients with pancreatic cancer. Furthermore, the expression of immune checkpoint genes in the TCGA cohort was investigated.

Short hairpin (sh) RNA lentiviral plasmids construction

A shRNA sequence targeting *TNFRSF6B* (GenBank: NM-003823) (5'-CGCTGGTTTCTGCTTGGAGCAC-3') was subcloned into a lentiviral vector (sh-*TNFRSF6B*), and a lentiviral vector containing a random sequence was used as a negative control (sh-NC). The viral supernatant was added at a multiplicity of infection (MOI) of 5 per well with 5 mg/mL polybrene.

Microarray and data analysis

Sh-*TNFRSF6B* cells (n=3) and sh-NC cells (n=3) were collected for total RNA extraction. The Affymetrix gene chip array (Thermo Fisher Science, Massachusetts, USA) was used to detect the genome-wide expression changes in the extracted RNA samples according to the manufacturer's instructions. Differential genes were screened according to P value <0.05 and absolute fold change >2. The ingenuity pathway analysis (IPA) software was used to classify differentially expressed genes into specific biological functions and pathways related to *TNFRSF6B* genes.

Clone formation assay

Sh-*TNFRSF6B* and sh-NC cells were seeded into 6-well plates in a density of 500 cells/well. Then they were placed into the constant temperature chamber in an atmosphere of 5% CO₂ at 37 °C for 2 weeks incubation with media changes every 2 or 3 days. Cells were then fixed with 4% paraformaldehyde and stained with 1% crystal violet. Cell colonies containing at least 20 cells were counted using an inverted microscope at 40× magnification. The experiments were performed three times.

Cell invasion assay

Cell migration assays were performed using 8- μ m Transwell® cell culture chambers (Corning, NY, USA, pore size of 8 μ m). Cells (5×10^5 cells/mL) were resuspended in serum-free Dulbecco's Modified Eagle Medium (DMEM) and seeded into the upper well of the Transwell chamber. The well below the Transwell chamber was filled with DMEM supplemented with 10% fetal bovine serum (FBS). After incubation for 48 hours at 37 °C, cells that had migrated to the bottom surface of the chamber were fixed with methanol and stained with 0.1% crystal violet. The membranes containing the cells were then imaged under light microscopy and the number of cells from at least five random fields of view were counted.

Western blot

Total proteins were extracted from cells with lysis buffer. Protein samples were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly vinylidene fluoride (PVDF) filter membranes (Roche, Switzerland). After blocking in phosphate buffered saline (PBS) containing 0.05% Tween-20 and 5% non-fat milk powder, the membranes were incubated with primary antibodies at 4 °C overnight (DcR3 and CEACAM1 at 1:1,000 dilution; and glyceraldehyde 3-phosphate dehydrogenase [GAPDH] at 1:5,000 dilution). Membranes were then washed and incubated at room temperature for 2 h with a secondary horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000) or goat anti-rabbit IgG (1:5,000). The protein bands on the membranes were detected using an enhanced chemiluminescence (ECL) detection system (FDbio, China). All experiments were performed according to the manufacturer's instructions.

Co-immunoprecipitation (CO-IP)

Cells were washed with PBS, centrifuged at 15,000 g at 4 °C for 10 minutes, and the supernatant was collected and incubated with the primary antibody or mouse IgG control at 4 °C for 4 hours. Subsequently, 20 μ L of pre-washed protein A/G sepharose beads was incubated with 400 μ g of sample overnight at 4 °C. After incubation, the immune complex was washed 3 times with lysis buffer, the protein was collected after centrifugation, and western blot analysis was performed.

Immunofluorescence (IF) staining

Cells were cultured and fixed on glass slides. After reaching confluence, cells were incubated with 0.1% Triton X-100 solution in PBS for 10 minutes to permeabilize the cells. Cells were then incubated with primary antibodies against β -catenin, followed by addition of the secondary antibodies. The slides were dyed with DAPI (4,6-diamidino-2-phenylindole) and examined with confocal microscopy (Nikon Corporation, Tokyo, Japan).

EdU (5-Ethynyl-2'-deoxyuridine) assay

The Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, Guangzhou, China) was used to perform proliferation assays according to the manufacturer's protocol. Briefly, cells were incubated with EdU for 2 hours, followed by fixation with 4% paraformaldehyde. Cells were then stained with Apollo Dye Solution and mounted with Hoechst 33342. EdU positive cells were photographed and counted under light microscopy.

Statistical analysis

Statistical analysis was performed using SPSS version 19.0 software. All numerical data are presented as mean $\pm$ standard deviation (SD). Parametric variables were compared using t -tests. Survival was assessed according to the Kaplan-Meier method and compared using the log-rank test. Differences were considered statistically significant when $P < 0.05$.

Results

TNFRSF6B mRNA expression

The expression of *TNFRSF6B* mRNA was analyzed using the TCGA database. *TNFRSF6B* expression was significantly elevated in several solid tumors including pancreatic adenocarcinoma (PAAD), colon adenocarcinoma (COAD), head and neck squamous cell carcinoma (HNSC), and stomach adenocarcinoma (STAD) (Figure 1A). The ONCOMINE database analysis of 19 datasets revealed that *TNFRSF6B* mRNA expression was increased in colorectal cancer, gastric cancer, kidney cancer, and pancreatic cancer compared to normal tissues (Figure 1B). Meta-analysis of *TNFRSF6B* expression in the ONCOMINE database revealed that the median rank was 704 and the P value was 1.97×10^{-7} (Figure 1C). Subsequent experiments

Figure 1 *TNFRSF6B* expression levels in human cancers. (A) The expression of *TNFRSF6B* in different tumor types from the TCGA database were determined by GEPIA (P value cutoff: 0.0001; red represents upregulated expression and green represents downregulated expression). (B) *TNFRSF6B* expression in data sets of different cancers in the ONCOMINE database. This graph generated by ONCOMINE indicates the number of datasets with statistically significant mRNA overexpression (red) of *TNFRSF6B* (cancer tissues vs. corresponding normal tissues). The threshold was defined with the following parameters: P value of $1E-4$, fold change of 2, and gene ranking of 10%. (C) Meta-analysis of *TNFRSF6B* mRNA expression in 19 analyses in the ONCOMINE database. (D) The GEPIA database verified that *TNFRSF6B* expression was significantly upregulated in COAD, STAD, and PAAD compared with normal tissues. *, $P < 0.05$. (E) The relationship between levels of *TNFRSF6B* expression and prognosis of COAD, STAD, and PAAD. *TNFRSF6B*, tumor necrosis factor receptor superfamily member 6b; TCGA, The Cancer Genome Atlas; GEPIA, Gene Expression Profiling Interactive Analysis; COAD, colon adenocarcinoma; STAD, stomach adenocarcinoma; PAAD, pancreatic adenocarcinoma.

focused on gastrointestinal tumors. The GEPIA database further confirmed that *TNFRSF6B* was upregulated in PAAD, COAD, and STAD compared to normal tissues (Figure 1D). Kaplan-Meier plots showed that increased expression of *TNFRSF6B* mRNA was significantly associated with OS in PAAD patients ($P=0.013$), but not in COAD patients ($P=0.87$), nor in STAD patients ($P=0.05$) (Figure 1E). Therefore, the involvement of *TNFRSF6B* in the network regulation of pancreatic cancer was further investigated.

The interaction network analysis of *TNFRSF6B*

To further study the biological function of *TNFRSF6B* in pancreatic cancer and the role of the regulatory network, genes related to *TNFRSF6B* were analyzed. The heat map shows the top 50 important genes that were positively or negatively correlated with *TNFRSF6B* (Figure 2A). GO and KEGG analyses of these genes were carried out to explore the potential functional pathways related to *TNFRSF6B*. The results demonstrated that *TNFRSF6B*-related genes were mainly involved in cytokine-mediated signaling pathways, myeloid leukocyte activation, ECM organization, response to type I interferon, cellular response to tumor necrosis factor, apoptotic signaling pathways, and actin filament-based processes (Figure 2B). The ClusterProfiler package was used for GSEA analysis of *TNFRSF6B*-related genes, including GO, KEGG, and Reactome analyses. The GO results revealed that *TNFRSF6B* was mainly involved in immune effector processes, immune responses, regulation of immune system processes, signaling receptor binding, cell adhesion, positive regulation of signal transduction, cell proliferation, regulation of cell death, apoptotic processes, and cell cycle processes. (Figure 2C). The KEGG results showed that *TNFRSF6B* was enriched in the cell cycle, the p53 signaling pathway, ECM-receptor interaction, the NF-kappa B signaling pathway, apoptosis, and the NOD-like receptor signaling pathway (Figure 2D). The Reactome results demonstrated that *TNFRSF6B* was widely involved in ECM organization, cell cycle checkpoints, degradation of the ECM, cell cycle, apoptosis, cytokine signaling in the immune system, and signaling by the interleukin innate immune system. (Figure 2E). These results suggested that *TNFRSF6B* is involved in the cell cycle, apoptosis, tumor-related signal transduction, and immune regulation in pancreatic cancer.

Construction of the weighted gene co-expression network analysis (WGCNA) and analysis of hub genes

The optimal soft threshold of mRNA co-expression network analysis was 11 (Figure 3A), and the systematic clustering tree of genes was obtained by using the WGCNA according to the heterogeneity between genes (Figure 3B). According to the dynamic mixed shearing method, the core gene cluster was identified and five gene modules were obtained (Figure 3C). Among these modules, the MEbrown module was found to be closely related to OS time ($r=-0.25$, $P=0.001$), DSS time ($r=-0.25$, $P=0.001$), PFI time ($r=-0.29$, $P=2e-04$), and DFI ($r=-0.33$, $P=2e-05$).

A total of 30 genes were extracted from the MEbrown module, and 10 of the most meaningful hub genes were screened by cytoHubba, namely, *S100 calcium binding protein A11* (*S100A11*), *gap junction protein beta 3* (*GJB3*), *tumor associated calcium signal transducer 2* (*TACSTD2*), *keratin 7* (*KRT7*), *potassium calcium-activated channel subfamily N member 4* (*KCNN4*), *capping actin protein, gelsolin like* (*CAPG*), *integrin subunit beta 4* (*ITGB4*), *laminin subunit beta 3* (*LAMB3*), *stratifin* (*SFN*), and *keratin 19* (*KRT19*). GeneMANIA was used to construct the PPI network of *TNFRSF6B* and the hub genes (Figure 3D). CLUEGO was used for biological function annotation analysis. The results demonstrated that these 10 hub genes were widely involved in ECM-receptor interaction, cell adhesion, cell cycle, and other processes (Figure 3E).

A heat map of the 10 hub genes was constructed under conditions of high and low *TNFRSF6B* expression (Figure S1A). GEPIA analysis showed that the expression of these 10 key genes in pancreatic cancer was higher than that in normal tissues (Figure S1B). With the exception of *ITGB4* ($P=0.078$) and *TACSTD2* ($P=0.087$), the other eight genes showed significant correlation with prognosis (Figure S1C), and thus, may function as potential prognostic markers for pancreatic cancer.

TNFRSF6B and immune cell infiltration

Above network analysis revealed that *TNFRSF6B* is highly related to the immune response. According to the median value of *TNFRSF6B*, immune cells were divided into the high expression group and the low expression group. A heat map of 29 types of immune cells was constructed (Figure 4A), and the correlation between *TNFRSF6B* and these immune cells was analyzed (Figure 4B). The results

Figure 2 Interaction network analysis of *TNFRSF6B*. (A) Heat maps showing the top 50 genes that were positively and negatively correlated with *TNFRSF6B* in PAAD samples. (B) A heatmap of Gene Ontology analysis including BP, CC, and MF. (C) Gene Ontology analysis, (D) KEGG pathway analysis, and (E) Reactome analysis of genes correlated with *TNFRSF6B* using Clusterprofiler. *TNFRSF6B*, tumor necrosis factor receptor superfamily member 6b; PAAD, pancreatic adenocarcinoma; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Figure 3 Weighted gene co-expression network analysis of gene expression data. (A) Analysis of scale-free fit index and the mean connectivity for various soft-thresholding powers. (B) Hierarchical clustering dendrogram of genes with dissimilarity based on topological overlap. Modules are the branches of the clustering tree. (C) Correlation between module eigengenes and clinical traits. Each row corresponds to a module eigengene and columns represent clinical traits. Each cell contains the correlation and P value. (D) Protein-protein interaction network of *TNFRSF6B* and hub genes by GeneMANIA. (E) The biological process analysis of hub genes was performed using the ClueGO plug-in. $P < 0.05$ was considered to be statistically significant. *TNFRSF6B*, tumor necrosis factor receptor superfamily member 6b.

Figure 4 The relationship between *TNFRSF6B* expression and immune cell infiltration. (A) The correlation between *TNFRSF6B* expression and immune cells. Red color represents positive correlation and blue color represents negative correlation. (B) The correlation between *TNFRSF6B* and 29 immune cell types. The color and the values indicate the Spearman correlation coefficient. (C) *TNFRSF6B* expression is correlated with immune cells in the TCGA database. (D) Correlation between *TNFRSF6B* expression and immune cells in the TIMER database. *TNFRSF6B*, tumor necrosis factor receptor superfamily member 6b; TCGA, The Cancer Genome Atlas; TIMER, Tumor Immune Estimation Resource.

revealed that *TNFRSF6B* was related to Type-II interferon-response, CD8+ T cells, mast cells, and neutrophils (Figure S2). Circle mapping was performed on immune cell types with a Pearson's correlation coefficient $r > 0.15$. *TNFRSF6B* was negatively correlated with CD8+ T cells ($r = -0.1814$, $P = 0.025$), CD4+ memory T cells ($r = -0.4430$, $P < 0.001$), and macrophages (M0; $r = -0.2102$, $P = 0.009$), and positively correlated with regulatory T cells (Treg; $r = 0.2136$, $P = 0.008$) (Figure 4C). Analysis of the TIMER database demonstrated that *TNFRSF6B* was negatively correlated with CD8+ T cells ($r = -0.4$, $P < 0.001$) and macrophages ($r = -0.19$, $P = 0.013$) (Figure 4D, Figure S3). The relationship between *TNFRSF6B* and immune checkpoints was further examined and shown in heatmap (Figure 5A). The expression of Fas ligand (FASLG), CD40 ligand (CD40LG), inducible T Cell costimulator ligand (ICOSLG), and CD28 decreased significantly in the *TNFRSF6B* high expression group compared to the low expression group ($P = 0.015$, $P = 0.002$, $P = 0.018$, and $P = 0.018$, respectively) (Figure 5B).

The effects of *TNFRSF6B* on cell proliferation and invasion in vitro

To verify the results of the bioinformatics analysis, shRNA was used to knock out *TNFRSF6B* gene expression in the PANC-1 pancreatic cancer cell line. EdU assays revealed that cell proliferation was significantly impaired in PANC-1 cells transfected with sh-*TNFRSF6B* (sh-*TNFRSF6B* cells) compared with cells transfected with the negative control shRNA (sh-NC cells) ($P < 0.01$, Figure 6A). A similar result was observed in the colony formation assays ($P < 0.0001$, Figure 6B). Transwell migration assays demonstrated that knockout of the *TNFRSF6B* gene significantly reduced the invasion ability of pancreatic cancer cells ($P < 0.01$, Figure 6C). These results suggested that knockout of the *TNFRSF6B* gene inhibited the proliferation and clone formation ability of pancreatic cancer cells.

Interaction network analysis of *TNFRSF6B* targeted genes

The gene chip data of pancreatic cancer samples from the *TNFRSF6B* down-regulation cells and the negative control cells were analyzed. A heat map of the expression levels was constructed, with red representing high expression and green representing low expression (Figure 7A). The downstream genes of *TNFRSF6B* were identified and the network regulation in sh-*TNFRSF6B* and sh-NC cells were examined. The GO results showed that these genes

were enriched in the Type 1 interferon signaling pathway, cellular response to type 1 interferon, response to type 1 interferon, and the cyclin-dependent protein kinase holoenzyme complex (Figure 7B). The KEGG analysis revealed that the important pathways included the JAK-STAT signaling pathway, the NOD-like receptor signaling pathway, necroptosis, the prolactin signaling pathway, the Toll-like receptor signaling pathway, the C-type lectin receptor signaling pathway, the TNF signaling pathway, the retinoic acid-inducible gene I (RIG-I)-like receptor signaling pathway, the p53 signaling pathway, and the NF-kappa B signaling pathway (Figure 7C). Based on the IPA, the regulatory effects of *TNFRSF6B* were analyzed and the possible pathways of the upstream regulatory networks and the downstream functions involving the differential genes were identified. The results revealed that upstream regulators including interferon (INF)- α , INF- β , INF- γ , and STAT1 were involved in the regulation of glomerular cell apoptosis, cryostasis of tumor cell lines, and the antiviral response of cells through *cyclin D1* (*CCND1*), *cyclin dependent kinase inhibitor 1A* (*CDKN1A*), *carcinoembryonic antigen-related cell adhesion molecule 1* (*CEACAM1*), *interferon regulatory factor 1* (*IRF1*) and other genes (Figure 7D).

The relationship between *TNFRSF6B* and *CEACAM1* was analyzed using UCSC Xena. Consistent correlation patterns were observed between these two genes in different patients (Figure 8A). This positive correlation was substantiated by analysis via the TIMER2.0 database (Figure 8B). Western blot assays confirmed that *CEACAM1* was regulated by *TNFRSF6B*, and the expression of the *CEACAM1* protein was down-regulated after knockout of the *TNFRSF6B* gene (Figure 8C). *TNFRSF6B* and *CEACAM1* were found to be enriched in circadian entrainment, gap junction, tyrosine metabolism, phenylalanine metabolism, and other pathways (Figure 8D). Immunofluorescence analysis showed that *TNFRSF6B* and *CEACAM1* were mainly expressed in the cytoplasm (Figure 8E). The interactions between *TNFRSF6B* and *CEACAM1* were also confirmed with immune co-precipitation assays (Figure 8F).

Discussion

Pancreatic cancer remains highly malignant with poor patient prognosis, which is largely due to high invasiveness, late diagnosis and low resection rates (2). Furthermore, pancreatic cancer is insensitive to radiotherapy and chemotherapy (25,26). Compared to conventional therapies, gene therapy is an emerging and dynamic area

Figure 5 The correlation between *TNFRSF6B* expression and immune checkpoints. (A) A heatmap representation of the correlation between *TNFRSF6B* expression and checkpoint-associated genes. Red color represents positive correlation and blue color represents negative correlation. (B) The correlation between *TNFRSF6B* expression and the four immune checkpoints FASLG, CD40LG, ICOSLG, and CD28. *TNFRSF6B*, tumor necrosis factor receptor superfamily member 6b; FASLG, Fas ligand; CD40LG, CD40 ligand; ICOSLG, inducible T cell costimulator ligand.

Figure 6 *TNFRSF6B* exhibits oncogenic roles in pancreatic cancer cells. (A) Cell proliferation was evaluated using EdU incorporation assays. **, $P < 0.01$. (B) Colony formation assays were performed on pancreatic cancer cells transfected with sh-*TNFRSF6B* or the negative control (sh-NC). ****, $P < 0.0001$. (C) The migration and invasion ability of *TNFRSF6B* cells were evaluated by Transwell assays. **, $P < 0.01$. *TNFRSF6B*, tumor necrosis factor receptor superfamily member 6b; sh, short hairpin; NC, negative control.

of research, in which enhanced immune surveillance of tumor cells has attracted considerable attention. According to American Society of Clinical Oncology (ASCO) guideline, Poly ADP-ribose Polymerase (PRAP) inhibitor olaparib is recommended for treatment of patients with pancreatic cancer who have a germline BRCA1 or BRCA2 mutation. Besides, in patients with tumors harboring neurotrophic tyrosine kinase (NTRK) fusions, treatment with larotrectinib or entrectinib is recommended. Although olaparib, larotrectinib and entrectinib are treatment options after first-line therapy, more clinical trials are needed for confirmation of therapeutic effect of these drugs (27).

In this study, the expression levels of *TNFRSF6B* in PC was first analyzed using the TCGA and ONCOMINE

databases. *TNFRSF6B* was found to be upregulated in PAAD samples compared to normal tissues, and *TNFRSF6B* expression was correlated with OS of patients with pancreatic cancer. Enrichment analysis of genes related to *TNFRSF6B* revealed important networks, including the tumor necrosis factor signaling pathway, the p53 signaling pathway, the interferon response pathway, and the apoptosis signaling pathway. In addition, *TNFRSF6B* was found to be involved in the regulation of immune responses, immune process regulation, cell proliferation, cell cycle, and apoptosis. To confirm the accuracy of the cancer database, the expression of *TNFRSF6B* was silenced in PANC-1 using shRNA, and the results were consistent with the prediction of the TCGA and ONCOMINE databases. Furthermore,

Figure 7 Analysis of the pathways and biological interaction networks of genes targeted by *TNFRSF6B*. (A) A heatmap representation of the differential genes in sh-*TNFRSF6B* cells and sh-NC cells. (B) Gene Ontology (GO) analysis indicated that genes regulated by *TNFRSF6B* were involved in biological processes (BP), cellular components (CC), and molecular function (MF). $P < 0.05$. (C) A bar chart showing the KEGG pathway analysis. (D) Analysis of the regulatory effect based on IPA. *TNFRSF6B*, tumor necrosis factor receptor superfamily member 6b; sh, short hairpin; NC, negative control; KEGG, Kyoto Encyclopedia of Genes and Genomes; IPA, ingenuity pathway analysis.

this study revealed that *TNFRSF6B* can promote proliferation, colony formation capacity, and invasion of pancreatic cancer cells.

The *TNFRSF6B* gene (1,114 bp) is located on human chromosome 20q13.3. It encodes the soluble protein DcR3

which consists of 271 amino acid residues, including four cysteine residue rich regions (CRDs) and an N-glycosylation site at the asparagine residue at position 173. *TNFRSF6B* is overexpressed in a variety of tumor tissues and cells, including esophageal cancer (28), gastric cancer (14),

Figure 8 Expression of *TNFRSF6B* and *CEACAM1* were highly correlated. (A) A heatmap of *TNFRSF6B* and *CEACAM1* expression across PAAD samples in the TCGA database using UCSC Xena. (B) The correlation between *TNFRSF6B* and *CEACAM1* expression was assessed using the TIMER database. (C) *TNFRSF6B* and *CEACAM1* protein expression in sh-NC and sh-*TNFRSF6B* cells was determined by Western blotting. (D) Interacting proteins of *TNFRSF6B* and *CEACAM1* were analyzed using KOBAS 3.0. (E) Immunofluorescence staining of *TNFRSF6B* and *CEACAM1* in PANC-1 cells. The scale bars represent 50 μ m. (F) Co-immunoprecipitation (CoIP) was used to identify the interactive proteins. TL1A was used as the positive internal reference. *TNFRSF6B*, tumor necrosis factor receptor superfamily member 6b; *CEACAM1*, carcinoembryonic antigen-related cell adhesion molecule 1; PAAD, pancreatic adenocarcinoma; TCGA, The Cancer Genome Atlas; TIMER, Tumor Immune Estimation Resource; sh, short hairpin; NC, negative control; KOBAS, Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology-Based Annotation System; TL1A, tumor necrosis factor-like cytokine 1A.

colon cancer (17,18), pancreatic cancer (15,16), lung cancer (12,13), glioblastoma multiforme (29), renal cell carcinoma (30), ovarian cancer (31), virus-associated lymphoma (32), diffuse B-cell lymphoma (33), multiple myeloma (34), oral malignancies (35) and hepatocellular carcinoma (36). Overexpression of *TNFRSF6B* can inhibit apoptosis and promote immune escape of tumor cells. *TNFRSF6B* can act both as a “decoy” and a “non-decoy”. As a decoy, *TNFRSF6B* competitively binds with FasL, TL1A, and LIGHT that are expressed on T cells (13), blocking their ability to induce apoptosis and clear tumor cells, thereby contributing to the pathogenesis of tumors (14). In its “non-decoy” function, *TNFRSF6B* plays an important role in cellular regulation (37) by affecting the activity and differentiation of T lymphocytes, macrophages, and dendritic cells (DC). It can also downregulate the immune system and facilitate tumor angiogenesis (38-41).

Indeed, analysis of the TCGA and ONCOMINE databases revealed that *TNFRSF6B* was related to immune cells such as CD8+ T cells, mast cells, neutrophils, and macrophages. There are several possible mechanisms by which *TNFRSF6B* can downregulate T lymphocytes. First, *TNFRSF6B* can inhibit the polymerization of actin in T cells stimulated by mitogen, inhibit the formation of pseudopodia, and inhibit the interaction between T cells and antigen presenting cells (42). Second, LIGHT provides T lymphocytes with CD28-independent costimulatory signals and induces the secretion of T helper type 1 (Th1) cytokines such as IFN- γ to participate in the tumor immune response. TL1A induces the activation of NF- κ B, which acts as a co-stimulating factor for the initiation of T lymphocytes. *TNFRSF6B* can bind competitively with both LIGHT and TL1A to block the activation of T cells by LIGHT and TL1A (43). Third, *TNFRSF6B* can downregulate the costimulatory molecules of T lymphocytes such as CD80, CD40, and CD54 (44). Furthermore, *TNFRSF6B* can regulate the differentiation of CD14+ monocytes into mature dendritic cells, thus inhibiting the proliferation of CD4+ T cells and promoting the secretion of IL-4. This suggests that *TNFRSF6B* may be an effector molecule capable of directing the T cell response to the T helper type 2 (Th2) phenotype (45).

Gene chip detection was performed to analyze the downstream genes of *TNFRSF6B* and predict the networks regulated by *TNFRSF6B*. The results were similar to those predicted by the TCGA and ONCOMINE databases. Interestingly, IPA demonstrated that *CEACAM1* plays an important role in the regulatory network of *TNFRSF6B*.

CEACAM1 is a transmembrane glycoprotein located on the cell surface. It is a member of the carcinoembryonic antigen family, which belongs to the immunoglobulin superfamily adhesion molecules (46). Previous studies have shown that *CEACAM1* can promote a variety of malignant tumors such as non-small cell lung cancer (47), colon cancer (48), and pancreatic cancer (49). Possible mechanisms include the prevention of T lymphocyte activation, stimulation of B lymphocyte proliferation, inhibition of T cell and NK cell cytotoxicity, promotion of tumor cells invasion, enhancement of endothelial cell viability, promotion of blood vessel formation, and regulation of vascular remodeling (48,50). Therefore, *CEACAM1* has many important biological functions. The positive correlation between *CEACAM1* and *TNFRSF6B* in pancreatic cancer cells suggested that the mechanism of *TNFRSF6B* may involve inhibiting the activation and proliferation of T lymphocytes, thereby promoting the rapid proliferation of cancer cells. Therapies which target these two collaborative genes may be an efficient way to treat pancreatic cancer.

In summary, this study demonstrated that *TNFRSF6B* is overexpressed in pancreatic cancer tissues compared to healthy pancreatic tissues. Furthermore, elevated *TNFRSF6B* expression can promote the growth and invasion of tumor cells, and is related to the OS of patients with pancreatic cancer. In addition, this report demonstrated that *TNFRSF6B* is widely involved in the regulation of the tumor necrosis factor signaling pathway, the p53 signaling pathway, response to type I interferons, the apoptosis signaling pathway, the immune response, the regulation of immune processes, cell proliferation, the cell cycle, and apoptosis. Therefore, this investigation revealed novel insights into the molecular mechanisms underlying the regulation of *TNFRSF6B* in pancreatic cancer cells and proposed that *TNFRSF6B* may be a promising molecular target for the diagnosis and treatment of patients with pancreatic cancer.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/jgo-21-303>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Since all data were extracted from publicly available database, approval by the ethics committee and patient consent were waived.

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Figure S1 The expression and prognostic value of 10 hub genes in PAAD. (A) Heatmap representation of the correlation between *TNFRSF6B* expression and 10 hub genes. Red color represents positive correlation and blue color represents negative correlation; (B) the expression of 10 hub genes (*S100A11*, *GJB3*, *TACSTD2*, *KRT7*, *KCNN4*, *CAPG*, *ITGB4*, *LAMB3*, *SFN*, *KRT19*) in PAAD; (C) the overall survival curve of *S100A11*, *GJB3*, *TACSTD2*, *KRT7*, *KCNN4*, *CAPG*, *ITGB4*, *LAMB3*, *SFN*, *KRT19*. PAAD, pancreatic adenocarcinoma.

Figure S2 Associations between *TNFRSF6B* gene expressions and immune cells in PAAD. *TNFRSF6B* has a negative correlation with Type-II INF-Response (A), mast cells (B), CD8(+) T cell (C) and neutrophils (D). *TNFRSF6B*, tumor necrosis factor receptor superfamily member 6b; PAAD, pancreatic adenocarcinoma; INF, interferon.

Figure S3 Correlation between *TNFRSF6B* expression and CD8(+) T cell in PAAD. *TNFRSF6B* was negatively correlated with CD8(+) T cell. *TNFRSF6B*, tumor necrosis factor receptor superfamily member 6b; PAAD, pancreatic adenocarcinoma.