

GDF11 restrains tumor growth by promoting apoptosis in pancreatic cancer

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Background: Growth differentiation factor (GDF) acted as a factor that regulated proliferation, apoptosis and differentiation in several tumors. However, the effects of growth differentiation factor (GDF11) in pancreatic cancer remain unclear.

Purpose: To investigate the expression and significance of GDF11 in pancreatic cancer.

Patients and methods: Pancreatic cancer and corresponding paracancerous tissues (n=28) were collected from the Department of Hepatobiliary and Pancreatic Surgical Oncology of Chinese PLA General Hospital. Tissue microarray was obtained from Outdo Biotech Co., Ltd. (Shanghai, People's Republic of China). GDF11 mRNA and protein expressions in pancreatic cancer samples and cell lines were detected using qRT-PCR, Western-Blot and immunohistochemistry. Overexpression and knockdown of GDF11 were performed with lentiviral transduction system and siRNA technique in PANC-1 cell line and CFPAC-1 cell line. Proliferation, migration and invasion of pancreatic cancer cell lines were examined by MTS and transwell assay, respectively. Flow cytometry was used for cell apoptosis analysis.

Results: The results of this study indicated that GDF11 was significantly down-regulated in pancreatic cancer tissues compared with adjacent tissues of pancreatic cancer. GDF11 was also associated with low expression in pancreatic cancer cell lines when compared with normal pancreatic cell line. In a cohort of 63 pancreatic cancer patients, high GDF11 expression levels was associated with favorable perineural invasion, T classification, N classification and overall survival (OS). Cox proportional hazards model revealed that high GDF11 expression was an independent predictor of favorable prognosis (HR: 0.496; 95% CI: 0.255–0.967; *P*=0.040). Overexpression of GDF11 in PANC-1 cells repressed the proliferation, migration and invasion abilities in vitro. Inhibition of GDF11 in CFPAC-1 showed inverse results. Furthermore, enhanced GDF11 expression promoted apoptosis and down-regulated GDF11 expression inhibited apoptosis in pancreatic cancer cell lines.

Conclusion: These findings suggested that GDF11 acted as a tumor suppressor gene for pancreatic cancer.

Keywords: GDF11, pancreatic cancer, proliferation, apoptosis, prognosis

Introduction

Pancreatic cancer is a digestive system tumor with high degree of malignancy and poor prognosis, accounting for the sixth leading cause of cancer mortality rate in People's Republic of China.^{1,2} The incidence and mortality rates of pancreatic cancer show a sustained increase in the world, with the overall 5-year survival rate less than 8%.³ Its low 5-year survival rate is mainly due to the early metastasis to regional lymph nodes and terminal hematogenous metastasis to distant organs.⁴ Lack of early observable symptoms, higher staging at the time of diagnosis, and few effective therapies are largely responsible for the poor prognosis of pancreatic cancer. Recently, more

researches have focused on the tumor biomarkers that were associated with pancreatic cancer prognosis.^{5,6} Therefore, it is imperative to identify novel prognostic molecules for improving the outcomes of pancreatic cancer patients.

The growth differentiation factor 11 (GDF11), also called bone morphogenetic protein 11 (BMP11), is a secreted protein in TGF- β superfamily and BMP subfamily, which regulates diverse developmental processes from anterior/posterior patterning to myogenesis.^{7,8} It is closely regulated to a negative regulator of muscle growth, GDF8 (myostatin).⁹ With regard to cancer, BMP acted as a factor that regulated cell proliferation, apoptosis, and differentiation^{10–12} and was also shown to be correlated with various cancers such as esophageal cancer,¹³ prostate cancer,¹⁴ and breast cancer.¹⁵ GDF11 has been measured in different tissues, such as the pancreas, intestine, kidney, skeletal muscle, heart and developing nervous system, olfactory system, and retina.¹⁶ Abnormal expression of GDF11 has been found in colorectal carcinoma, and high GDF11 expression was related to poorer prognosis.¹⁷ A recent study has demonstrated that GDF11 showed tumor-suppressor function in triple-negative breast cancer.¹⁸ However, to our knowledge, there is no information regarding GDF11 in pancreatic cancer, and the biological function of GDF11 in cancer cells remains largely unknown.

In this study, we first examined the expression of GDF11 in pancreatic cancer. We identified GDF11 as a novel suppressor of pancreatic cancer growth and metastasis through *in vitro* studies. We also evaluated its association with clinicopathological characteristics and then assessed its prognostic value in pancreatic cancer patients.

Patients and methods

Patients

This study was conducted after approval by the Ethic Committee of Chinese PLA General Hospital, and written informed consent was obtained from each patient. Tissue samples including tumor tissues and adjacent tissues of cancer were obtained from a total of 28 patients who had undergone surgical resection and confirmed the diagnosis of pancreatic cancer by postoperative pathology between December 2016 and March 2017 in Chinese PLA General Hospital, Beijing, People's Republic of China. All the tissues were put into liquid nitrogen for frozen immediately after resection and stored at -80°C for RT-PCR analysis and Western blot.

Cell lines and culture

Pancreatic cancer cell lines AsPC-1, BxPC-3, CFPAC-1, PANC-1, and SW1990 and human pancreatic duct epithelial cell line HPDE6-C7 were purchased from China

Infrastructure of Cell Line Resources (Beijing, People's Republic of China) and cultured in Dulbecco's Modified Eagle's Medium (Hyclone Co., Logan, UT, USA) and supplemented with 10% FBS (Hyclone Co.) at 37°C in a humidified 5% CO_2 air incubator.

RNA extraction and qRT-PCR

Total RNA was extracted from fresh tumor tissues and adjacent tissues of cancer of 28 patients using TRIzol reagent (Qiagen, Hilden, Germany) and was reverse-transcribed using a High Capacity cDNA Reverse Transcription kit (TaKaRa, Kusatsu, Japan). Levels of the corresponding peptidyl prolyl isomerase A (PPIA) and GDF11 mRNA were detected by qRT-PCR using the ABI7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Housekeeping gene PPIA was used as internal standard for GDF11 mRNA. The sequences of the qRT-PCR primer were as follows: GDF11 forward, 5'-GATCTTGAGCAAAGTGCAGC-3'; GDF11 reverse, 5'-TGAAGTCGATGCTCTGCCAA-3'; PPIA forward, 5'-TCATCTGCACTGCCAAGACTG-3'; PPIA reverse, 5'-CATGCCTTCTTTCAC TTTGCC-3'.

Western blot analysis

Five pancreatic cancer cell lines, a human pancreatic duct epithelial cell line, eight samples of pancreatic cancer tissue and paired nontumor tissue were used for Western blot analysis. Proteins were extracted from cells and tissues using RIPA lysis buffer with proteinase inhibitor. Then, proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk and 0.01% Tween-20 in tris-buffered saline (TBS; pH 7.6) and incubated with GDF11 antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) diluted at 1:200 in TBS overnight at 4°C . Subsequently, a 1:5,000 dilution of the goat antimouse IgG alkaline phosphatase-linked secondary antibody (Sigma-Aldrich Co., St Louis, MO, USA) was incubated at room temperature. The antigen-antibody complex was visualized by ImmobilonTM Western Chemiluminescence HRP Substrate (EMD Millipore, Billerica, MA, USA).

Tissue microarray and immunohistochemistry (IHC)

Tissue microarray was obtained from Outdo Biotech Co., Ltd. (Shanghai, People's Republic of China). IHC studies of GDF11 were performed on pancreatic cancer samples of tissue microarray. IHC was performed as previously described.¹⁹ Rabbit antihuman polyclonal GDF11

antibody (Abcam Co., Cambridge, MA, USA) was used at a 1:500 dilution. Quantitative analysis of the staining was performed based on the percentage of positive cells and staining density by three experienced pathologists using 12 standard points. For data analysis, staining scores <9 were defined as low expression, and scores of 9–12 indicated high expression.

Flow cytometry

For cell apoptosis analysis, 1×10^6 cells in PBS were stained with FITC-Annexin V and PI and incubated in the dark at room temperature. Annexin V/PI staining assays were performed following the manufacturer's protocol (BD Biosciences, San Jose, CA, USA), and the samples were assessed using flow cytometry. Data acquisition and analysis were performed on an FC500 MPL system (Beckman Coulter, Inc., Brea, CA, USA).

Plasmid construction

V5-tagged GDF11 was constructed by cloning the full-length cDNA into pLV-sfGFP-Puro vectors, yielding lentiviral vectors (LV)-GDF11. Empty vector with no cDNA fragment was used as the negative control (NC), termed LV-Control.

RNAi treatment

siRNAs targeting GDF11 and related NC were chemically synthesized by GenePharma (Shanghai, People's Republic of China). The sequences of the GDF11 siRNA are as follows: 5'-CCGAGACCGUCAUUAGCAUTT-3' (sense) and 5'-AUGCUGAAUGACGGUCUCGGTT-3' (antisense). The sequences of siNC are as follows:

5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense). Transfection was carried out using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol.

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

Cell growth was measured by MTS assays (Promega, Madison, WI, USA) in 96-well plates (1,000 cells per well) following manufacturer instructions.

Transwell migration and invasion assay

Cells in medium with no FBS were seeded into upper chambers of Matrigel-coated (8 μ m pore size; HTSTM Fluoroblok invasion system, BD Biosciences) or -uncoated polycarbonate membrane filters (8 μ m pore size; Corning

Incorporated, Corning, NY, USA). The lower chamber was filled with medium containing 10% FBS. Then, cells were incubated in 37°C. After 24 and 48 hours, cells that migrated through the membrane and attached to the bottom of the membrane were fixed and stained with 0.05% crystal violet for 30 minutes. Images were captured from five random fields under microscope. All experiments were performed in triplicate.

Statistical analysis

SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Data were expressed as the mean \pm standard deviation (SD). Student's *t*-test was used to compare data between two groups. Pearson chi-squared test or Fisher's exact test was used to analyze the relationship between GDF11 expression and clinicopathological characteristics. Kaplan–Meier analysis was performed to assess the differences in survival rates. Each test was two sided, and $P < 0.05$ was considered statistically significant.

Results

GDF11 mRNA and protein expressions in pancreatic cancer samples and cell lines

To validate the protein and mRNA expression level of GDF11, 28 pairs of tissues including pancreatic cancer samples and matched paracarcinoma tissue samples were detected by qRT-PCR and eight pairs were detected by Western blot in this study. The mRNA and protein levels of GDF11 were decreased in pancreatic cancer samples compared to normal tissues (Figure 1A and B). Western blot and qRT-PCR analyses revealed that GDF11 expression was downregulated in pancreatic cancer cell lines than HPDE6-C7 cell line (Figure 1C and D). Moreover, IHC staining of 28 pairs of pancreatic adenocarcinoma tissues and normal pancreas tissues revealed that GDF11 was localized in cytoplasm. High-level expression of GDF11 was detected in 24/28 (85.7%) of adjacent paracarcinoma tissues, but only 10/28 (35.7%) in cancerous tissues (Figure 1E). The results acquired demonstrated that GDF11 might serve as a tumor suppressor in pancreatic cancer.

The association between GDF11 expression and clinicopathological characteristics

The correlation of GDF11 expression with clinicopathological characteristics in tissue microarray of 63 pancreatic cancer patients is further summarized in Table 1. Same as above,

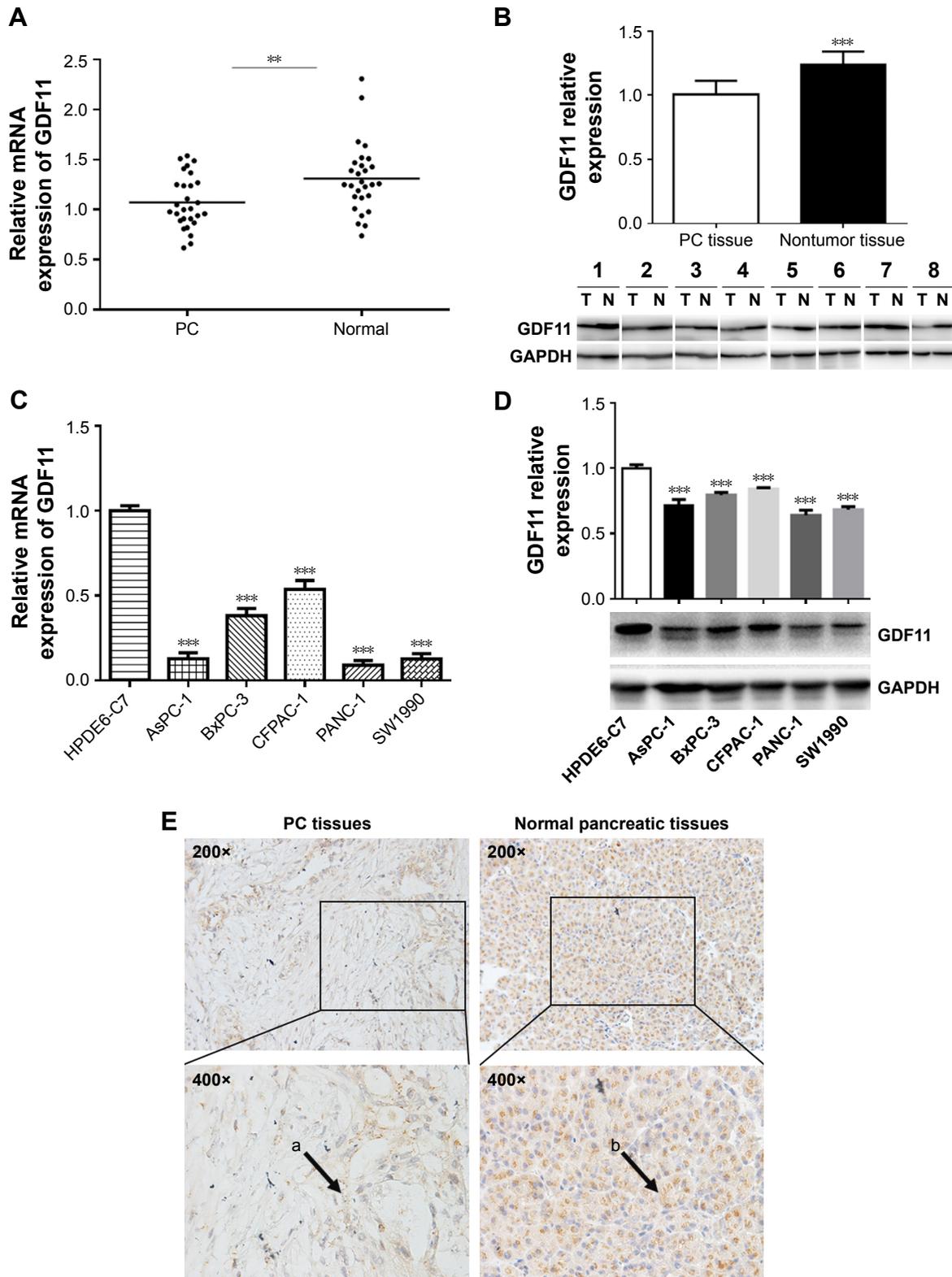


Figure 1 The mRNA and protein expression of GDF11 in pancreatic cancer cell lines and clinical samples.

Notes: (A) qRT-PCR analysis of GDF11 levels in pancreatic cancer tissues and normal pancreatic tissues (n=28). (B) Protein levels of GDF11 were detected by Western blot in eight pairs of pancreatic cancer tissues and normal pancreatic tissues. (C) The mRNA expressions of GDF11 in the normal pancreatic cell line HPDE6-C7 and five pancreatic cancer cell lines were examined by qRT-PCR. (D) Western blot analysis of GDF11 in the HPDE6-C7 and five pancreatic cancer cell lines. (E) Representative images and its regional magnification of GDF11 IHC in pancreatic cancer tissues and their paired normal tissues. a: Low expression of GDF11 in pancreatic cancer tissues; b: high expression of GDF11 in the cytoplasm of normal pancreatic tissues. Data shown represent the mean \pm SD. ** $P < 0.01$. *** $P < 0.001$.

Abbreviations: GDF11, growth differentiation factor 11; IHC, immunohistochemistry; PC, pancreatic cancer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1 The relationship between GDF11 expression and clinicopathological characteristics in tissue microarray of 63 pancreatic cancer patients

Characteristics	N	GDF11		P-value
		High expression	Low expression	
Gender				0.212
Male	36	13	23	
Female	27	14	13	
Age (years)				0.229
≤65	12	7	5	
>65	51	20	31	
Differentiation				0.941
Well/moderate	37	16	21	
Poor	26	11	15	
Perineural invasion				0.019
No	36	20	16	
Yes	27	7	20	
T classification				0.010
T1–T2	28	17	11	
T3–T4	35	10	25	
N classification				0.029
N0	32	18	14	
N1	31	9	22	

Abbreviation: GDF11, growth differentiation factor 11.

IHC staining showed that the high-level expression rate of GDF11 was lower in cancerous tissues than that in normal tissues (Figure 2A). Kaplan–Meier method for overall survival (OS) indicated that the patients with high GDF11 expression had significantly better survival rates compared to patients with low GDF11 expression (Figure 2B). Univariate analysis revealed that GDF11 expression ($P=0.001$), differentiation

($P=0.026$), T classification ($P=0.024$), and N classification ($P<0.001$) were significant prognostic factors for OS. Multivariate analysis further indicated high GDF11 expression as an independent biomarker of favorable prognosis (HR: 0.496; 95% CI: 0.255–0.967; $P=0.040$). However, age, gender, and perineural invasion were not associated with OS (Table 2).

Overexpression of GDF11 suppressed proliferation, migration, and invasion while knockdown of GDF11 facilitated these functions in pancreatic cancer cell lines

Lentiviral transduction system was used to elevate the expression level of GDF11 in PANC-1 cell line and study biological functions of GDF11 in pancreatic cancer cell. Moreover, we knocked down GDF11 expression using the siRNA technique in CFPAC-1 cell line for same functional experiments. Overexpression and knockdown of GDF11 were confirmed by qRT-PCR and Western blot (Figure 3A). Enhanced GDF11 expression suppressed cell proliferation in PANC-1 cells while CFPAC-1 cell transfected with GDF11–siRNA showed boosted tumor growth (Figure 3B). In addition, migration and invasion assays showed a decrease of cell migration and invasion in group with high GDF11 expression, and these functions were accelerated in GDF11 downregulated group (Figure 3C and D). These results suggest that GDF11 might act as a tumor suppressor and inhibit the proliferation, migration, and invasion of pancreatic cancer cells.

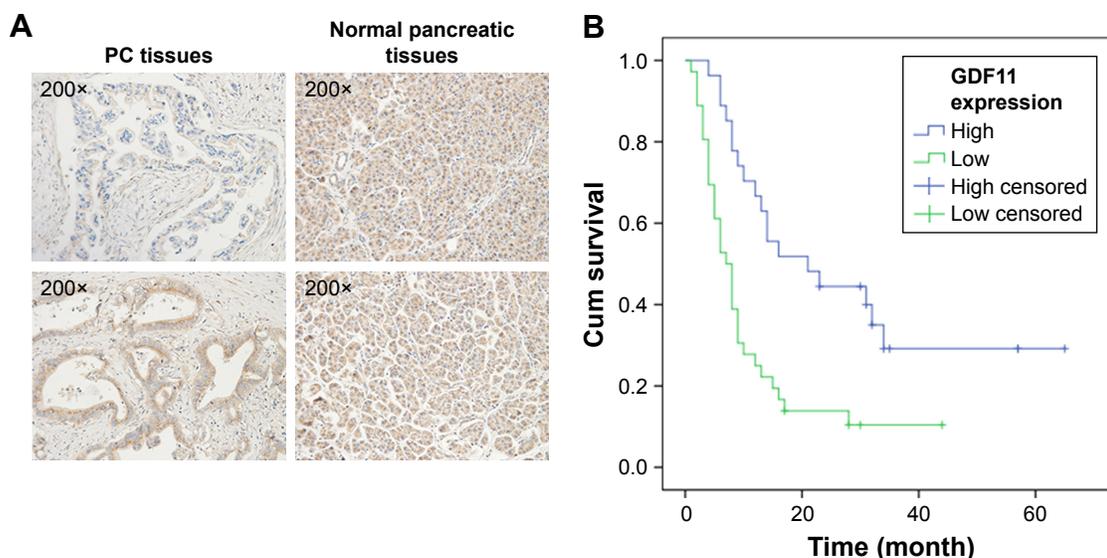


Figure 2 Relationship between GDF11 expression and OS in tissue microarray of pancreatic cancer.

Notes: (A) Representative IHC staining images of GDF11 of paired tumor and nontumor tissues. (B) Kaplan–Meier analysis showed that patients with low expression of GDF11 had a poorer OS than those with high expression of GDF11. $P=0.012$, log-rank test.

Abbreviations: GDF11, growth differentiation factor 11; IHC, immunohistochemistry; OS, overall survival; PC, pancreatic cancer.

Table 2 Univariate and multivariate Cox regression analyses of factors associated with overall survival

Variables	Univariate analysis			Multivariate analysis		
	P-value	HR	95% CI	P-value	HR	95% CI
Gender (female vs male)	0.507	0.826	0.470–1.452			
Age (years) (>65 vs ≤65)	0.835	1.076	0.537–2.156			
Differentiation (poor vs well/moderate)	0.026	1.898	1.080–3.336	0.115	1.597	0.892–2.862
Perineural invasion (yes vs no)	0.335	0.757	0.429–1.334			
T classification (T3–T4 vs T1–T2)	0.024	1.922	1.088–3.395	0.067	1.748	0.961–3.181
N classification (N1 vs N0)	<0.001	3.256	1.793–5.914	0.005	2.544	1.325–4.882
GDF11 (high vs low)	0.001	0.364	0.200–0.662	0.040	0.496	0.255–0.967

Abbreviation: GDF11, growth differentiation factor 11.

Enhanced GDF11 expression promoted apoptosis and downregulated GDF11 expression inhibited apoptosis in pancreatic cancer cell lines

To further investigate GDF11's role in pancreatic cancer, we examined the apoptosis rates in aforementioned cell lines with overexpression and knockdown of GDF11. PANC-1 cell line with enhanced GDF11 expression showed significantly higher apoptosis rates (Figure 4A). In accordance with

the former result, CFPAC-1 cell line with downregulated GDF11 expression showed significantly lower apoptosis rates (Figure 4B).

Discussion

Pancreatic cancer is a complex and heterogeneous disease of the digestive system, which is related to various gene deletions, mutations, and amplifications.²⁰ Its morbidity and mortality have risen significantly in recent years.³ The 5-year survival rate for pancreatic cancer is one of the worst

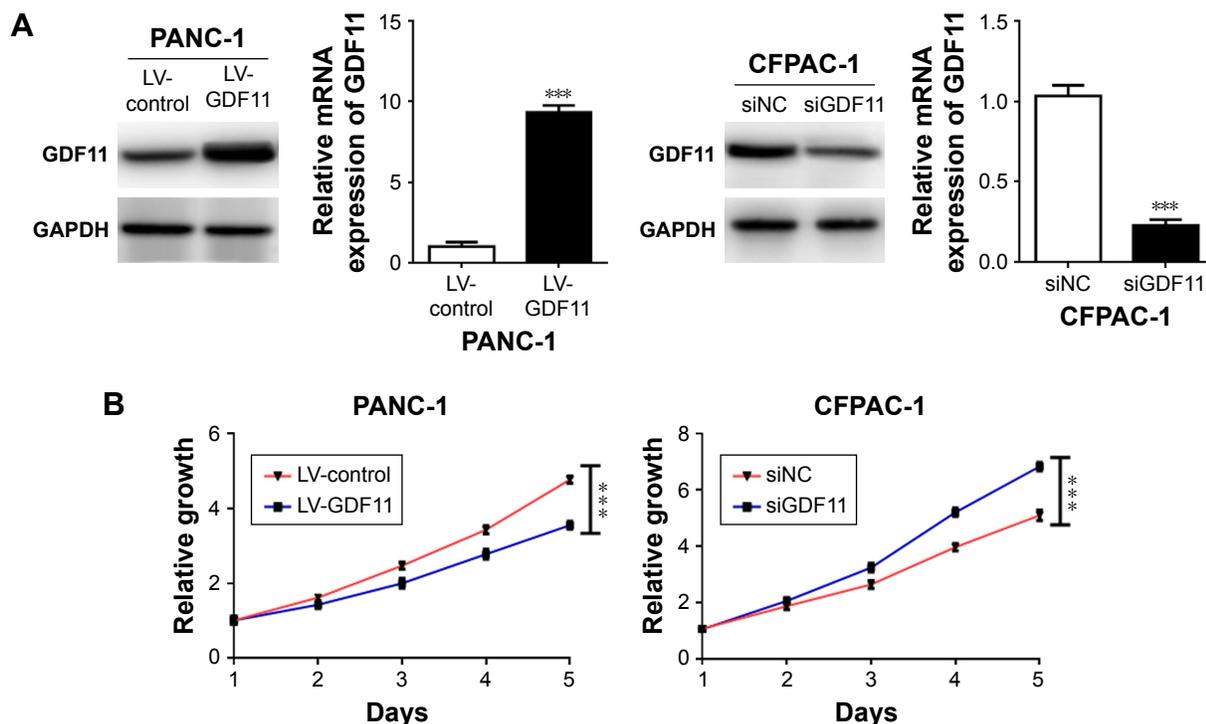


Figure 3 (Continued)

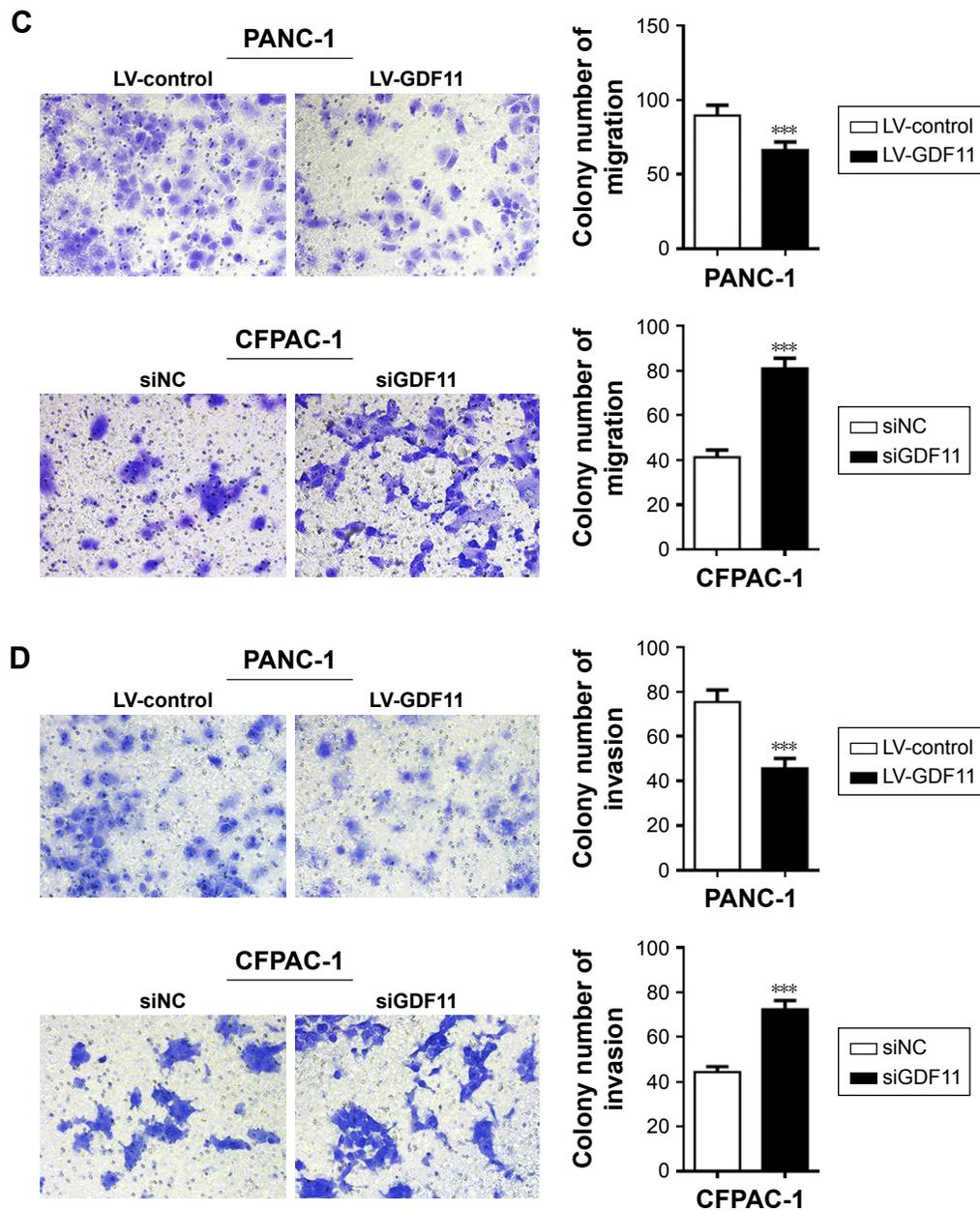


Figure 3 Overexpression of GDF11 suppresses tumor growth and metastasis while knockdown of GDF11 promoted these functions in pancreatic cancer cell line.

Notes: PANC-1 cell was transfected using a lentiviral transduction system to enhance GDF11 expression, and CFPAC-1 cell was treated using siRNA to inhibit GDF11 expression. (A) Elevated and downregulated GDF11 expression was examined by Western blot and qRT-PCR analyses. (B) MTS assay revealed the effects of GDF11 overexpression and knockdown on the proliferation of pancreatic cancer cell lines. (C, D) Representative photographs and cell quantifications of transwell migration and invasion assays of elevated and downregulated GDF11 in pancreatic cancer cell lines. Data shown represent the mean \pm SD. *** P <0.001.

Abbreviations: GDF11, growth differentiation factor 11; LV, lentiviral vectors; NC, negative control; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

prognosis of malignant tumors, at only 5%.²¹ Risk factors for pancreatic cancer include cigarette smoking, obesity, type 2 diabetes mellitus, alcohol consumption, and chronic pancreatitis.⁴ The early stages of the disease are usually asymptomatic, signifying it is difficult for early diagnosis and treatment. Changing in a series of genes involved in specific pathways and processes contributes to the occurrence and development of pancreatic cancer. However, there are

currently no validated biomarkers to predict clinical behavior of this cancer.⁴

In the current study, we first investigated the expression level of GDF11 in tissues and cell lines and then evaluated the relationship between GDF11 protein expression and the clinicopathological features in pancreatic cancer. Results showed that GDF11 expression was significantly lower in pancreatic cancer tissues than that in normal pancreatic tissues. Similar

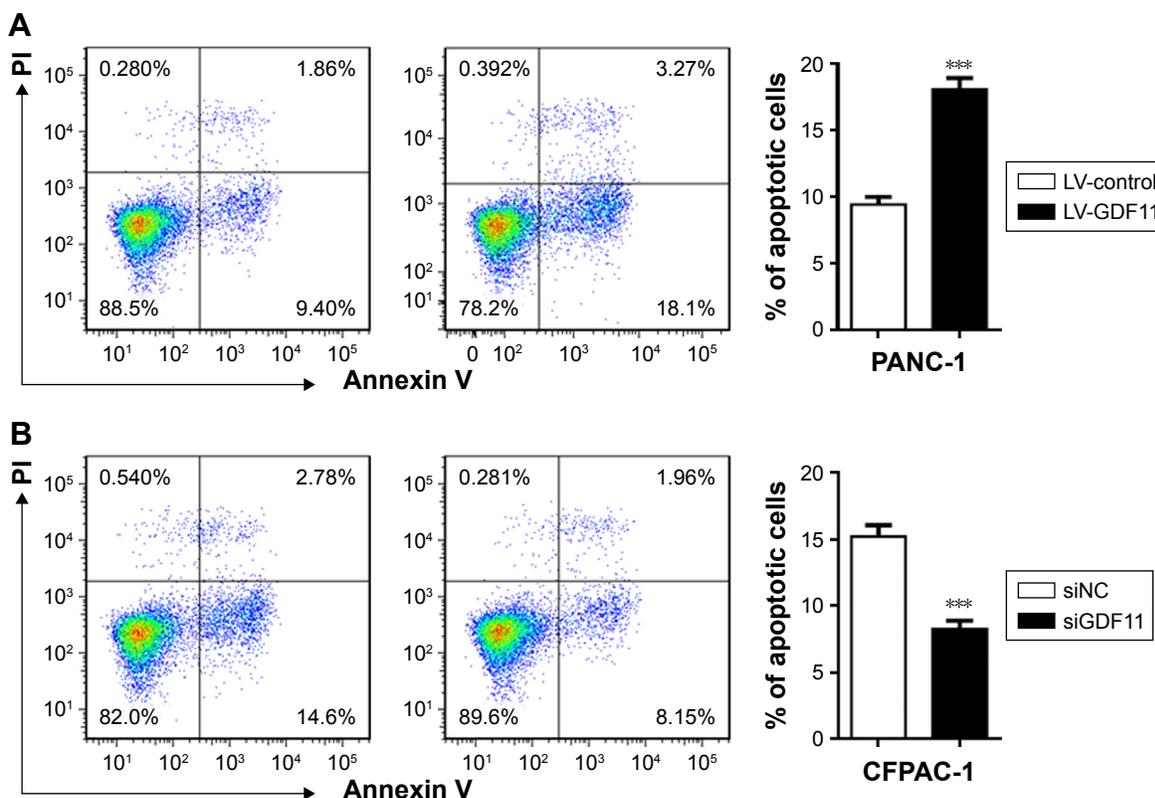


Figure 4 Enhanced GDF11 expression promoted apoptosis and downregulated GDF11 expression inhibited apoptosis in pancreatic cancer cell lines.

Notes: (A) The effect of GDF11 overexpression on the apoptosis of pancreatic cancer cell lines by flow cytometry. (B) The effect of knockdown GDF11 on the apoptosis of pancreatic cancer cell line by flow cytometry. Data shown represent the mean \pm SD. *** $P < 0.001$.

Abbreviations: GDF11, growth differentiation factor 11; LV, lentiviral vectors; NC, negative control.

results were demonstrated from the GDF11 expression examination in pancreatic cancer cell lines and human pancreatic duct epithelial cell line. In the cohort of pancreatic cancer patients, high expression of GDF11 in the tumor tissue was found associated with favorable prognosis. Patients with high GDF11 expression showed a significantly higher OS rate. These results are in accordance with prognostic summary of GDF11 in pancreatic cancer in the Human Protein Atlas database.²² Moreover, our data demonstrated that high GDF11 protein expression was associated with mild clinical features, such as perineural invasion, T classification, and N classification. These findings indicated that the high expression of GDF11 may play important roles in the growth, invasion, and metastasis of pancreatic cancer.

Accordingly, we further explored the functional mechanism of GDF11 expression in pancreatic cancer cells. We enhanced GDF11 expression in PANC-1 cell line using a lentiviral transduction system and attenuated GDF11 expression in CFPAC-1 cell line using RNAi treatment. Functional studies indicated that elevated GDF11 expression promoted apoptosis while proliferation, migration, and invasion were suppressed in PANC-1 cell. Instead, inhibited apoptosis, accelerating cell proliferation, migration, and invasion were

relevant to lower expression of GDF11 in CFPAC-1 cell. Bokobza et al²³ found that GDF9 promoted growth and proliferation of prostate cancer cells by protecting tumor cells from caspase-3-mediated apoptosis. The results of functional experiment showed a tumor-inhibiting effect of GDF11 in pancreatic cancer through restraining tumor growth by promotion of apoptosis.

TGF- β superfamily was known as the dual role in cancer. TGF- β signaling promotes progression, invasion, and metastasis in advanced cancers while its tumor-suppressing effects were found in normal cells and early cancers.^{24–26} Some reports have revealed the prognostic significance of GDF11 expression in several types of tumors.^{17,18,27–29} Yokoe et al¹⁷ found that GDF11 mRNA expression in colorectal cancer tissue was significantly higher than in normal tissue. Patients with high GDF11 expression have a high frequency of lymph node metastasis and a significantly poorer OS, indicating that GDF11 might be a novel diagnostic and prognostic biomarker in colorectal cancer patients. However, a recent study showed that GDF11 exerted tumor-suppressing functions in triple-negative breast cancer and its tumor suppression was lost through defective subcellular processing.¹⁸ In liver cancer, the mRNA and protein expression of GDF11 is downregulated,

and it plays a tumor-suppressing role through Smad2/3-signaling pathway.²⁷ The present study suggested that the aberrant expression of GDF11 was significantly correlated with prognosis of pancreatic cancer patients. Kaplan–Meier method showed that patients with low GDF11 expression had worse OS rate than those with high GDF11 expression.

In summary, we demonstrated that GDF11 protein expression significantly decreased in pancreatic cancer tissues and was correlated with tumor progression in pancreatic cancer. The higher expression of GDF11 was significantly correlated with better OS rate of pancreatic cancer patients. Furthermore, overexpression of GDF11 suppressed aggressive behaviors of pancreatic cancer cells may result from its apoptosis-promoting effect on pancreatic cancer cells. Therefore, GDF11 may be a valuable predictive biomarker in assessing the severity and prognosis of pancreatic cancer patients.

Acknowledgments

This study was approved by the ethics committee of Chinese PLA General Hospital and was carried out in accordance with the Declaration of Helsinki of the World Medical Association. All enrolled patients provided written informed consent.

Disclosure

The authors declare no conflicts of interest in this work.

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