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Significance of IL28RA in diagnosis of early pancreatic cancer and its regulation to pancreatic cancer cells by JAK/STAT signaling pathway – effects of IL28RA on pancreatic cancer

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Abstract. – OBJECTIVE: To explore the significance of IL28RA in diagnosis of early pancreatic cancer and its regulation to pancreatic cancer cells by the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway.

PATIENTS AND METHODS: A total of 81 patients with early pancreatic cancer were enrolled as a pancreatic cancer group, and 81 patients with benign pancreatic diseases were enrolled as a benign disease group. Western blot was adopted to analyze the serum IL28RA expression of the two groups and its diagnostic value in early pancreatic cancer. A pancreatic cancer cell model was constructed, and the IL28RA expression in pancreatic cancer cells, PANC-1 and BXPC-3, was up-regulated to explore the biological function of pancreatic cancer cells after up-regulation of IL28RA and the effects on JAK-STAT signaling pathway.

RESULTS: Lowly expressed in serum of patients with pancreatic cancer, IL28RA showed a sensitivity of 80.25%, specificity of 75.31%, and area under the curve (AUC) of 0.846 in diagnosis of early pancreatic cancer. It was found that up-regulation of IL28RA expression in pancreatic cancer cells inhibited proliferation and invasion abilities of pancreatic cancer cells, increased apoptosis rate and expression of pro-apoptotic protein bax, decreased expression of anti-apoptosis protein bcl-2, and significantly inhibited phosphorylation level of JAK2 and STAT3 proteins.

CONCLUSIONS: IL28RA is lowly expressed in pancreatic cancer patients, and has certain diagnostic value for early pancreatic cancer. Its up-regulated expression can inhibit the proliferation and invasion of pancreatic cancer cells, and promote their apoptosis by inhibiting the activation of JAK-STAT signaling pathway.

Key Words:
IL28RA, Pancreatic cancer, Diagnostic significance, JAK/STAT signal, Biological function.

Introduction

Pancreatic cancer, a high-incidence digestive system malignant tumor, shows a very high mortality[1]. Compared with the gradual rise of survival rate of other malignant tumors, the rise of survival rate of pancreatic cancer is very slow, and the 5-year survival rate of it is only about 8% so far[2]. One of the reasons for the low survival rate of pancreatic cancer is the lack of accurate and effective methods for diagnosis of early pancreatic cancer. At present, pancreatic cancer is mainly diagnosed by both imaging and pathology, but this method lacks certain timeliness. Therefore, many patients are often already in advanced stage at diagnosis, and have lost the best treatment period[3,4]. The reason for high mortality of pancreatic cancer is that the high malignant degree and strong invasiveness of pancreatic cancer cause low sensitivity to most treatments such as radiotherapy and chemotherapy, and to commonly used molecular targeted therapy[5]. Therefore, in addition to finding an effective diagnosis method for early pancreatic cancer, finding an effective treatment method for pancreatic cancer is also one of the urgent problems in clinical practice.

Interleukin 28 receptor α (IL28RA) is a common receptor of type III interferons, IL-28A, IL-28B, and IL-29[6]. With similar structures and

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functions, type III interferons and type I interferons have different receptor complexes, and they can all inhibit the activity of viruses, including hepatitis B virus and cytomegalovirus[7] by regulating Janus kinase/signal transducer and activator of the transcription (JAK/STAT) signaling pathway. Vitale et al[8] have found that the interaction between type I interferons and their receptors has certain inhibitory effect on pancreatic cancer cells. In addition, IL28RA[9] is detected to be highly expressed in tissues of human pancreas, skeletal muscle, prostate, and others. Mucha et al[10] show that IL28RA is abnormally expressed in tumor tissues, including breast cancer tissues. IL28RA, a new factor discovered in recent years, has certain connection with the development of pancreatic cancer. We suspected that IL28RA may be taken as a new molecular marker for early diagnosis of pancreatic cancer, and its mechanism on pancreatic cancer cells had not been explored yet. Therefore, we conducted the following research to seek for a new molecular direction for diagnosis and treatment of pancreatic cancer.

Patients and Methods

General Materials

A total of 81 patients [42 males and 39 females, with an average age of (57.59±4.19) years] with early pancreatic cancer admitted to our hospital from August 2015 to November 2018 were enrolled as a pancreatic cancer group, and 81 patients with benign pancreatic diseases admitted to our hospital during the same period were enrolled as a benign disease group. The inclusion criteria were as follows: patients diagnosed with stage I-II pancreatic cancer based on imaging and pathology.

Exclusion criteria were as follows: patients with other comorbid malignant tumors, severe hepatic renal dysfunction, severe immune system diseases, cognition disorder or communication obstacle, and those unwilling to cooperate for the study. All patients and their families agreed to participate in the research and signed an informed consent. The investigation has been approved by the Ethics Committee of the Hospital.

Experimental Materials and Reagents

Human pancreatic cancer cell lines, SW1990, CFPAC-1, PANC-1, and BXPC-3, and normal pancreatic epithelial cell HPNE (Shanghai Cell Bank of Chinese Academy of Sciences); fetal bovine serum (FBS) and phosphate-buffered saline (PBS; Gibco, Rockville, MD, USA); trypsin, ristocetin-induced platelet agglutination (RIPA), and bicinchoninic acid (BCA protein kits; Thermo Fisher Scientific Company, Waltham, MA, USA); RIPA lysate (China Beyotime Biotechnology Co., Jiangsu, China Ltd.); IL28RA, β-actin antibody, p-JAK2 antibody, p-STAT3 antibody, bac antibody, bcl-2 antibody, and goat anti-rabbit secondary antibody (Abcam, Cambridge, MA, USA); IL28RA recombinant plasmid (Origene Technologies; Rockville, MD, USA); Annexin V-FITC/PI apoptosis kit (Jiangsu KeyGEN BioTECH Corp., Ltd.); lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Detection of Protein Expression in Serum and Cells by Western Blot

Venous blood was sampled from all patients within 24 h after their admission, centrifuged at 2000/min for 10 min. Then, its serum was taken for subsequent determination. Serum and related cells were collected, and then their total protein was extracted using the RIPA lysis method. The concentration of the total protein was determined using the bicinchoninic acid (BCA) method, and then adjusted to 4 μg/μ. The total protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to the polyvinylidene difluoride (PVDF) membrane, stained, socked in Phosphate-Buffered Saline with Tween-20 (PBST) for 5 min for washing, blocked with 5% skim milk powder for 2 h, added with mouse monoclonal antibodies of primary antibody, IL28RA (1:500), P-JAK 2 (1:100), p-STAT3 (1:2000) and β-actin (1:1000) after the membrane was transferred, and then sealed overnight at 4°C. The membrane was washed to remove the primary antibody, added with horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (1: 5000), incubated at 37°C for 1 h, rinsed with phosphate-buffered saline (PBS) 3 times, and absorbed to remove excess liquid after rinsing, and then made to be luminescent with enhanced chemiluminescence (ECL) and developed.

Cell Transfection

IL28RA expression in PANC-1 and BXPC-3 cells was relatively low in the above detection, so PANC-1 and BXPC-3 cells were selected for subsequent experiment. First, the cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine

serum (FBS) and 1% penicillin-streptomycin, and cultured under 5% CO_2 at 37°C. Next, the cells at logarithmic phase were taken for subsequent transfection. The cells at logarithmic phase were seeded in a 24-well plate, and the concentration was adjusted to 5×10^3. When the fusion of cell adherent growth reached 80%, 25% pancreatin was added into the cells for digestion, and then the cells were continually cultured in culture medium after digestion for passage. After the passage, each cell line was divided into blank group, negative control group, and IL28RA group. Negative control group and IL28RA group were transfected with IL28RA-NC and IL28RA, respectively. The two groups were added with Lipofectamine 3000 according to the instruction of Lipofectamine 3000 manufacturer kit, respectively, mixed, incubated at room temperature for 5 min, mixed with cells well, and transfected under 5% CO_2 at 37°C.

Detection of Cell Proliferation by MTT Assay

PANC-1 and BXPC-3 cells transfected for 48 h were seeded into a 96-well plate, 100 μl of enchylema in each well at cell density of 2×10^3 cell/ml. Each well was added with 20 μl of MTT solution after being incubated at 37°C for 24 h, 48 h and 72 h, respectively, cultured at 37°C for 4 h, then added with 200 μl of dimethyl sulfoxide (DMSO), and incubated at 37°C for 30 min. Next, the absorbance of the cells was measured at 490 nm using an enzyme mark instrument to detect cell proliferation. The experiment was repeated three times.

Detection of In Vitro Invasion Ability of Cells by Transwell Chamber

Matrigel gel was placed at 4°C overnight for liquefaction and the liquefied Matrigel matrix gelatin was diluted at a ratio of 1:6. The transfected PANC-1 and BXPC-3 cells were resuspended with DMEM without FBS, and their cell density was adjusted to 2×10^5 cells/ml. Then, the cells were seeded in transwell chambers of a 24-well plate. Each chamber was added with about 200 μl of cell suspension, and the lower chamber of the 24-well plate was added with 600 μl of DMEM with 10% FBS, and cultured in an incubator at 37°C for 24 h. After culture, the supernatant was removed with cotton swabs, and the chamber was taken out and washed with PBS. The cells in the lower chamber were fixed with 95% ethanol solution for 30 min, taken out, and washed with PBS again, and then each well was added with 600 ul of 0.1% crystal violet for staining. The number of migrating cells in 5 randomly selected wells was calculated, respectively, with a microscope after staining, and their average value was calculated. The experiment was repeated three times.

Cell Apoptosis Experiment

The Annexin V-FITC/PI double staining combined with flow cytometry was adopted to detect cell apoptosis. The transfected cells were seeded in a 6-well plate at 3×10^5 cells /well, and digested with 0.25% trypsin. After digestion, the cells were washed with PBS for 2 times, and then added with 100 μL of binding buffer to prepare $2x10^5$ cells /mL suspension. The suspension was added with AnnexinV-FITC and PI in order, incubated at room temperature for 5 min in the dark, and finally detected in apoptosis using the flow cytometry. The average value was calculated, and the experiment was repeated for 3 times.

Statistical Analysis

Data were analyzed using SPSS 20.0 software (IBM, Armonk, NY, USA), and visualized into figure using the GraphPad Prism 6 software. Measurement data were expressed in the mean ± standard deviation (means ± SD), and checked by *t*-test. Inter-group comparison groups were carried out using the independent-samples *t*-test, and expressed by *t*. Comparison between multiple groups was performed using the one-way (ANOVA), and post-hoc pairwise comparison was performed using the least significant difference-*t* (LSD-*t*). The diagnostic value of IL28RA for pancreatic cancer was analyzed using a receiver operating characteristic (ROC). $p<0.05$ indicated a significant difference.

Results

General Data

There was no significant difference in sex, age, and body mass index (BMI) between pancreatic cancer group and benign disease group (all $p>0.05$). More details are shown in Table I.

Serum IL28RA Expression in the Two Groups and its Diagnostic Value for Pancreatic Cancer

Serum IL28RA expression in pancreatic cancer group was significantly lower than that in benign disease group (0.62 ± 0.24 *vs.* 1.04 ±

Table I. General data.

Factors	Pancreatic cancer group n = 81	Benign disease group n = 81	t/χ^2	p
Gender			0.099	0.753
Male	42 (51.85)	40 (49.38)		
Female	39 (48.15)	41 (50.62)		
Age (Y)			0.025	0.875
≤ 57	38 (46.91)	37 (45.68)		
> 57	43 (53.09)	44 (54.32)		
BMI (kg/m^2)			0.026	0.873
≤ 22	33 (40.74)	32 (39.51)		
> 22	48 (59.26)	49 (60.49)		
Staging				
Stage I	34 (41.98)	–		
Stage II	47 (58.02)	–		
Pathological type				
Ductal adenocarcinoma	18 (22.22)	–		
Acinic cell carcinoma	19 (23.46)	–		
Small gland carcinoma	21 (25.93)	–		
Small cell carcinoma	23 (28.40)	–		
Alanine aminotransferase (IU/L)	26.94 ± 1.41	26.84 ± 1.43	0.448	0.655
Aspartate aminotransferase (IU/L)	21.31 ± 1.12	21.29 ± 1.11	0.114	0.909
Creatinine (umol/L)	64.46 ± 4.64	64.23 ± 4.65	0.315	0.753

0.35, $p<0.05$). Figure 1-A: sensitivity, specificity, and area-under-the-curve (AUC) of IL28RA for diagnosis of pancreatic cancer were 80.25%, 75.31%, and 0.846, respectively. More details are shown in Figure 1-B.

IL28RA in Each Cell Lines

Human pancreatic cancer cell lines, SW1990, CFPAC-1, PANC-1, and BXPC-3, showed significantly lower IL28RA expression than normal pancreatic epithelial cell HPNE ($p<0.05$), and

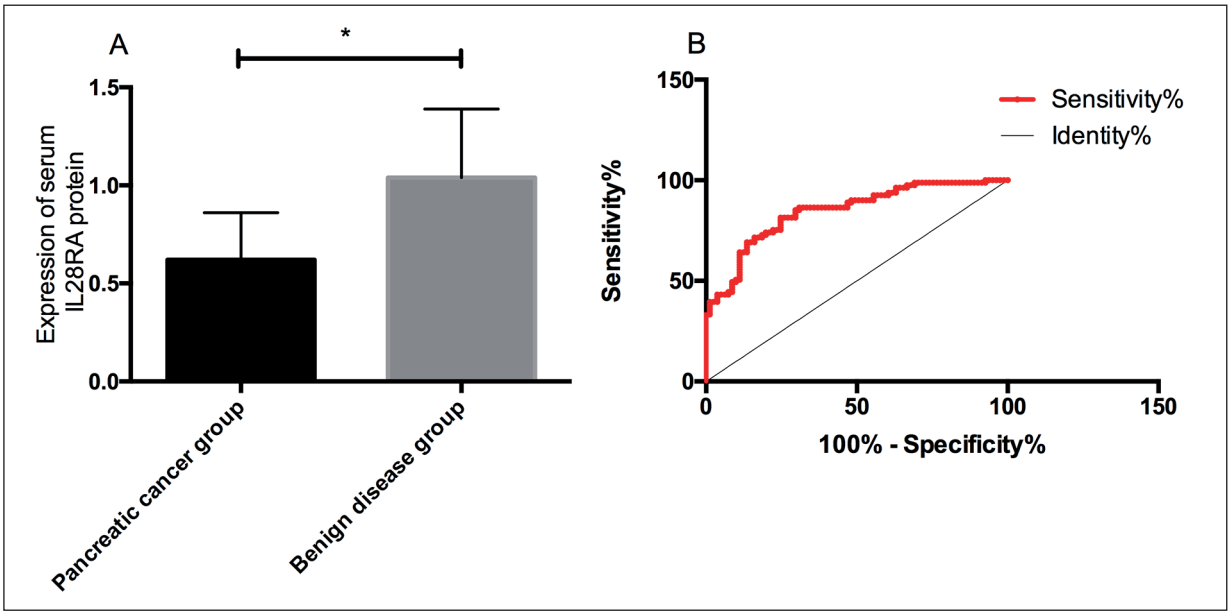

Figure 1. **A**, Serum IL28RA expression of the pancreatic cancer group was significantly lower than that of the benign disease group; **B**, Sensitivity, specificity and AUC of IL28RA for diagnosis of pancreatic cancer were 80.25%, 75.31% and 0.846 respectively. *Indicates $p<0.05$.

PANC-1 and BXPC-3 showed relatively low IL-28RA expression, so PANC-1 and BXPC-3 were used for subsequent experiments. More details are shown in Figure 2.

Protein Expression of IL28RA, p-JAK2, and p-STAT3 in Cells of Each Group after Transfection

Compared with blank group and negative control group, PANC-1 and BXPC-3 cells transfected with IL28RA showed significantly increased IL-28RA, and significantly down-regulated p-JAK2 and p-STAT3 protein expression (Figure 3, all $p<0.05$).

Effects of Up-Regulated IL28RA Expression on Cell Proliferation, Invasion, and Apoptosis

To study the effects of up-regulated IL28RA expression on proliferation, invasion, and apoptosis of pancreatic cancer cells, PANC-1 and BXPC-3 cells were transfected with IL28RA or

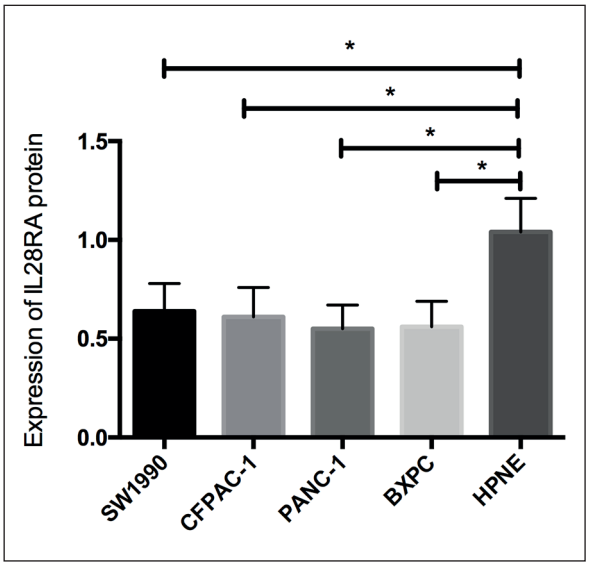

Figure 2. Cell lines including human pancreatic cancer cell lines, SW1990, CFPAC-1, PANC-1, and BXPC-3 showed significantly lower IL28RA expression than normal pancreatic epithelial cell IL28RA.*Indicates $p<0.05$.

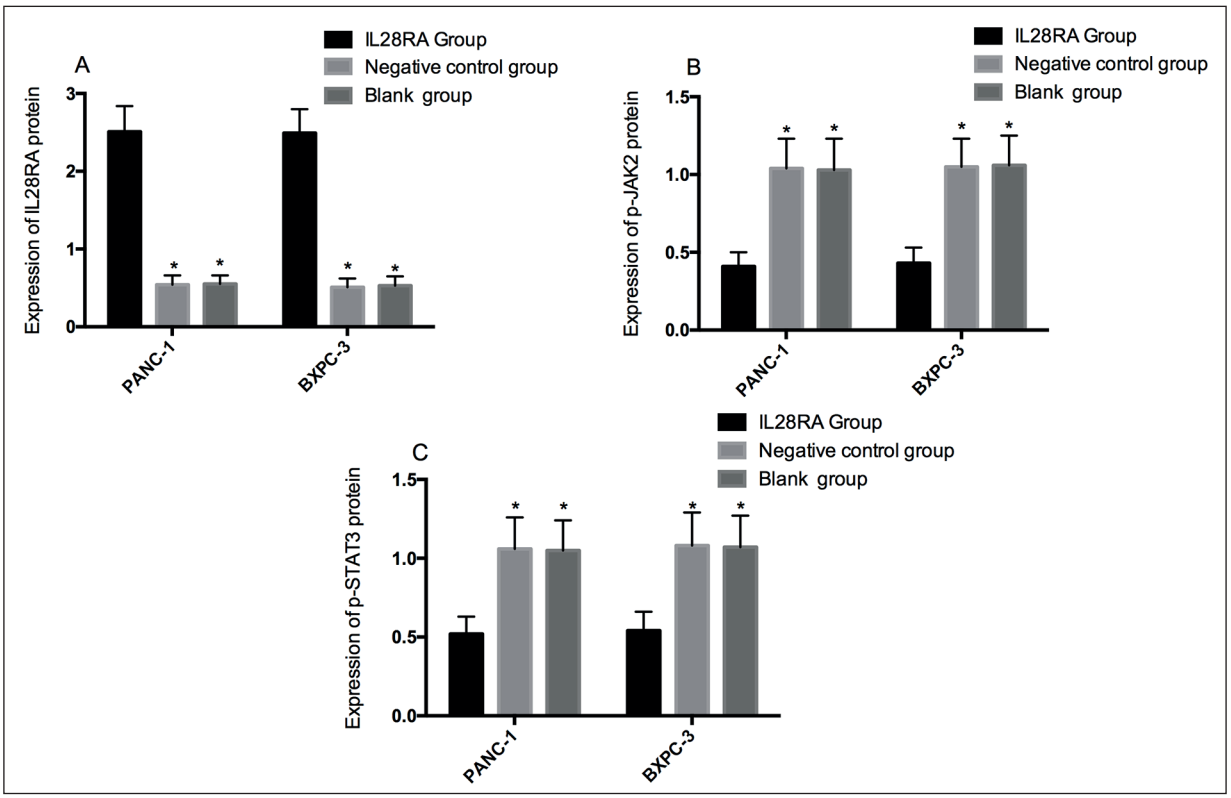

Figure 3. Protein expression of IL28RA, p-JAK2, and p-STAT3 in cells of each group after transfection. **A**, Compared with the blank group and negative control group, PANC-1 and BXPC-3 cells transfected with IL28RA showed significantly increased IL28RA; **B**, Compared with the blank group and negative control group, PANC-1 and BXPC-3 cells transfected with IL28RA showed significantly down-regulated p-JAK2 protein expression; **C**, Compared with the blank group and negative control group, PANC-1 and BXPC-3 cells transfected with IL28RA showed significantly down-regulated p-STAT3 protein expression. *Indicates that in comparison with the IL28RA group, $p<0.05$.

IL28RA-NC, and then determined in the way of MTT assay, and cell invasion and flow apoptosis assay. Up-regulated IL28RA expression significantly inhibited the proliferation and invasion of PANC-1 and BXPC-3 cells (both $p<0.05$) and promoted their apoptosis. Western blot experiment revealed that PANC-1 and BXPC-3 cells with up-regulated IL28RA expression showed increased expression of pro-apoptotic protein bax and down-regulated expression of anti-apoptosis protein bcl-2. More details are shown in Figure 4.

Discussion

Pancreatic cancer is a common malignant tumor, which is characterized by unobvious early symptoms and rapid progression, so most patients are already in the middle or advanced stage at diagnosis and have lost a good treatment time[11,12]. In addition to difficulty in early diagnosis, pancreatic cancer has strong invasiveness and metastasis, which leads to unsatisfactory curative effects of conventional radiotherapy and chemotherapy, and targeted therapy[13], suggesting that we need to seek for more diagnostic and therapeutic methods for pancreatic cancer to overcome the difficulty in early diagnosis and poor curative effect of pancreatic cancer.

IL28RA, the receptor of type III interferons, is a new factor discovered in recent years that is closely related to tumor development. Dumoutier et al[14] reported that IL28RA expression in tumor tissues was related to the prognosis of tumor patients, and Yang et al[15] even pointed out that low IL28RA expression was related to accelerated tumor growth and low survival rate of patients. Based on the above, we speculated that IL28RA may also play a certain role in diagnosis and treatment of pancreatic cancer. Therefore, we determined the serum IL28RA expression in patients with pancreatic cancer and patients with benign pancreatic diseases, and it turned out that the serum IL28RA expression in patients with pancreatic cancer decreased significantly. In addition, we drew a ROC curve and found that IL28RA had certain diagnostic value to early pancreatic cancer, which indicated that IL28RA may be a new molecular marker for the diagnosis of pancreatic cancer. Subsequently, to further explore

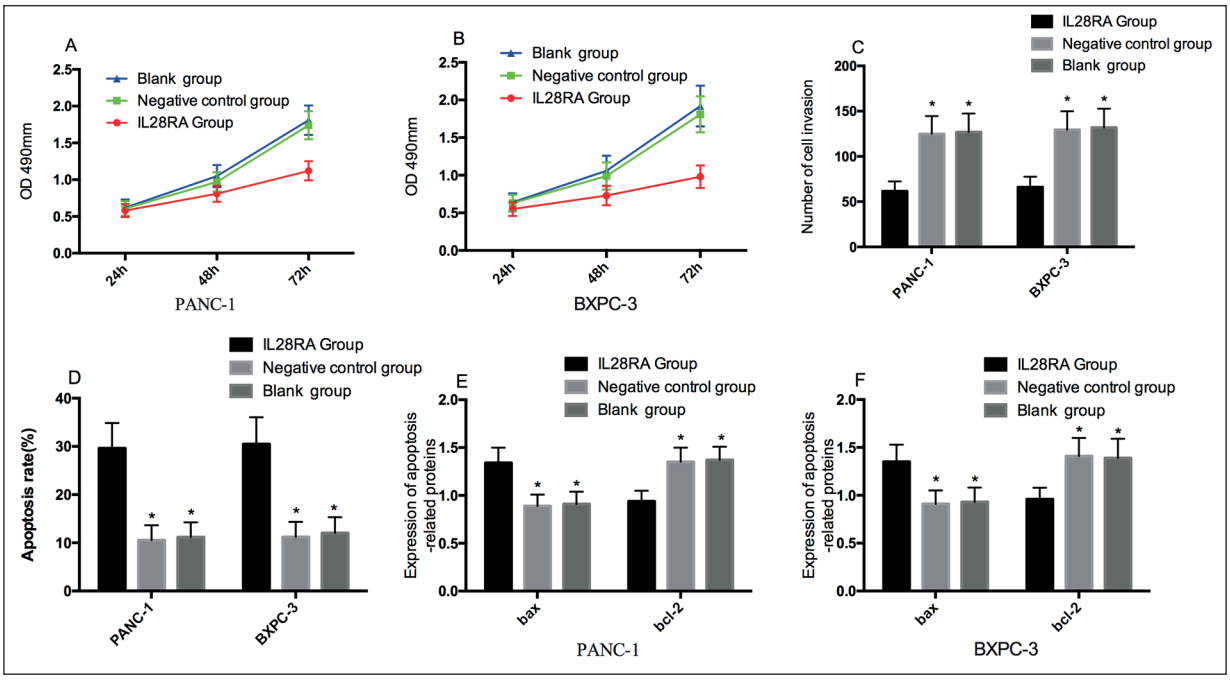

Figure 4. Effects of up-regulated IL28RA expression on cell proliferation, invasion and apoptosis A and B: Up-regulated IL28RA expression significantly inhibited the proliferation of PANC-1 and BXPC-3 cells ($p<0.05$). **C**, Up-regulated IL28RA expression significantly inhibited the invasion of PANC-1 and BXPC-3 cells; **D**, Up-regulated IL28RA expression significantly promoted apoptosis of PANC-1 and BXPC-3 cells; **E** and **F**, PANC-1 and BXPC-3 cells with up-regulated IL28RA expression showed increased expression of pro apoptotic protein bax and down-regulated expression of anti-apoptosis protein bcl-2. *Indicates that in comparison with the IL28RA group, $p<0.05$.

the mechanism of IL28RA in pancreatic cancer, we up-regulated the IL28RA expression in pancreatic cancer cells, and explored their biological function, which revealed that up-regulated IL-28RA expression inhibited the proliferation and apoptosis of pancreatic cancer cells, but caused increased apoptosis rate, increased expression of pro-apoptotic protein bax, and decreased expression of anti-apoptosis protein. There are relatively few investigations on IL28RA and pancreatic cancer, but there are researches based on the fact that type III interferons and type I interferons have similar signaling pathways[16]. We speculated that type III interferons may have inhibitory effects on tumor growth. Luo and Jia et al[17] considered that type III interferons could inhibit JAK-STAT signal conduction by binding with IL-28RA/IL10RB receptor complex, and eventually inhibit tumor cell proliferation. In addition, Chen et al[18] pointed out that lowering the expression of IL28RA, one of the components of type III interferon receptor complex, could promote cell proliferation and inhibit cell apoptosis. Tsai et al[19] explored mice with myocardial injury and found that IL28RA level in their myocardial cells increased and the expression of anti-apoptosis protein bcl-2 decreased, from which we inferred that IL28RA played an important role in promoting cell apoptosis. In addition, Jian et al[20] found that lowering IL28RA expression could promote the activation of JAK-STAT, thus inhibiting the apoptosis of myocardial cells. It can also confirm our finding that up-regulated IL28RA expression increased the apoptosis rate of pancreatic cancer cells, but this finding has not been observed in other tumor cells.

Since IL28RA was closed related to JAK-STAT signaling pathway in terms of its effects on cell growth and apoptosis, and it was well known that JAK-STAT signaling pathway is involved in tumorigenesis and development[21], we speculated that IL28RA may have an effect on pancreatic cancer cells by regulating JAK-STAT signaling pathway. When the body is in a normal state, JAK and STAT can maintain a relatively stable state through feedback regulation. When the body shows pathological changes, JAK protein phosphorylation and signal transduction pathway will be activated, which will in turn lead to phosphorylation of STAT protein[22,23]. In the development of pancreatic cancer, activation of JAK2-STAT3 signaling pathway can promote cell proliferation, thus promoting the development of pancreatic cancer[24]. We also found that up-regulated IL28RA expression in pancreatic cancer cells lowered the phosphorylation level of JAK2 and STAT3, which suggested that IL28RA could inhibit the activation of JAK-STAT signaling pathway. In recent years, there are relatively more researches on JAK-STAT signaling pathway on pancreatic cancer. For example, Huang et al[25] found that inhibiting the activation of JAK-STAT signaling pathway in pancreatic cancer could inhibit the growth of pancreatic cancer cells, and Nagaraju et al[26] pointed out that activation of JAK-STAT signaling pathway would trigger phosphorylation of JAK1 and STAT3 protein, and promote proliferation of pancreatic cancer cells and inhibit their apoptosis. The above investigations indicated that JAK-STAT signaling pathway can regulate the biological function of pancreatic cancer cells.

Conclusions

To sum up, IL28RA is lowly expressed in pancreatic cancer patients and has certain diagnostic value for early pancreatic cancer. Its up-regulated expression can inhibit the proliferation and invasion of pancreatic cancer cells and promote their apoptosis by inhibiting the activation of JAK-STAT signaling pathway. However, this study also has certain limitations. As there are relatively few relevant investigations on IL28RA in tumors, we cannot provide more documents to confirm our experimental conclusion. In addition, although IL28RA has the ability of regulating JAK-STAT signaling pathway, whether IL28RA can regulate other pathways to affect pancreatic cancer cells remains to be further explored.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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