Original Article

The *EFNA4* gene is a potential prognostic biomarker in pancreatic cancer: a bioinformatics analysis

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Background: Pancreatic cancer is a highly aggressive malignancy with poor prognosis, and there is an urgent need to understand its molecular mechanisms for early diagnosis and treatment. Despite surgical resection being the only effective treatment, most patients are diagnosed at an advanced stage, missing the optimal window for therapy. Identifying novel biomarkers is crucial for prognostic assessment, treatment planning, and early intervention. Ephrin A4 (*EFNA4*), a member of the receptor tyrosine kinase family, is involved in vascular and epithelial development via regulation of cell migration and rejection. However, the role of *EFNA4* in pancreatic cancer has not been reported. Therefore, our study aimed to clarify the role of *EFNA4* in pancreatic cancer through bioinformatics analysis and vitro experiments.

Methods: The expression of *EFNA4* and its potential value as a diagnostic and prognostic biomarker in pancreatic cancer was analyzed using data from The Cancer Genome Atlas (TCGA) and the Gene Expression Profiling Interactive Analysis (GEPIA) database. According to the expression level of *EFNA4*, patients were divided into high expression group and low expression group, and the correlation between overall survival (OS) and disease-free survival (DFS) with different expression levels of EFNA4 and clinical parameters were analyzed. Subsequently, reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was performed to detect *EFNA4* expression. The proliferation, invasion, and cloning ability of the cells were detected via Cell Counting Kit 8 (CCK8), Transwell, and plate cloning assays, respectively.

Results: *EFNA4* is highly expressed in pancreatic cancer, and upregulation of *EFNA4* is associated with poor prognosis. In this study, *EFNA4* expression was correlated with T stage and TNM (tumor-node-metastasis) stage of pancreatic cancer, and the median survival time and progression-free survival (PFS) were worse in those with high *EFNA4* expression (394 days) than in those with low expression (525 days) [hazard ratio (HR): 1.47, 95% confidence interval (CI): 1.00–2.16, P=0.047]. In addition, *EFNA4* was also found to be involved in the regulation of signal pathways such as cell adhesion, cyclic AMP, insulin secretion, pancreatic secretion, and protein digestion and absorption. *In vitro* experiments demonstrated that *EFNA4*

knockdown significantly inhibited the proliferation, cloning ability, and invasiveness of the PANC-1 and SW1990 pancreatic cancer cell lines.

Conclusions: The abnormal expression of *EFNA4* in pancreatic cancer is associated with poor prognosis. Knockout of *EFNA4* gene could significantly inhibit the proliferation and invasion of pancreatic cancer cells. Therefore, *EFNA4* may be one of the molecular targets for poor prognosis of patients with pancreatic cancer.

Keywords: Pancreatic cancer; ephrin A4 (*EFNA4*); bioinformatics analysis

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Introduction

Pancreatic cancer is a deadly and aggressive malignancy with a 5-year survival rate of only 2–9%. The mortality rate of those with pancreatic cancer is all rising, and by 2030 it is expected to be the second leading cancer-related cause of death after lung cancer, surpassing colorectal and breast cancer (1-3). Currently, surgical resection is the only effective treatment, through which the 5-year survival rate can be significantly increased to 20–30%. However, fewer than 20% of patients are eligible for resection because most patients are diagnosed at an advanced stage when they have metastases (4). This poor prognosis is mainly attributable to the rapid tumor progression and postoperative recurrence and metastasis (5). Therefore, it is necessary to study the molecular mechanisms of pancreatic cancer proliferation, invasion, and metastasis and to identify novel biomarkers to accurately monitor the development and progression of this disease. Several studies have shown that the carbohydrate antigen 19-9 (CA 19-9) and the carcinoembryonic antigen (CEA) can predict outcomes in various cancers, including pancreatic cancer, yet these biomarkers lack the specificity and sensitivity required for optimal use in pancreatic cancer (6-9). Despite the identification of numerous potential biomarkers through high-throughput sequencing, few emerge as promising candidates for pancreatic cancer (10).

Ephrin A4 (*EFNA4*) is a ligand of the ephrin (EFN) family and is involved in the development of blood vessels and epithelium through the regulation of cell migration and repulsion (11). *EFNA4* participates in the regulation of many signaling pathways, including the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB, also known as AKT) signaling pathway and EFN/EFN receptor (Eph) signaling pathway, among others (12). Previous study has shown that *EFNA4* directly interacts with EFN receptor A2 (EPHA2) and promotes its phosphorylation at Ser897, recruits phosphoinositol-3-kinase regulatory subunit 2 (PIK3R2), and activates the glycogen synthase kinase-3β (GSK3β)/β-catenin signaling pathway. In addition, the overexpression of β-catenin further promotes the expression of PIK3R2, forming a positive feedback loop (11).

Highlight box

Key findings

- The study found that ephrin A4 (*EFNA4*) expression plays a role in promoting tumor growth and metastasis in pancreatic cancer cell lines, similar to its role in breast cancer, liver cancer, and glioblastoma. The research suggests that *EFNA4* not only has diagnostic value for pancreatic cancer but also may serve as a critical therapeutic target.

What is known and what is new?

- Previous studies have identified *EFNA4* as a factor involved in tumor growth and metastasis in various cancer types including breast, liver, and glioblastoma.
- This manuscript adds new evidence that *EFNA4* similarly affects pancreatic cancer cell lines, supporting its potential as a therapeutic target. Also, the study specifically identifies the effects of *EFNA4* on proliferation, cloning, and invasion abilities of pancreatic cancer cells.

What is the implication, and what should change now?

- The findings of this study suggest that targeting *EFNA4* could be a promising strategy in treating pancreatic cancer. Consequently, a deeper understanding of *EFNA4*'s role in pancreatic cancer could lead to the development of new diagnostic and therapeutic approaches.
- Further research is needed, particularly *in vivo* studies, to ascertain *EFNA4*'s impact on the biological behavior of pancreatic cancer cells and to validate its potential as a therapeutic target. This could eventually lead to changes in the clinical management of pancreatic cancer, including the development of EFNA4-inhibiting drugs or related therapies.

Additionally, a study has shown that *EFNA4* is the upstream gene of the PI3K/Akt signaling pathway and promotes cell proliferation and tumor metastasis of hepatocellular carcinoma through the PIK3R2/GSK3β/β-catenin positive feedback loop. EFNA4 expression also affects overall survival (OS) and progression-free survival (PFS) in patients with breast cancer mainly because its expression increases breast cancer resistance to chemotherapy (13). Higher *EFNA4* expression in gastric cancer cells compared to normal cells has been correlated with the level of tumor-infiltrating immune cells in gastric cancer (14). In one study, a high serum level of soluble *EFNA4* isotype was positively correlated with peripheral blood lymphocyte count and lymph node enlargement. These findings suggest that Eph/EFN may be associated with normal B-cell biology and may represent a new potential prognostic marker and therapeutic target for chronic lymphocytic leukemia (15). In addition, the expression level of *EFNA4* has also been found to be increased in liver cancer and glioblastoma (16,17). Overall, these findings suggest that *EFNA4* plays an important role in the occurrence and development of multiple tumors. However, its role and prognostic significance in pancreatic cancer remain underexplored.

As Yu *et al.* highlighted the immune microenvironment plays a pivotal role in pancreatic cancer, and *EFNA4*, being a regulator of cell migration, could potentially influence tumor-immune interactions (18). The potential for immunotherapy in the pancreatic cancer perioperative setting, as discussed by Springfeld, further underscores the importance of understanding the tumor's molecular landscape, including the role of *EFNA4*, which may modulate the tumor's interaction with the immune system (19). The question of whether locally advanced pancreatic cancer should be considered a resectable disease, as posed by Rangelova, is directly relevant to the identification of prognostic markers that can guide treatment decisions (20). Moreover, the contributions of hypoxia to poor outcomes in pancreatic cancer, as outlined by Bijlsma, may intersect with the role of *EFNA4*, given its involvement in cellular responses to microenvironmental cues (21).

Furthermore, the correlation between *EFNA4* expression levels and critical clinical features of pancreatic cancer, such as tumor staging, degree of differentiation, neural invasion, and lymphatic metastasis, is of significant interest. These factors are known to profoundly influence patient prognosis and treatment strategies. Understanding the relationship between *EFNA4* expression and these clinical parameters could provide insights into the biological behavior of pancreatic cancer and identify opportunities for therapeutic intervention.

Bioinformatics analysis allows us for the integration and interpretation of large-scale genomic data to uncover molecular patterns associated with disease progression and outcome. Additionally, bioinformatics analysis provides us with a cost-effective and time-saving preliminary experimental proof method to screen and validate the suitability of *EFNA4* as a potential biomarker. In our study, we evaluated *EFNA4* expression in pancreatic cancer using RNA data from The Cancer Genome Atlas (TCGA). The effect of abnormal *EFNA4* expression on the prognosis of pancreatic cancer was studied using the Gene Expression Profiling Interactive Analysis (GEPIA) database. We then performed functional enrichment and pathway analysis to clarify the role of *EFNA4* in the pathogenesis of pancreatic cancer. Subsequently, the bioinformatics results were verified through *in vitro* experiments. This preliminary study on the potential mechanism of *EFNA4* in pancreatic cancer suggests that *EFNA4* can be used as a prognostic marker for pancreatic cancer. We present this article in accordance with the MDAR reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-227/rc).

Methods

Our study utilized TCGA and GEPIA databases for bioinformatics analysis of *EFNA4* expression in pancreatic cancer. This was followed by *in vitro* experiments on PANC-1 and SW1990 cell lines to assess *EFNA4*'s role in cell proliferation, invasion, and cloning using RT-qPCR, Cell Counting Kit 8 (CCK8), Transwell, and cloning assays. Additionally, we performed enrichment and pathway analyses to explore the biological significance of *EFNA4*-related differentially expressed genes (DEGs), correlating these with our experimental data to substantiate *EFNA4*'s potential as a prognostic biomarker and therapeutic target. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Expression of EFNA4 in pancreatic cancer

In this study, the transcriptome data, clinical medical records, and survival and prognostic information of 179 patients with pancreatic adenocarcinoma were obtained from TCGA (https://tcga.xenahubs.net) database (22). The filter module and analysis conditions on TCGA platform were set as

Table 1 Primers and sequences used

Primer name	Primer sequences
GAPDH-F	CCCATCACCATCTTCCAGG
GAPDH-R	CATCACGCCACAGTTTCCC
EFNA4-F	TGTGCTCCCTGCCCTTT
EFNA4-R	CCCTCGCCACCCTGATG

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EFNA4, ephrin A4; F, forward primer; R, reverse primer.

follows: in the "Expression on Box Plots" module, "*EFNA4*", "PAAD", and "Match TCGA normal and GTEx data" were selected; OS and PFS were selected as survival data, while age, sex, smoking history, drinking history, chronic pancreatitis history, diabetes history, and TNM (tumor-node-metastasis) stage were selected as the clinicopathological parameters.

Correlation analysis of EFNA4 expression and clinicopathological parameters with prognosis in patients with pancreatic cancer

A total of 179 cases with a complete *EFNA4* expression profile and clinicopathological parameters were included in the TCGA dataset. Due to the short OS, a total of 175 cases with an OS greater than 30 days were screened; among these cases, 95 were male, 80 were female, 54 were younger than or equal to 60 years old, and 121 cases were over 60 years old. According to the median expression level of *EFNA4*, the patients were divided into a high-expression group and a low-expression group. The correlation of *EFNA4* expression and age, sex, smoking history, drinking history, chronic pancreatitis history, diabetes history, TNM stage with OS and DFS was analyzed.

Enrichment analysis of EFNA4-related DEGs

The "limma" package in R (The R Foundation of Statistical Computing) was applied to analyze the significantly DEGs of the *EFNA4* high- and low-expression groups in pancreatic cancer (13). Subsequently, the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.ncifcrf.gov/), an online analytical database of DEGs, was used to conduct Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. According to the median value of *EFNA*4 expression, the 183 TCGA data samples were divided into high- and low-expression groups, with 91 in the high-expression group and 92 in the low-expression group.

Detection of EFNA4 mRNA expression and experimental cell lines screening in pancreatic cancer cell lines

Four cell lines, including bxpc-3 (human pancreatic cancer cells *in situ*), PANC-1 (human pancreatic cancer cells), HPDE6-c7 (human normal pancreatic ductal epithelial cells), and SW1990 (human pancreatic cancer cells) were provided by the Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China: sh-*EFNA4*-001, sh-*EFNA4*-002, and sh-*EFNA4*-003, the expression of *EFNA4* in each group of cells was detected via quantitative polymerase chain reaction (qPCR), and the optimal interference chain was screened to extract the total RNA of each group of cells. Subsequently, reverse-transcription qPCR (RT-qPCR) was conducted with a 2× Universal Blue SYBR Green qPCR Master Mix kit (Thermo Fisher Scientific, Waltham, MA, USA) using the following reaction program: 40 cycles of predenaturation at 95 ℃ for 1 minute, denaturation at 95 ℃ for 20 seconds, annealing at 55 ℃ for 20 seconds, and extension at 72 ℃ for 30 seconds. Cells were stored at 4 ℃. The primers used are listed in *Table 1*.

Transfection of pancreatic cancer cells

The optimal interference chain selected (sh-*EFNA4*-002) and the negative control sequence (sh-NC) were lentiviral packaged with 293T cells. Two centrifuge tubes were respectively prepared with the plasmid and diluent of the transfection reagent. Centrifuge tube 1 included 5 μg of lentiviral vector and 500 μL of Dulbecco's Modified Eagle Medium (DMEM); meanwhile, centrifuge tube 2 included 20 μL of polyfect-V transfection reagent and 40 μL of DMEM. The diluent of the transfection reagent in centrifuge in tube 2 was added and thoroughly mixed into the plasmid DNA solution of centrifuge tube 1, and the transfection mixture was incubated at room temperature for 15 minutes. The transfection mixture (1 mL) was added to the cell culture dish drop by drop and cultured at 37 ℃. After 4–6 hours, the liquid was replaced with 10 mL of fresh 293T medium. At 24 hours after transfection, 10 mL of virus medium was used to replace the fluid. Cell culture supernatant was collected 48 hours after transfection and centrifuged at 500 ×g for 10 minutes to remove cell debris. The successfully lentivirus-infected cells were

further cultured, and puromycin was added to the medium for screening. The screening lasted for 1 week and was observed under a fluorescence microscope until the infection efficiency reached 80% of the optimal screening concentration of purinomycin.

qPCR detection of EFNA4 expression in each group of cells

The total RNA of cells in each group was extracted, and then RT-qPCR was conducted with a 2× Universal Blue SYBR Green qPCR Master Mix kit (Thermo Fisher Scientific). The reaction program was as follows: 40 cycles of predenaturation at 95 ℃ for 1 minutes, denaturation at 95 ℃ for 20 seconds, annealing at 55 ℃ for 20 seconds, and extension at 72 ℃ for 30 seconds. The cells were stored at 4 ℃.

CCK8 assay and clonogenic detection

PANC-1 and SW1990 cells were transfected after 48 hours. Cells were digested and collected with 0.25% pancreatic enzyme, and the cells were resuspended and counted with culture supernatants. Following this, 1×10^3 cells were inoculated into 96-well plates, with five repeats per group. A total of four 96-well plates were inoculated and placed back in the incubator for further culture and measured once at 0, 24, 48, 72, and 96 hours. Subsequently, 100 µL of diluted CCK8 solution was added to each well, and the wells were incubated for 2 hours away from light. The absorbance was measured at 450 nm with an enzyme labeling instrument. PANC-1 and SW1990 cells were inoculated in 6-well plates at a rate of 200 cells/well and cultured in an incubator in humid air with a 5% CO_2 atmosphere at 37 ℃ for 14 days until the colonies were visible to the naked eye. The colonies were washed twice with phosphate-buffered saline (PBS) (Solarbio) and stained with 0.25% crystal violet for 1 hour. The colonies were counted and statistically analyzed using ImageJ software (US National Institutes of Health).

Transwell assay

For the Transwell assay, we used a Transwell chamber (Corning) with a pore size of 8 µm, which was completely covered by Matrigel matrix adhesive. PANC-1 and SW1990 cells were transfected for 24 hours. Following this, 500 µL of serum-free medium was added to the 24-well plate and just touched the bottom of the Transwell chamber. Next, 1×10^5 cells/well were suspended into 200 µL of complete medium containing 10% fetal bovine serum (FBS), inoculated in the upper chamber of the Transwell, and cultured in an incubator for 5 hours. When the cells had adhered to the wall, the medium was changed, the upper chamber was cleaned with PBS, serum-free medium was added, and the lower chamber was cultured with complete medium containing 10% FBS. After 24 hours, the cells were removed and fixed with methanol for 10 minutes, stained with 0.4% crystal violet solution for 20 minutes, and counted under a light microscope (Nikon, Tokyo, Japan) in several randomly selected areas.

Statistical analysis

The difference in messenger RNA (mRNA) expression of *EFNA4* between the pancreatic cancer group and the normal control group was statistically analyzed using $\log_2$ [transcripts per million (TPM) +1] in GEPIA, with the significant difference expression criteria being $|\log_2$ fold change$| \geq 1$ and $P<0.05$. The correlation between clinicopathological features of pancreatic cancer and *EFNA4* was determined with the chi-squared test using SPSS 21 statistical software (IBM Corp.). The correlation between *EFNA4* expression and the prognosis of pancreatic cancer patients was analyzed with Kaplan-Meier and univariate Cox survival analyses. A P value <0.05 was considered statistically significant.

Results

EFNA4 expression in the pancreatic cancer and normal control groups

In this study, the GEPIA database was used to analyze the expression level of *EFNA4* in pancreatic cancer and normal pancreatic tissues, including 179 cases in the pancreatic cancer group and 171 cases in the normal control group. The results showed that the expression of *EFNA4* in pancreatic cancer tissue was significantly higher than that in normal tissue ($P<0.05$; *Figure 1*).

Analysis of EFNA4 expression and clinicopathological parameters in patients with pancreatic cancer

In the clinical data of TCGA dataset, cases were divided into *EFNA4* high- and low-expression groups according to the median value of 8.3676. The *EFNA4* mRNA expression level was significantly correlated with history of drinking ($P=0.04$), tumor stage ($P<0.01$), and TNM pathological

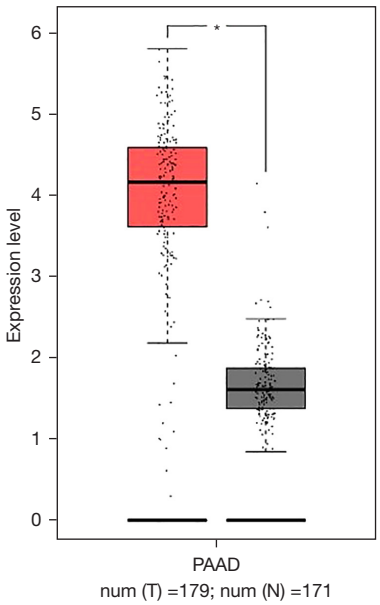

Figure 1 The expression of *EFNA4* in pancreatic cancer tissue was significantly higher than that in normal tissue. *, P<0.05; PAAD, pancreatic adenocarcinoma; num(T), number(Tumor); num(N), number(Normal); *EFNA4*, ephrin A4.

stage of pancreatic cancer (P=0.007), but was not significantly correlated with age, sex, history of smoking, history of chronic pancreatitis, history of diabetes, lymph node metastasis, or distant metastasis (P>0.05) (*Table 2*).

EFNA4 expression and survival analysis of patients with pancreatic cancer

The results showed that the OS time of patients with a low expression of *EFNA4* in pancreatic cancer was 666 days, compared with 545 days in patients with a high expression of *EFNA4*. The progression-free survival times of patients with low *EFNA4* expression was 525 days, which was longer than that of patients with high *EFNA4* expression [hazard ratio (HR): 1.47, 95% confidence interval (CI): 1.00–2.16, P=0.047] (*Table 3* and *Figure 2*).

Bioinformatics analysis of EFNA4

To further explore the function of *EFNA4* in pancreatic cancer, we performed GO and KEGG functional enrichment analyses on 1,420 DEGs (*EFNA4* high-expression group and low-expression groups). Among these, 530 genes were upregulated and 890 genes were downregulated, and the DEGs were drawn in a volcano map (*Figure 3*). GO analysis and KEGG pathway analyses of these DEGs conducted via the DAVID online database

Table 2 Correlation of clinicopathological features with *EFNA4* expression in pancreatic cancer

Clinical variables	N	*EFNA4*		χ^2	P value
		Low	High		
Gender				0.960	0.33
Male	95	44	51		
Female	80	43	37		
Age (years)				0.003	0.96
≤60	54	27	27		
>60	121	60	61		
Drinking				4.331	0.04
Yes	100	42	58		
No	63	37	26		
Chronic pancreatitis				1.226	0.27
Yes	13	4	9		
No	126	59	67		
Diabetes				1.350	0.25
Yes	36	19	17		
No	108	45	63		
Smoking				0.868	0.35
Yes	54	24	30		
No	121	63	58		
M stage				0.213	>0.99
M0	79	39	40		
M1	5	3	2		
N stage				0.106	0.75
N0	47	22	25		
> N0	123	61	62		
T stage				6.287	0.01
T1 + T2	28	20	8		
T3 + T4	147	67	80		
TNM stage				7.286	0.007
I + II	19	15	4		
III + IV	156	72	84		

EFNA4, ephrin A4; TNM, tumor-node-metastasis.

Table 3 Association of *EFNA4* expression with the survival of patients with pancreatic cancer

EFNA4	OS			PFS		
	MST (days)	95% CI	P value	MST (days)	95% CI	P value
Low (N=87)	666	1.44 (0.96–2.18)	0.08	525	1.47 (1.00–2.16)	0.047
High (N=88)	545			394		

EFNA4, ephrin A4; OS, overall survival; PFS, progression-free survival; MST, median survival time; CI, confidence interval.

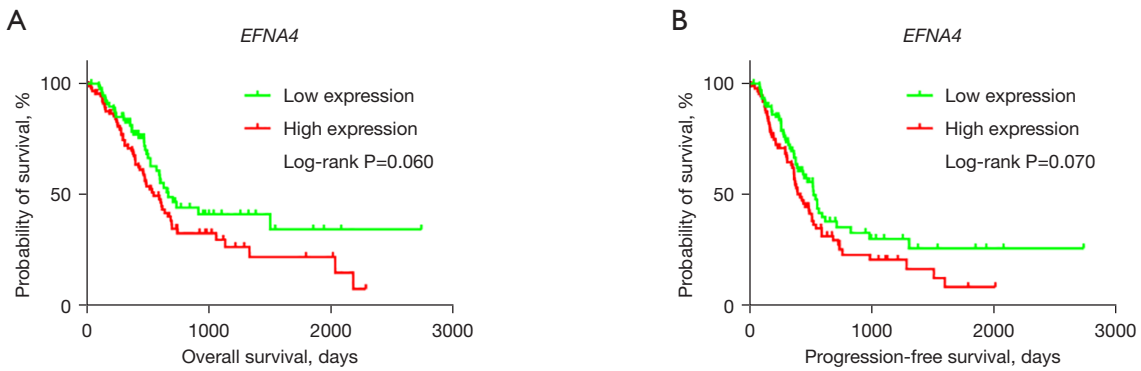

Figure 2 Kaplan-Meier plotter survival analysis of patients with pancreatic cancer. (A) Overall survival curve; (B) progression-free survival curve. *EFNA4*, ephrin A4.

indicated that the biological processes of the DEGs were mainly involved in signal transduction, cell adhesion, nervous system development, axon guidance, chemical synaptic transmission, cytosolic calcium ion concentration, positive regulation of potassium ion transmembrane transport, regulation of ion transmembrane transport, potassium ion transport, regulation of insulin secretion, etc. Cytological composition analysis indicated that these genes are involved in the composition of extracellular plasma membrane, neuron cell body, and outer nucleus. Molecular function analysis mainly indicated that these genes are involved in calcium ion binding, signal receptor activity, heparin binding, transmembrane signal receptor activity, carbohydrate binding, serine-type endopeptidase activity, etc. KEGG pathway enrichment analysis indicated that these DEGs are mainly involved in neuroactive ligand-receptor interaction, cyclic AMP signaling pathway, cell adhesion molecules, insulin secretion, pancreatic secretion, protein digestion and absorption, calcium signaling pathway, etc. (*Figure 3*).

Construction of short hairpin RNA of the EFNA4 gene

PANC-1 and SW1990 with high expression of *EFNA4* were selected for subsequent cell experiments (*Figure 4A*). The expression of *EFNA4* in sh-*EFNA4*-001, sh-*EFNA4*-002, sh-*EFNA4*-003 and transfected PANC-1 cells was detected with qPCR, and the optimal interference chain was screened. The qPCR detection results revealed that the sh-*EFNA4*-002 group had the best interference effect, and sh-*EFNA4*-002 was selected for follow-up experiments (*Figure 4B*).

Expression levels of EFNA4 after the small interfering RNA transfection

EFNA4 mRNA expression was not significantly decreased in PANC-1 and SW1990 cells transfected with the negative control sequence (sh-NC) compared to those cells without transfection (control group). The expression level of *EFNA4* in the scrambled group sh-*EFNA4*-002 in both cell lines was significantly reduced compared to its negative control group sh-NC (*Figure 4B*,*4C*).

Effect of downregulation of EFNA4 expression on the proliferative capacity of pancreatic cancer cells

The absorbance values of pancreatic cancer cells PANC-1 and SW1990 were detected via CCK-8 assay, and cell

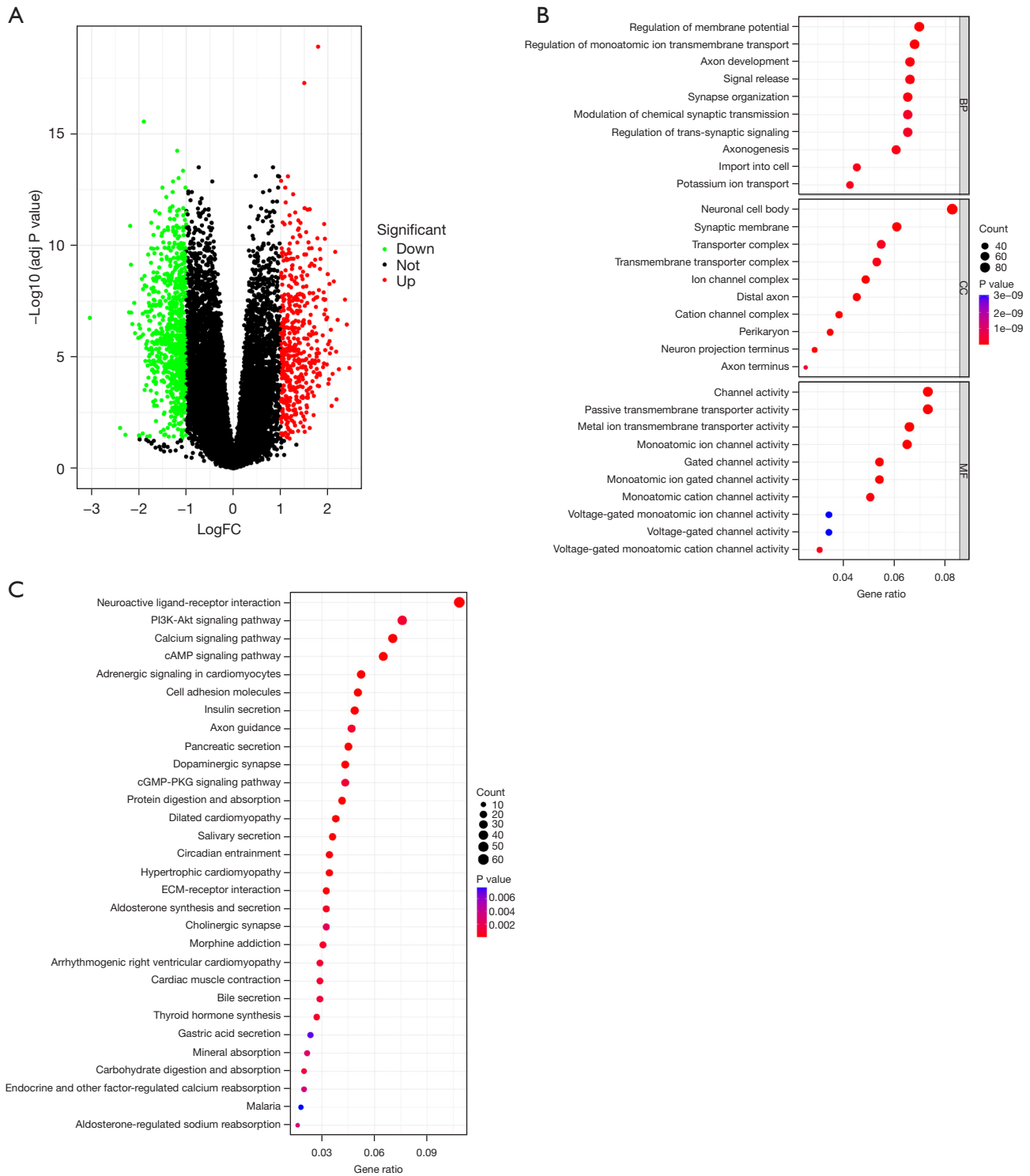

Figure 3 Functional enrichment analysis. (A) DEGs analysis between the *EFNA4* high-expression group and low-expression group; (B) GO term analysis of DEGs; (C) KEGG pathway analysis of DEGs. FC, fold change; BP, biological process; CC, cellular component; MF, molecular function; ECM, extracellular matrix; DEGs, differentially expressed genes; EFNA4, ephrin-A4; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

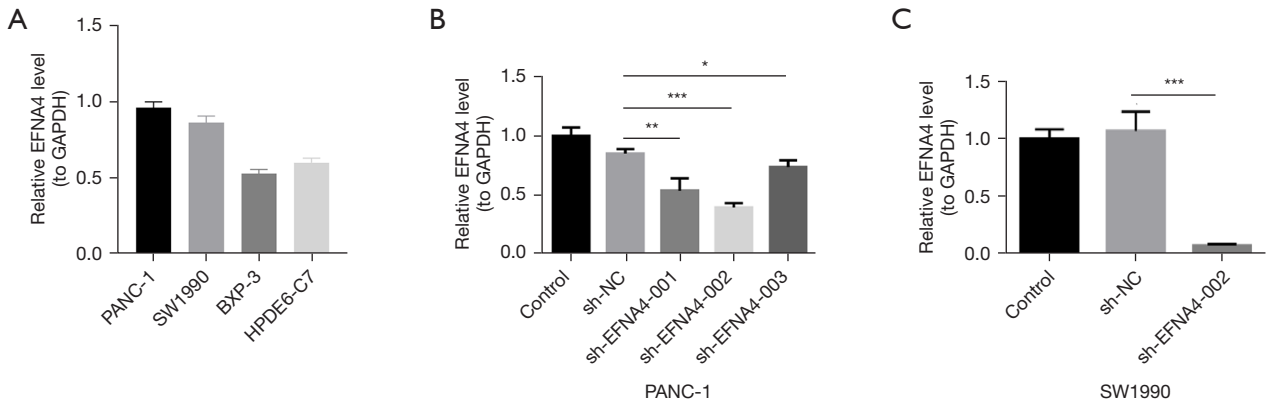

Figure 4 Experimental cell line screen and knockdown for *EFNA4*. (A) EFNA4 mRNA express in different cell lines of pancreatic cancer; (B) *EFNA4* knockdown in PANC-1 cells; (C) *EFNA4* knockdown in SW1990 cells. *, P<0.05; **, P<0.01; ***, P<0.001. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EFNA4, ephrin A4; NC, negative control; PANC-1, Human Pancreatic Cancer Cells PANC-1; SW1990, Human Pancreatic Cancer Cells SW1990.

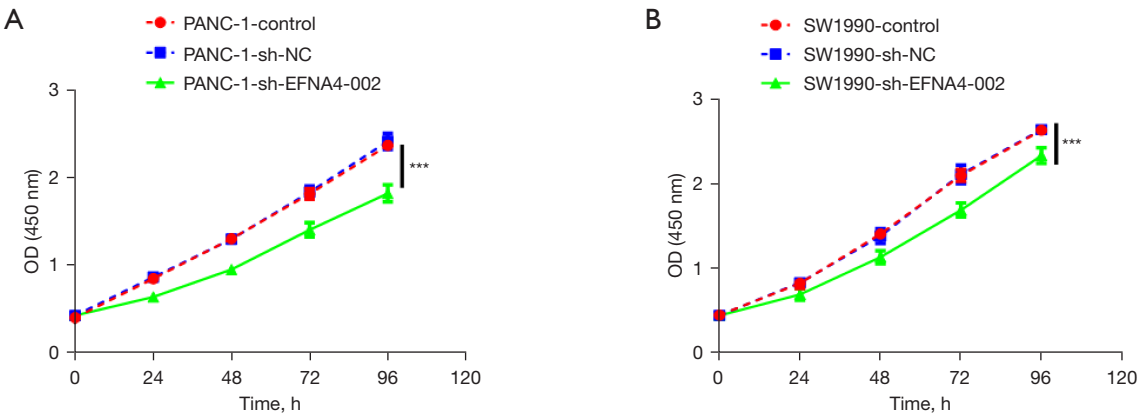

Figure 5 CCK-8 assays detected the cell proliferation viability of PANC-1 and SW1990. (A) Detection of PANC-1 cell proliferation viability; (B) detection of SW1990 cell proliferation viability. ***, P<0.001. OD, optical density; NC, negative control; CCK-8, Cell Counting Kit-8; EFNA4, Ephrin-A4; PANC-1, Human Pancreatic Cancer Cells PANC-1; SW1990, Human Pancreatic Cancer Cells SW1990.

proliferation curves were drawn according to the absorbance values (*Figure 5*). The optical density (OD) values for the sh-*EFNA4* group were distinctly lower than those of both the control group and the sh-NC group at 24 hours. According to the OD values at 96 hours, the disparity became more pronounced for the SW1990 sh-*EFNA4* group being 2.35±0.09, significantly lower compared to the control (2.65±0.05) and sh-NC groups (2.65±0.05) (P<0.0001). The PANC-1 sh-*EFNA4* group exhibited OD values of 1.83±0.10, which was also substantially reduced when contrasted with control group (2.38±0.04) and the sh-NC group (2.43±0.09) (P<0.0001). This suggested that downregulating *EFNA4* expression significantly reduced the proliferative capacity of the PANC-1 and SW1990 cells.

Clone formation ability of cells

We tested the cloning ability of pancreatic cancer cell line PANC-1 through a plate cloning assay. The experimental results showed that compared with the cell migration number of the sh-NC group (72.96±7.54), that of the sh-*EFNA4* group (56.35±5.31) was significantly lower (P<0.01; *Figure 6A,6B*).

Similarly, for SW1990, the cell migration number of the sh-NC group (72.57±3.46) was significantly lower than that of the sh-*EFNA4* group (56.69±2.97) (P<0.05; *Figure 6C,6D*).

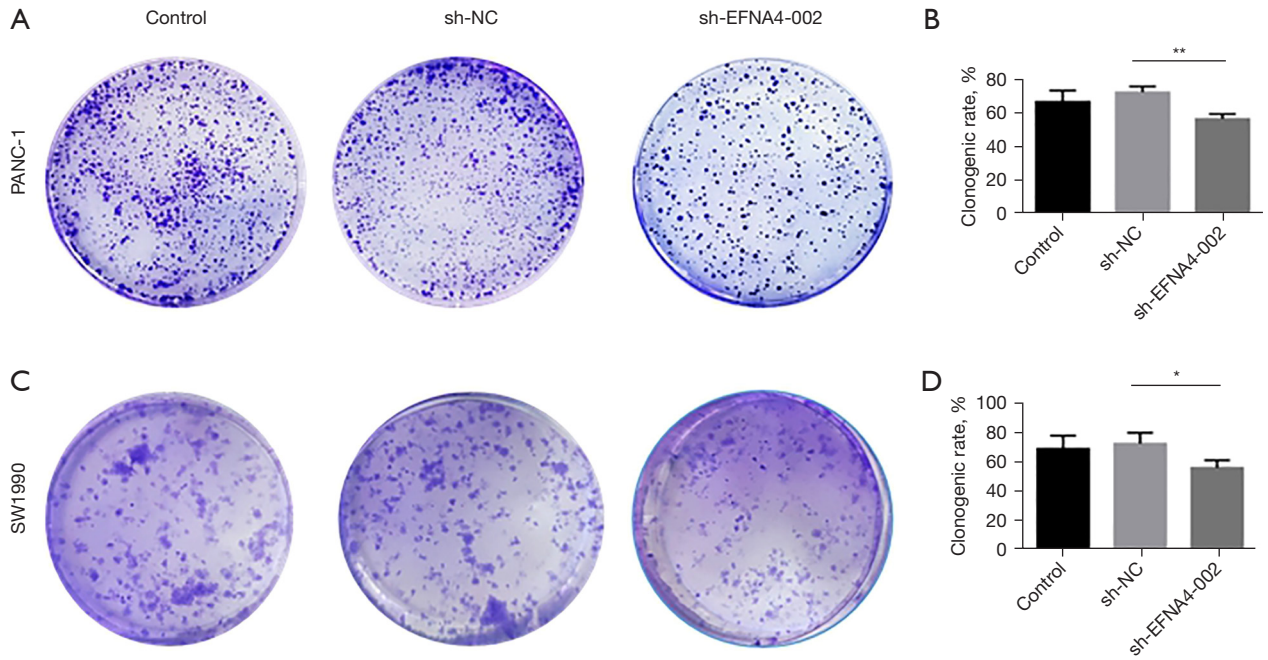

Figure 6 Colony formation ability of pancreatic cancer cells. (A,B) Colony formation ability detection of PANC-1; (C,D) colony formation ability detection of SW1990. Crystal violet staining (magnification: 1×). *, P<0.05; **, P<0.01. NC, negative control; PANC-1, Human Pancreatic Cancer Cells PANC-1; SW1990, Human Pancreatic Cancer Cells SW1990.

Experiments assessing the clonogenic ability of the NC-1 and SW1990 cells indicated that the clonogenic ability of the interference group, sh-*EFNA4*-002, in the two cell lines was significantly lower than that of the control group, sh-NC. This suggested that the expression level of the *EFNA4* gene may be positively correlated with the clone formation ability of the PANC-1 and SW1990 cell lines.

Effect of downregulating EFNA4 expression on the invasive ability of pancreatic cancer cells

Transwell migration assay was used to detect the invasion of pancreatic cancer cell line SW1990. It was found that the cell migration number of the sh-*EFNA4* group (36±7) was significantly lower than that of sh-NC group (102±20) (P<0.01; *Figure 7A*,*7B*).

Similar results were obtained for the invasion ability of PANC-1. Compared with the cell migration number of the sh-NC group (145±28), that of the sh-*EFNA4* group (51±4) was significantly lower (P<0.01; *Figure 7C*,*7D*)

Regarding the clonogenic ability of PANC-1 and SW1990 cells, the invasion ability of sh-*EFNA4*-002 in the interference group was significantly lower than that of sh-NC in the control group. These results indicated that the *EFNA4* gene expression level may be positively correlated with the invasion ability of the PANC-1 and SW1990 cell lines.

Discussion

In the field of pancreatic cancer treatment, novel therapies targeting specific molecular targets are continuously emerging. KRAS inhibitors are targeted at the most common *KRAS* gene mutations found in pancreatic cancer, with Sotorasib and Adagrasib being two drugs under investigation (23,24). Additionally, PARP inhibitors such as Olaparib, which target *BRCA1/2* mutations, have demonstrated efficacy in clinical trials for patients with pancreatic cancer and have been approved by the FDA for this use (25). Although these have shown effectiveness in some clinical trials, drug resistance remains an issue (26). Patients with *HER2*-overexpressing pancreatic cancer may respond to HER2-targeted therapies such as trastuzumab (27). Furthermore, therapies targeting the tumor microenvironment, including angiogenesis inhibitors and immunomodulatory agents (28), and therapeutic strategies targeting metabolic pathways are currently under development (29). Despite the progress

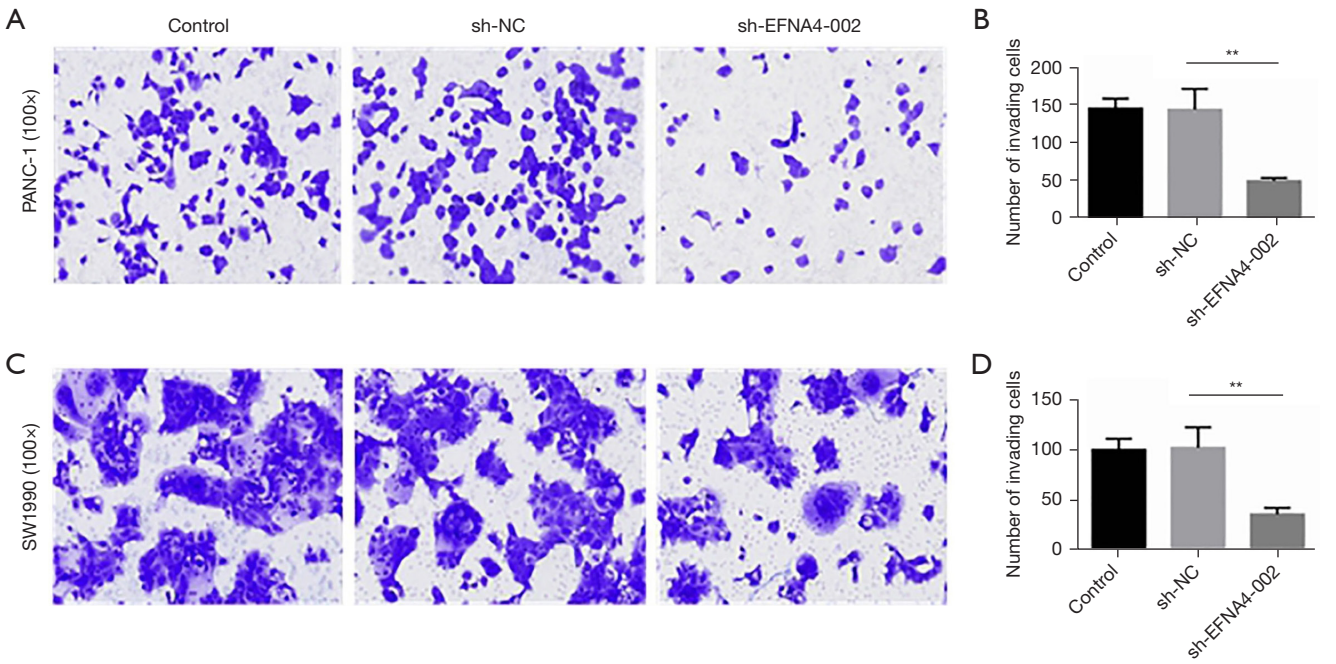

Figure 7 Invasion assays of pancreatic cancer cells. (A,B) Invasion ability detection of PANC-1 cells; (C,D) invasion ability detection of SW1990 cells. Crystal violet staining. **P<0.01. NC, negative control; PANC-1, Human Pancreatic Cancer Cells PANC-1; SW1990, Human Pancreatic Cancer Cells SW1990.

made, the clinical application of targeted therapy for pancreatic cancer still faces challenges, including limited treatment efficacy, issues of drug resistance, and adverse drug reactions. Future research needs to address these challenges and explore new targets and combination treatment strategies to improve treatment outcomes and patient prognosis. *EFNA4*, a molecule that plays a role in various cancers, is being actively investigated for its potential in pancreatic cancer treatment. High expression of *EFNA4* is associated with tumor invasiveness and metastasis, making it an attractive therapeutic target. In previous studies, *EFNA4* has been shown to function as an oncogene in a variety of malignancies and participate in the regulation of tumor cell growth and metastasis (8-12). In this study, through TCGA database analysis, we verified that *EFNA4* is highly expressed in pancreatic cancer. Correlation enrichment analysis of pancreatic cancer DEGs in the *EFNA4* high- and low-expression group indicated that *EFNA4*-related DEGs are involved in the regulation of cell adhesion, signal transduction, cyclic AMP, and other signaling pathways and were related to cancer metastasis and metabolism (30,31). Regulation of ion transmembrane transport has also been implicated in cystic fibrosis of the pancreas, a multistage process of carcinogenesis (32). In the correlation analysis of *EFNA4* expression and the baseline characteristics of patients with pancreatic cancer, *EFNA4* expression was significantly correlated with T stage and TNM stage, suggesting that *EFNA4* may play an important role in the occurrence, development, metastasis, and spread of pancreatic cancer. Therefore, examining the role of *EFNA4* expression in pancreatic cancer cells is crucial to clarifying its function.

We also constructed *EFNA4* shRNA to transfect two pancreatic cancer cells to downregulate *EFNA4* expression, and the interference of shRNA on *EFNA4* expression was successfully confirmed via RT-qPCR experiments at the gene level. In Transwell invasion assay, we observed that the cloning and invasion ability of pancreatic cancer cell lines PANC-1 and SW1990 were significantly decreased when *EFNA4* was downregulated. Therefore, it can be assumed that *EFNA4* plays an important role in the invasion of pancreatic cancer. These findings are consistent with our analysis of the baseline characteristics of patients with pancreatic cancer and *EFNA4* expression. Therefore, the macroscopic phenomenon can be explained from a microscopic perspective: overexpression of *EFNA4* greatly increases the invasion and migration ability of pancreatic

cancer cells, resulting in an increase in the breakthrough of vascular intima and distant spread, thus precipitating lymph node metastasis and distant metastasis.

A malignant tumor is a new organism with the biological characteristics of abnormal cell differentiation, abnormal proliferation, infinite growth, invasion, and metastasis (33,34). The proliferation of tumor is the prelude to metastasis and spread (35-37). Hence, we investigated the effect of *EFNA4* expression on the proliferation ability of pancreatic cancer cells via cloning and CCK8 assays. Similar to the results of Transwell invasion assay, the CCK8 assay showed that the proliferation and cloning ability of the PANC-1 and SW1990 pancreatic cancer cells with downregulated *EFNA4* expression were significantly decreased. This suggests that *EFNA4* plays an important role in the proliferation and cloning ability of pancreatic cancer.

A few limitations to this study should be mentioned. First, we did not investigate the upstream factors, such as transcription factors, that affect *EFNA4* expression. Second, we only explored the effect of *EFNA4* on the proliferation, cloning, and invasion ability of pancreatic cancer cells, and we will further explore the mechanism of the influence of *EFNA4* on the biological behavior of pancreatic cancer cells through *in vitro* and *in vivo* studies. Third, the pan-cancer crosstalk between *EFNA4* and the tumor microenvironment is a critical area of focus, as it pertains to the prognosis and response to immunotherapy in pancreatic cancer. *EFNA4* has been identified as a significant modulator within the tumor microenvironment in gastric cancer by not only facilitating the recruitment and infiltration of immune cells but also by potentially regulating immune responses through its effect on immune checkpoint expression (38). The exploration of *EFNA4*'s role within the tumor microenvironment is required to fully comprehend its implications for pancreatic cancer treatment and prognosis.

EFNA4 also plays the same role in promoting tumor growth and metastasis in pancreatic cancer cell lines as it does in breast cancer, liver cancer, and glioblastoma. Therefore, we believe that *EFNA4* expression in pancreatic cancer not only has diagnostic value but also holds the potential to be a critical therapeutic target.

Conclusions

Our study provides a better understanding of the molecular underpinnings of pancreatic cancer aggressiveness and prognosis. By identifying *EFNA4* overexpression as a marker for poor prognosis, this study offers valuable insights that may influence future research directions and patient management strategies. Moreover, the findings suggest potential therapeutic implications by targeting *EFNA4*, thereby opening new avenues for treatment modalities in a disease notably resistant to current therapeutic options.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-227/rc

Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-227/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-227/coif). 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).

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