

Original Article

The up-regulation of Axl is associated with a poor prognosis and promotes proliferation in pancreatic ductal adenocarcinoma

Weihua Yu, Xiaolong Ge, Xusheng Lai, Jiemin Lv, Yong Wang

Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang Province, China

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Abstract: Pancreatic cancer is one of the most aggressive tumors and has a very poor prognosis. Recent studies show that Axl plays a key role in the occurrence and development of pancreatic cancer tumors. However, the expression and role of Axl in pancreatic cancer has not been reported. This study aimed to reveal the clinical significance of Axl expression in patients with pancreatic cancer and determine its mechanisms. In this study, western blot and immunohistochemistry were used to show that the expression of Axl in pancreatic cancer cell lines and tissues is significantly higher than its expression in corresponding non-tumor, normal tissues. By statistically analyzing clinical and pathological data, we found that there is a correlation between Axl expression and TNM stages and T stages, and Axl positive expression indicates a worse prognosis. According to in vitro assays, the proliferation of pancreatic cancer cells decreased, and the apoptosis level increased with Axl knockdown. Meanwhile, the knockdown of Axl increased the sensitivity of pancreatic cancer to gemcitabine. Moreover, AKT and ERK1/2 pathway proteins decreased with Axl knockdown. In conclusion, our results suggest that Axl is highly expressed in pancreatic cancer and is a prognostic factor. It may also be a potential biomarker and therapy target for pancreatic cancer.

Keywords: Pancreatic cancer, prognosis, Axl, gemcitabine, proliferation

Introduction

Pancreatic cancer is one of the most lethal digestive tract malignancies, is difficult to detect early, and has a poor prognosis. The 5-year overall survival rate has remained relatively stable, at close to 8%, over the past decade [1, 2]. Most patients present with metastatic disease at the time of diagnosis, and only 20% of the patients are eligible for surgery [3, 4]. Thus, a further exploration of potential new biomarkers and an elucidation of the underlying mechanisms of pancreatic cancer are therefore urgently needed.

The tyrosine-protein kinase receptor UFO (Axl) belongs to TAM family of receptor tyrosine kinases, which consists of Axl, tyrosine-protein kinase receptor Tyro3, and tyrosin protein kinase Mer. Axl has been demonstrated to be an important factor in certain tumors including non-small cell lung carcinoma (NSCLC), breast cancer, prostate cancer, myeloid leukemia, and ovarian cancer [5-8]. The growth arrest specific

gene 6 (Gas6) is a ligand of Axl, and Axl activation and signaling by Gas6 have been implicated in multiple cellular responses, including angiogenesis, proliferation, the epithelial-to-mesenchymal transition (EMT), invasiveness, and survival [9-13].

The role of Axl in tumors has attracted researchers' attention. However, the expression and role of Axl have not been reported in pancreatic cancer. This study further explored the expression of Axl in pancreatic cancer and its correlation with patients' clinicopathological features and their prognoses, with the goal of understanding its role in the development and progression of pancreatic cancer.

Materials and methods

Cell culture

Human pancreatic cancer cell lines, including Panc-1, SW1990, CFPAC-1, MIAPaCa-2, and normal pancreatic ductal epithelial cells

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HPDE6-C7 were all obtained from the Chinese Academy of Sciences (Shanghai, China). The CFPAC-1 cell line was cultured in an IMDM medium with 10% fetal bovine serum (FBS, Gibco, New York, USA). PANC-1, SW1990, MIAPaCa-2 and HPDE6-C7 cell lines were cultured in DMEM medium with 10% FBS.

Western blot analysis

Total proteins were isolated, and the protein concentration was measured. Identical quantities of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filter membranes. After an incubation with antibodies against Axl (1:1000, 8661, Cell Signaling Technology), AKT (1:1000, ab192623, Abcam), p-AKT (1:1000, ab192623, Abcam), ERK (1:1000, 4695, Cell Signaling Technology), p-ERK (1:1000, 4370, Cell Signaling Technology) antibodies, and antibodies against GAPDH (1:5000, ab181602, Abcam) were used as an internal control. The samples were incubated with secondary goat anti-rabbit antibodies conjugated with horseradish peroxidase (1:5000, Pierce, USA) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Human tissue specimens and immunohistochemical analysis

A total of 51 cases of formalin-fixed and paraffin-embedded pancreatic ductal adenocarcinoma and adjacent normal pancreatic tissue specimens were collected from the Sir Run Run Shaw Hospital. The research protocol was reviewed and approved by the Research Ethics Committee of Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University. All participants or their guardians gave written consent for their tissue samples and medical information to be used for scientific research. All cases were confirmed by pathological diagnosis and were analyzed according to the 8th edition of the UICC 2017 TNM classification.

A two-step assay was used for the immunohistochemistry stain for Axl. Briefly, after baking at 65°C for 2 h, the sections (4 µm thickness) were dewaxed in xylene, hydrated using a graded series of alcohols (100%, 95% and 85%) and rinsed with PBS. Antigenic retrieval was per-

formed by submerging the samples in citric acid (pH 6.0) and microwaving. To block any nonspecific binding, the sections were treated using a 0.3% hydrogen peroxide solution for 15 min. Then, the sections were incubated overnight at 4°C using the Axl antibody (1:100, 8661, Cell Signaling Technology) as described previously and examined using HRP Envision Systems (Dako, Shanghai, China). Finally, the sections were visualized after counterstaining with hematoxylin. To score Axl as immunopositive staining, Axl expression was classified semi-quantitatively according to the following criteria: 0: negative, none or less than 5% positive cells), 1: 5-25% positive cells, 2: 26-50% positive cells, and 3: more than 50% positive cells. The dominant staining intensity was scored as: 0 indicates negative; 1 indicates weak; 2 indicates intermediate; and 3 indicates strong staining intensity. The expression was calculated by adding a density score (0-3) and an intensity score (0-3) before categorizing the samples into low and high expressions. High expression was defined as a score ≥ 3 .

Transient transfection

Axl siRNA (stQ0004790-1) and the matched negative controls were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Briefly, 1×10^5 cells were seeded onto 6-well culture plates. Cell lines were transfected in a serum-free medium, and transfection was performed using Lipofectamine 3000 following the manufacturer's protocol (Invitrogen, California, USA). After 24 h of transfection, the cells were kept in a culture medium containing 10% FBS for up to 48 h. The cells were then harvested and subject to PCR or a Western blot analysis.

RNA extraction and qRT-PCR

The total RNA of the cells was isolated and purified using a miRNeasy Mini Kit (Qiagen, Maryland, USA), following the manufacturer's instructions. The reverse transcription (RT) was performed using a PrimeScript RT reagent Kit (Takara, Otsu, Japan) following the manufacturer's instructions. The Axl and GAPDH primers were designed as follows: Axl forward 5'-TCAAGGTGGCTGTGAAGACGA-3' and reverse 5'-CGTTCAGAACCCTGGAACAGAC-3'; GAPDH forward 5'-CGGAGTCAACGGATTTGGTCGTAT-3', and reverse 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

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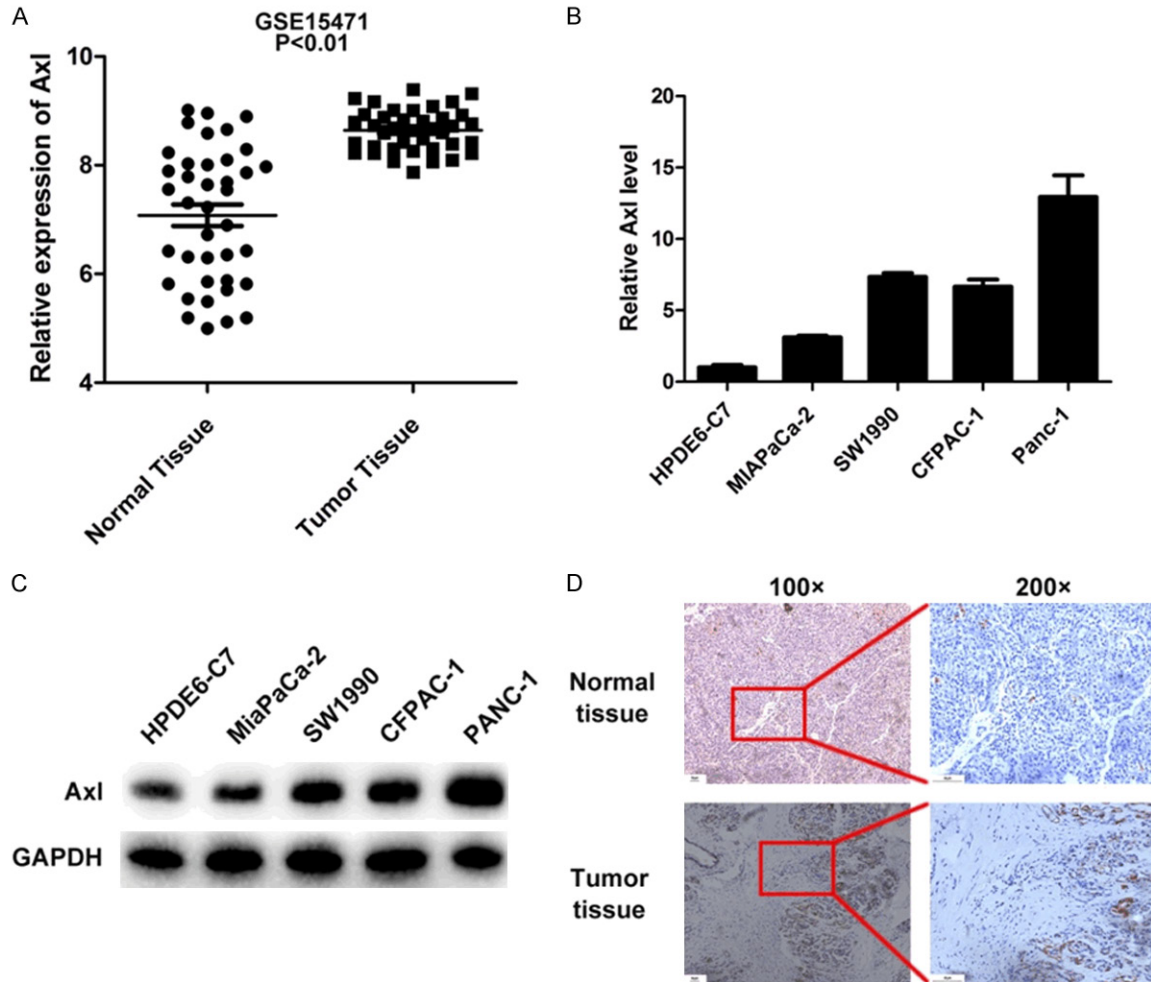


Figure 1. The expression of Axl in pancreatic cancer cell lines and tissues. A: Relative Axl mRNA expression in pancreatic cancer tissues and adjacent normal pancreatic tissues by analyzing GEO database GSE15471. B: The expression of Axl in pancreatic cancer cell lines was detected by qRT-PCR. C: The expression of Axl in pancreatic cancer cell lines was detected by Western blot. D: Immunohistochemistry was used to detect the expression of Axl in pancreatic cancer tissues and normal pancreatic tissues. Data are expressed as mean \pm SEM (n=3).

Cell proliferation assay

The cell proliferation was measured using a CCK-8 assay. The cells were seeded at a density of 5×10^3 /well into 96-well plates and cultured for 72 h. The cells were then incubated with 20 μ l CCK-8 for 2 h at 37°C. The absorbance at 490 nm was recorded.

Cell apoptosis assays

For the cell apoptosis analysis, the cells were collected by trypsinization and washed three times using PBS, and then the cells were treated with AnnexinV-PE and 7AAD for 30 min according to the instruction manual. The samples

were acquired on a BD FACSaria III Cell Sorting System (Becton Dickinson, New York, USA) before undergoing an analysis using the BD FACSDiva software 6.1.3 (Becton Dickinson).

Statistical analysis

The data were compiled with the software package SPSS, version 19.0. The data were presented as the mean \pm standard error (SE). Fisher's exact and χ^2 tests were used to assess the associations between the Axl expressions and the clinicopathological parameters. A univariate survival analysis was performed according to Kaplan-Meier, and differences in the survival curves were assessed with a log-rank test. A p

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Table 1. Relationship between Axl expression and clinicopathological features

Parameters	(n)	AXL expressions		P values
		-	+	
Age				
<60	17	6	11	0.834
≥60	34	11	23	
Gender				
Male	24	8	16	1.000
Female	27	9	18	
TNM stage				
I~II	29	13	16	0.041*
III~IV	22	4	18	
Pathological grade				
Poor	21	6	15	0.546
Middle and high	30	11	19	
T stage				
T1-2	13	9	4	0.005*
T3-4	38	8	30	
Lymph node metastasis				
No	26	8	18	0.692
Yes	25	9	16	
Distant metastasis				
M0	47	15	32	0.593
M1	4	2	2	

*P<0.05.

value <0.05 was considered statistically significant.

Results

The expression of Axl in pancreatic cancer cell lines and tissues

By analyzing gene expression data from the GEO database that included 36 pairs of pancreatic cancer and corresponding non-tumor normal tissues, we found that the mRNA expression of Axl was significantly higher in the pancreatic cancer tissues compared with the normal tissues (**Figure 1A**, $P<0.01$). We confirmed the high expression of Axl in the pancreatic cancer cell lines through PCR and western blot analyses when compared with normal pancreatic ductal epithelial cells HPDE6-C7 (**Figure 1B, 1C**). We also performed an immunohistochemical analysis of Axl in 51 cases of pancreatic cancer and normal pancreatic tissue specimens. It was shown that the Axl expression was mainly located on the cell membranes and in the cytoplasm (**Figure 1D**).

Correlation between Axl expression and the patient's clinicopathological features

The 51 patients were divided into two groups according to their Axl expression levels. By statistically analyzing the clinical and pathological data, we found that there is a correlation between the Axl expression level and TNM stage and T stage (**Table 1**). The TNM stage and T stage in the Axl positive expression group tend to be higher than the stages in the Axl negative expression group (**Figure 2A, 2B**). In addition, we followed up with the patients and found that the survival duration of the Axl positive expression group was significantly lower than the survival duration of the Axl negative expression group (**Figure 2C**, $P=0.005$). This indicates that Axl expression is an important factor affecting the prognosis of pancreatic cancer patients.

The knockdown of Axl inhibits cell proliferation and promotes cell apoptosis

We knocked down Axl by transfection of siRNA in the Panc-1 cell line. It was determined by PCR that the mRNA level was significantly decreased at 48 h after transfection (**Figure 3A**), and a Western blot assay determined that the protein level was also down-regulated. (**Figure 3B**). The cell counting kit-8 assay showed that the cell proliferation rate was decreased significantly after the Axl knockdown (**Figure 3C**). Moreover, the flow cytometry results indicated that the apoptosis level was increased after the Axl knockdown (**Figure 2D**). Based on these findings, the down-regulation of Axl inhibits cell proliferation in PDAC cells mainly via its effects on cell apoptosis.

Knockdown of Axl increases the sensitivity of pancreatic cancer to gemcitabine

By analyzing GSE80617 in the GEO database, we discovered that Axl expression was significantly increased in gemcitabine-resistant pancreatic cancer cell lines when compared with parental cells (**Figure 4A**). Based on our results, we hypothesized that the change in Axl expression may affect the chemosensitivity of gemcitabine in pancreatic cancer cells. Panc-1 cells were treated with gemcitabine following the knockdown of Axl, and the killing effect of gemcitabine was increased significantly (**Figure 4B**). Therefore, Axl may be involved in the devel-

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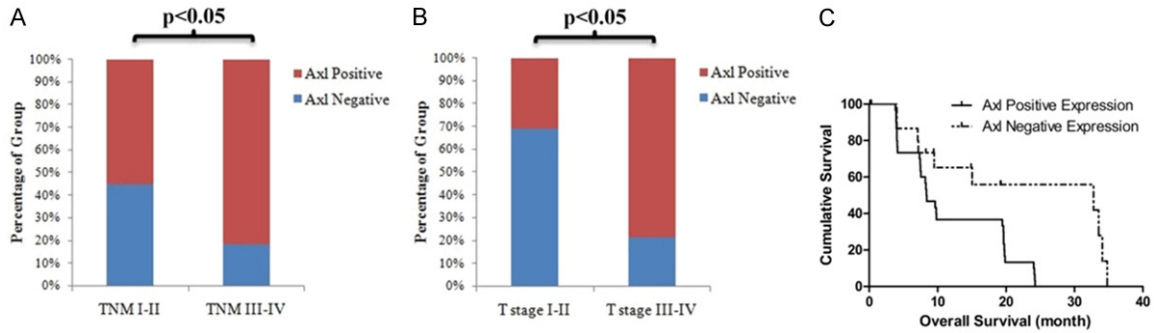


Figure 2. Correlation between Axl expression and clinicopathological features. A: In the Axl positive expression group, the TNM stages tend to be higher than the states in the Axl negative expression group. B: In the Axl positive expression group, the T stage tends to be higher than the stages in the Axl negative expression group. C: The effect of the Axl expression level on overall survival in 51 pancreatic cancer patients.

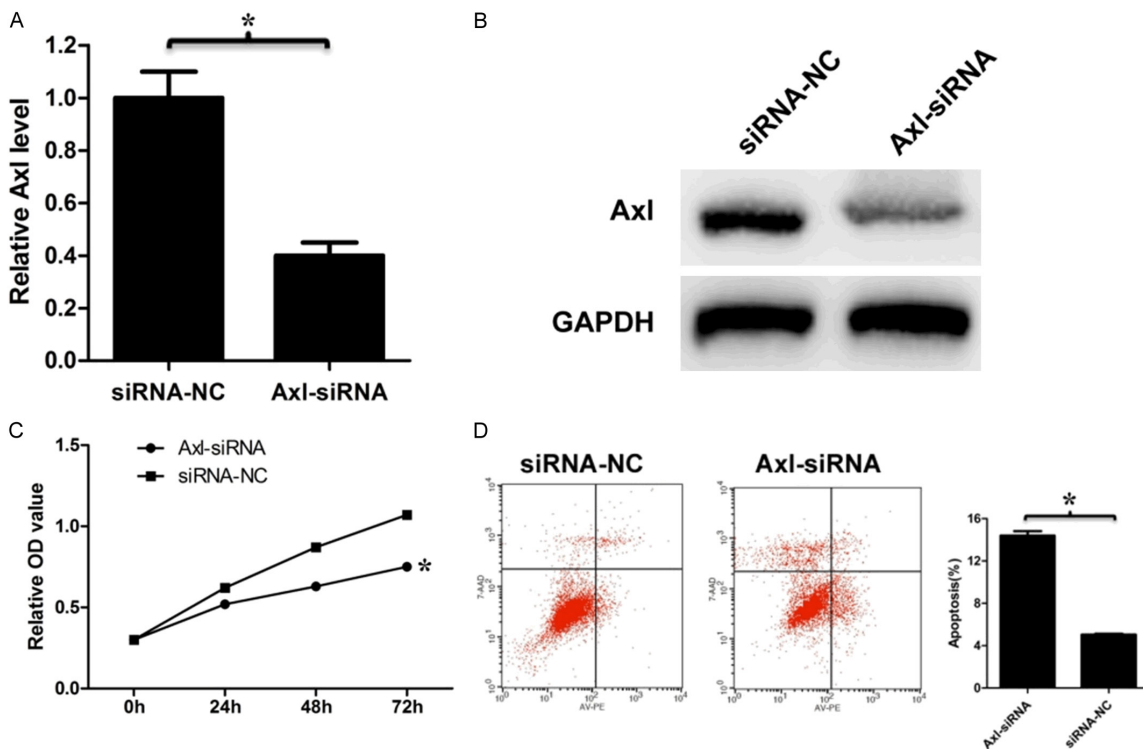


Figure 3. Knockdown of Axl inhibits cell proliferation and promotes cell apoptosis. A: Axl mRNA level in Panc-1 cell line transfected with siRNA or negative control. B: Axl protein level in Panc-1 cell line transfected with siRNA or negative control. C: CCK-8 assay showed Axl knockdown significantly suppressed the proliferation. D: Flow cytometric analysis showed apoptosis in Axl knockdown cells was increased compared to the control. Data are expressed as the mean \pm SEM (n=3). *indicated $P < 0.05$.

opment of gemcitabine resistance in pancreatic cancer.

The effect of Axl on the blockage of AKT and ERK phosphorylation

In order to explore the mechanism of Axl's involvement in the chemosensitivity of gem-

citabine, we detected the downstream signaling pathway using Western blot. We found that p-AKT and p-ERK expressions were significantly lower in the Axl knockdown cells compared with the controls (Figure 5A, 5B). Therefore, the decreased expression of Axl may increase the sensitivity of pancreatic cancer to gemcitabine by inhibiting AKT and ERK phosphorylation.

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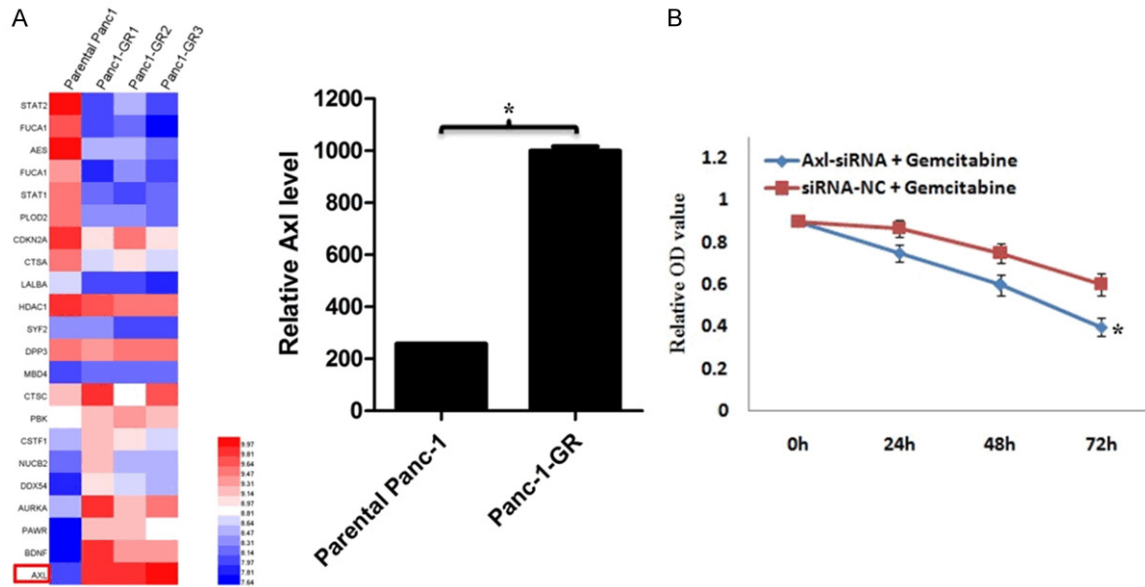


Figure 4. The knockdown of Axl increases the sensitivity of pancreatic cancer to gemcitabine. A: The expression of Axl in the gemcitabine-resistant pancreatic cancer cell line (Panc-1-GR) and the parental cell line based on the GEO databases GSE80617. B: The cytotoxic effect of gemcitabine increased significantly in Axl knockdown cells compared to the negative controls. Data are expressed as mean \pm SEM (n=3). *indicated P<0.05.

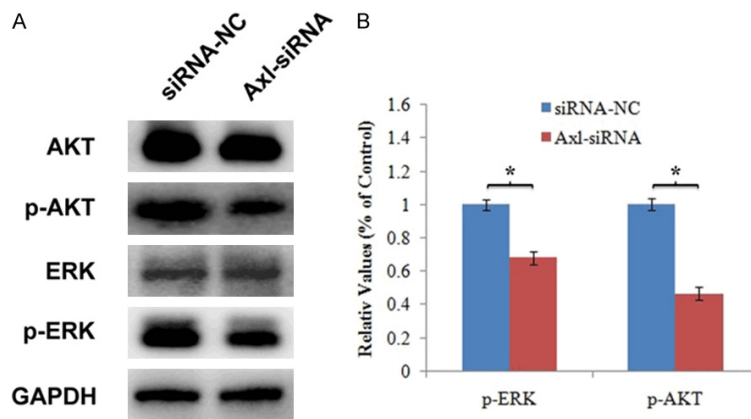


Figure 5. The Effect of Axl on the blockage of AKT and ERK phosphorylation. A, B: The expressions of the proteins in the AKT and ERK signaling pathways were detected in the Axl knockdown or control cells. Data are expressed as mean \pm SEM (n=3). *indicated P<0.05.

Discussion

In recent years, pancreatic cancer has become a malignant tumor with the fastest growing morbidity and mortality in the world and a 5-year survival rate of only about 8% [1]. An important feature of pancreatic cancer's malignant biological behavior is its significant potential for invasion and metastasis. Surgical treatment offers the only possible cure for pancreatic cancer, but 80% of the patients are inoperable

at diagnosis. In addition, even after surgery, the 5-year survival rate is only 15%-20% because of the cancer's high metastatic rate and local recurrence [14]. Therefore, understanding the molecular mechanisms of pancreatic cancer carcinogenesis and metastasis may help discover potential tumor biomarkers and effective therapeutic strategies.

The enhanced expression of Axl has been reported in different types of cancer, indicating that Axl may be important in the onset and progression of malignant tumors. However, the expression and role of Axl has not been reported in pancreatic cancer. In this study, we investigated the expression and functions of Axl in pancreatic cancer. We found that the expression of Axl was much higher in pancreatic cancer cell lines and tumor tissues than it was in the controls. In addition, through the analysis of the correlation between Axl expression and patients' clinicopathological features, it was observed that the TNM and T stages of the Axl

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positive expression group was significantly higher than the stages of the Axl negative expression group. This indicates that Axl expression is an important factor affecting the prognosis of pancreatic cancer patients. In addition, a number of studies have highlighted the significant role of Axl in cancer progression. Ou et al. reported that the activation of Axl can enhance cell proliferation in mesothelioma cells [15]. Papadakis et al. reported that Axl could down-regulate the pro-apoptotic Bcl-2 family members to promote survival in cutaneous squamous cell carcinoma [16]. However, how Axl influences the progression of pancreatic cancer still needs further exploration. The present study showed that the knockdown of Axl expression inhibits cell proliferation and promotes cell apoptosis, which is consistent with studies in other tumors.

Drug resistance is the main cause of cancer treatment failure. It has been widely reported that Axl serves a notable function in the drug resistance of a number of different cancer types, and that the suppression of Axl with genomic or pharmaceutical methods may restore the sensitivity of cancer cells to drugs. An early study demonstrated that Axl mediates the acquired resistance to EGFR-targeted therapy in lung cancer [17]. Also, Abbes Belkhir et al. reported that Axl plays an important role in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and cisplatin resistance in Ehrlich ascites carcinoma (EAC) cells [18]. However, the mechanisms of Axl in mediating the resistance to gemcitabine have not been fully investigated [19]. In this study we discovered that the knockdown of Axl increases the sensitivity of pancreatic cancer to gemcitabine. The activation of Axl regulates a number of signal transduction pathways, depending on the cell types. As we all know, the AKT pathway, which is an important downstream pathway of Axl, plays a crucial role in protein synthesis, cell survival and the proliferation of tumor cells [20]. It was shown that in pancreatic cancer the phosphorylation level of AKT and ERK were dramatically reduced after Axl knockdown. Consequently, the effect of Axl on cell proliferation and the increase in the sensitivity to gemcitabine probably works through the AKT/ERK pathway.

In conclusion, this study revealed that Axl is up-regulated in pancreatic cancer, correlates with

a poor prognosis, and promotes proliferation. Meanwhile, the knockdown of Axl can increase the sensitivity of pancreatic cancer to gemcitabine, which may work through the AKT/ERK pathway. The mechanisms involved in Axl may provide new insights into therapy strategies for pancreatic cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Yong Wang, Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310000, Zhejiang Province, China. Tel: (+86) 0571-86090073; Fax: (+86) +86-571-86090073; E-mail: 3313023@zju.edu.cn

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