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Role of miR-214-5p in the migration and invasion of pancreatic cancer cells

T.-H. CAO[1], X. LING[2], C. CHEN[1], W. TANG[3], D.-M. HU[3], G.-J. YIN[3]

[1]Department of Medical Oncology, Affiliated Wujiang Hospital Of Nantong University, Suzhou, China
[2]Department of Gastroenterology, Affiliated Wujiang Hospital Of Nantong University, Suzhou, China
[3]Department of Gastroenterology, the Second Affiliated Hospital of Soochow University, Suzhou, China

Tinghua Cao and *Xin Ling* contributed equally to this work

Abstract. – OBJECTIVE: To analyze the role of miR-214-5p in proliferation and metastasis of pancreatic cancer (PC) cells, as well as its underlying mechanism.

PATIENTS AND METHODS: 30 pairs of PC tissues and adjacent normal tissues were collected in our Department. The expression level of miR-214-5p was detected by quantitative Real-time-polymerase chain reaction (qRT-PCR). Biological information analysis and luciferase report gene assay were used to verify potential target genes of miR-214-5p. Cell counting kit-8 (CCK-8) and transwell methods were applied to observe the interference of miR-214-5p on invasion and migration of PC cells. Western blot (WB) assay was applied to determine the expression changes of Jagged 1 (JAG1) and epithelial-mesenchymal transition (EMT)-related genes in PC cells.

RESULTS: QRT-PCR results showed that the expression level of miR-214-5p is significantly down-regulated in PC tissues and cells. Bioinformatics software and luciferase report gene assay identified that JAG1 is a target gene of miR-214-5p. The negative correlation between protein expressions of miR-214-5p and JAG1 was assessed by Western Blot assay. Furthermore, miR-214-5p could suppress cell proliferation, invasion and migration, and it also blocked the EMT in PC cells *in vitro*. Meanwhile, JAG1 overexpression reversed the inhibitory effects of miR-214-5p on proliferation, invasion and migration of PC cells.

CONCLUSIONS: Overexpressing miR-214-5p could significantly inhibit malignant behavior of PC cells through targeted regulation of JAG1. Thus, miR-214-5p might be a potential therapeutic target for treatment of PC.

Key Words:
Pancreatic Cancer (PC), MiR-214-5p, Jagged 1 (JAG1), Invasion and Migration, Epithelial-Mesenchymal Transition (EMT).

Introduction

As one of the most common malignant tumors of the digestive tract, pancreatic cancer (PC) is the most destructive between all kinds of solid tumors. It has been reported that the mortality of PC ranks 8th in tumors around the world, and it is the 4th major cause of tumor death in the United States. It is estimated that the mortality of PC ranks 7th and its incidence ranks 6th in tumors in 48 regions of China. In recent years, the incidence of PC in China has shown an increasing trend year by year[1, 2]. In addition, the high invasion and early metastasis of PC result in an extremely short survival time of PC patients. However, effective markers for the early clinical diagnosis and prognosis are still lacked. Recent investigations[3, 4] have revealed that approximately 40-50% PC patients have been in the late stage at the time of diagnosis, and are often accompanied by distant metastasis and local extensive infiltration of tumors. In recent years, PC surgery has been applied more and more widely with the continuous improvement of surgical techniques. However, surgical treatment can be performed for only 10-20% in early-stage PC patients without metastasis, and the 5-year survival rate of patients definitely diagnosed with PC is only 5%[5]. Therefore, it is of great importance to investigate the pathogenesis of PC more deeply in improving the early diagnosis and effective treatment of PC patients. Micro ribonucleic acid (miRNA) is a kind of single-stranded, non-coding ribonucleic acid molecule with 19-22 nucleotides in length, which regulates the 3'-untranslated region (UTR) of target gene mainly through base complementary pairing. MiRNA regulates the transcription of downstream target gene and translation of target protein, thus playing an important role in biological processes, such as proliferation, migration,

Corresponding Author: Guojian Yin, MD; e-mail: ygj234@163.com

invasion, differentiation and apoptosis of tumor cells[6-8]. Moreover, miRNA, as the oncogene or tumor-suppressor gene, can exert different biological functions in the occurrence and development of tumor[9]. According to many previous researches[10-13], miRNA plays an important role in the malignant progression of PC cells, including the occurrence, proliferation, invasion and epithelial-mesenchymal transition (EMT) of PC. MiR-214-5p is a product of the miR-214 gene with 110 bp. As a member of the miRNA family, it plays a regulatory role in the development of human osteosarcoma and hepatocellular carcinoma[14,15]. However, there were few reports on the roles of miR-214-5p in the occurrence and development of PC, and its related molecular mechanism. In this study, the expression of miR-214-5p in PC tissues and cells was observed first, and its possible mechanism was further analyzed via the *in-vitro* experiments. We aim to provide new ideas for the research on molecular mechanism of the occurrence and development of PC.

Patients and Methods

Tissue Samples and Cell Lines

Carcinoma tissues and normal tissues adjacent to carcinoma tissues (more than 5 cm away from the tumor edge) were collected from 30 patients with first-onset PC receiving surgical treatment in our hospital from July 2016 to December 2017. None of patients underwent preoperative radiotherapy or chemotherapy. They were pathologically diagnosed with pancreatic ductal adenocarcinoma after surgery, and para-carcinoma tissues were negative in pathological examination. All patients signed the informed consent. This study was approved by the Ethics Committee of Affiliated Wujiang Hospital of Nantong University. Human PC cells (PANC-1) and normal pancreatic cells (HPDE6-C7) were provided by Nanjing KeyGen Biotech Co. (Nanjing, China), Ltd. PANC-1 cell line was cultured in the Dulbecco's modified Eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) under 5% CO_2 and saturated humidity at 37°C. Culture medium was replaced according to the cell growth density for subculture, and cells in the logarithmic phase were taken for the experiment. PANC-1 cells were transfected with miR-214-5p mimics, negative control Con-miR or siRNA according to instructions of the Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA). In the *in-vitro* experiment, cells were divided into NC group (negative control), Mimics group (PANC-1 cells transfected with miR-214-5p mimics) and Mimics + JAG1 group (PANC-1 cells transfected with miR-214-5p mimics and si-JAG1).

Target Gene Prediction

Prediction of miR-214-5p target genes was conducted via bioinformatics. The target genes of miR-214-5p were predicted using TargetScan, PicTar and Miranda databases. The binding site between 3'-UTR of target gene JAG1 and miR-214-5p was predicted using Miranda database.

Dual Luciferase Activity Assay

The mutation sequence (mutant-type 3'-UTR) of the binding site between 3'-UTR (wild-type 3'-UTR) of target gene JAG1 and miR-214-5p was constructed. The wild-type or mutant-type 3'-UTR of JAG1 was cloned into the pmirGLO vector to construct pmirGLO-JAG1-wt and pmirGLO-JAG1-mut. There were 4 groups in the experiment: 1) Scramble and pmirGLO-JAG1-wt, 2) Scramble and pmirGLO-JAG1-mut, 3) MiR-214-5p mimics and pmirGLO-JAG1-wt, and 4) miR-214-5p mimics and pmirGLO-JAG1-mut. PANC-1 cells were transfected based on the above groups. Firefly and Renilla luciferase activity were detected according to instructions of the Dual-luciferase kit (Thermo Fisher Scientific, Waltham, MA, USA). The Renilla luciferase activity was used as the internal reference control.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

The total RNA was extracted from the tissues using the RNA extraction kit (TaKaRa, Otsu, Shiga, Japan), and reversely transcribed using the TaqMan reverse transcription kit (TaKaRa, Otsu, Shiga, Japan) according to instructions. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as the internal reference in the detection of JAG1, while U6 was used as the internal reference in the detection of miR-214-5p. $2^{-\Delta\Delta Ct}$ method was adopted in the analysis. Primer sequences used in this study were as follows: JAG1, F: 5'-GGCCTGTATAGTGAGGCACCCG-3', R: 5'-CTTACGATACGCCACACTTG-3'; miR-214-5p, F: 5'-TCTCTTGCTATAGAAGCACAAC-3', R: 5'-TCCTCCACAATCATGCTGTGT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blotting Analysis

Cells were lysed with radioimmunoprecipitation assay (RIPA) lysis solution containing phenylmethanesulfonyl fluoride (PMSF) (Beyotime, Shanghai, China), After protein quantification *via* bicinchoninic acid (BCA, Pierce, Rockford, IL, USA). 30 g protein were taken for Western blotting. The protein was incubated with the primary antibodies (JAG1, E-cadherin, Vimentin and GAPDH (diluted at 1:1000, Cell Signaling Technology (CST) Inc.), Danvers, MA, USA) at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-labeled secondary antibody at room temperature for 1 h. The signal was detected using the Super electrochemiluminescence (ECL) Plus kit (Thermo Fisher Scientific, Waltham, MA, USA), with GAPDH used as the internal reference control.

Cell Proliferation

Cells in each group were digested, counted and prepared into the cell suspension in a concentration of 5×10^4/mL, and 100 μL cell suspension were added into each well of a 96-well plate. The 96-well plate was placed in an incubator with 5% CO_2 at 37°C for 24 h incubation. The medium was discarded, and cells were washed with phosphate-buffered saline (PBS) twice and diluted into the desired concentration using the complete medium, followed by cell culture for 24, 48 and 72 h, respectively. 10 μL cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) were added into each well, and the optical density (OD) value was determined (λ = 450 nm).

Cell Invasion and Migration

After the medium was removed, cells were starved for 24 h using the incomplete medium. Next, cells were digested and counted, and the cell density was adjusted to 5×10^5/mL using the incomplete medium. 100 μL cell suspension were added into the upper transwell chamber, and 500 μL medium containing 20% FBS were added into the lower chamber. The 24-well plate was placed in the incubator with 5% CO_2 at 37°C for incubation for 24 h, followed by cell counting. Cells in the upper chamber were wiped off with the cotton swab, transwell chamber was removed, inverted and air-dried. 500 μL 0.1% crystal violet was added into the chamber placed in the 24-well plate, and the membrane was soaked in the dye at 37°C. After 30 min, the chamber was taken and washed with phosphate buffered saline (PBS). 3 randomly selected fields of each chamber were selected, followed by photography (×200) and counting for migratory cells. Invasion assay: 30 μL diluted Matrigel were pre-coated into the transwell chamber, and the remaining procedures were the same as those in migration assay.

Statistical Analysis

Statistical analysis was performed with a Student's *t*-test or *F*-test. All *p*-values were two-sided and $p < 0.05$ were considered significant and analyzed by Prism 6.0 software (La Jolla, CA, USA).

Results

Expression Level of MiR-214-5p in PC

QRT-PCR results suggested that compared with the adjacent normal tissues, the expression level of miR-214-5p in PC tissues was significantly lower (Figure 1A). Meanwhile, the results at the cellular level were the same as those at the tissue level. The low expression of miR-214-5p in PC cell line (PANC-1) was noticeable by comparing with control cell lines (Figure 1B).

Target Gene Prediction Results

The possible downstream target genes of miR-214-5p were predicted using classical bioinformatics software (TargetScan, PicTar and Miranda). It was found that JAG1 was a target gene, and there was an effect binding site of miR-214-5p in JAG1 3'-UTR. The dual-luciferase reporter results revealed that increased expression of miR-214-5p results in the decrease of the luciferase activity of the wide-type JAG1 3'-UTR reporter gene, but it had no effect on mutant-type (Figure 2A). Besides, Western blot results showed that JAG1 expression significantly decreases in PANC-1 cells transfected with miR-214-5p mimics by comparing with control (Figure 2B). The above results indicated that miR-214-5p could bind to JAG1 3'-UTR to regulate the JAG1 expression, and JAG1 was a functional target of miR-214-5p.

Effects of MiR-214-5p on Cell Proliferation

The proliferation capacity of PC cells after transfection was detected via CCK8 assay, and the absorbance was detected at 24, 48 and 72 h, respectively. It was found that after transfection with miR-214-5p mimics, the cell growth rate

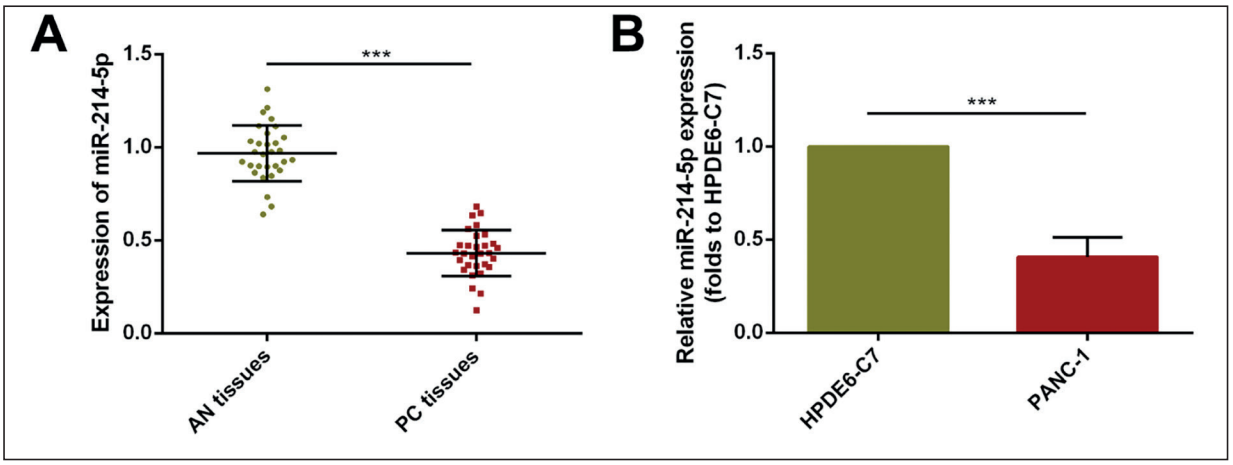

Figure 1. The expressions of miR-214-5p in PC tissue samples and cells. ***A,*** Expression of miR-214-5p in tumor tissues and adjacent normal tissues. (***$p < 0.001$). ***B,*** Expression of miR-214-5p in Human PC cells (PANC-1) and normal pancreatic cells (HPDE6-C7). (***$p < 0.001$).

declined, and it was significantly reduced at 72 h. However, the proliferation capacity of PANC1 cells co-transfected with miR-214-5p mimics and si-JAG1 had no statistically significant difference compared with that in NC group (Figure 3).

Effects of MiR-214-5p on Cell Invasion and Migration

Transwell assay manifested that the invasive and migratory capacities of PANC-1 cells were significantly inhibited in miR-214-5p transfection group, and the number of invasive and migratory cells was significantly less than that in NC group. On the contrary, the invasive and migratory capacities were intensive in miR-214-5p + si-JAG1 group, and the number of invasive and metastatic cells was significantly more than that in miR-214-5p mimics group (Figure 4). EMT refers to the process that epithelium-derived tumor cells lose the epithelial phenotype but obtain the mesen-

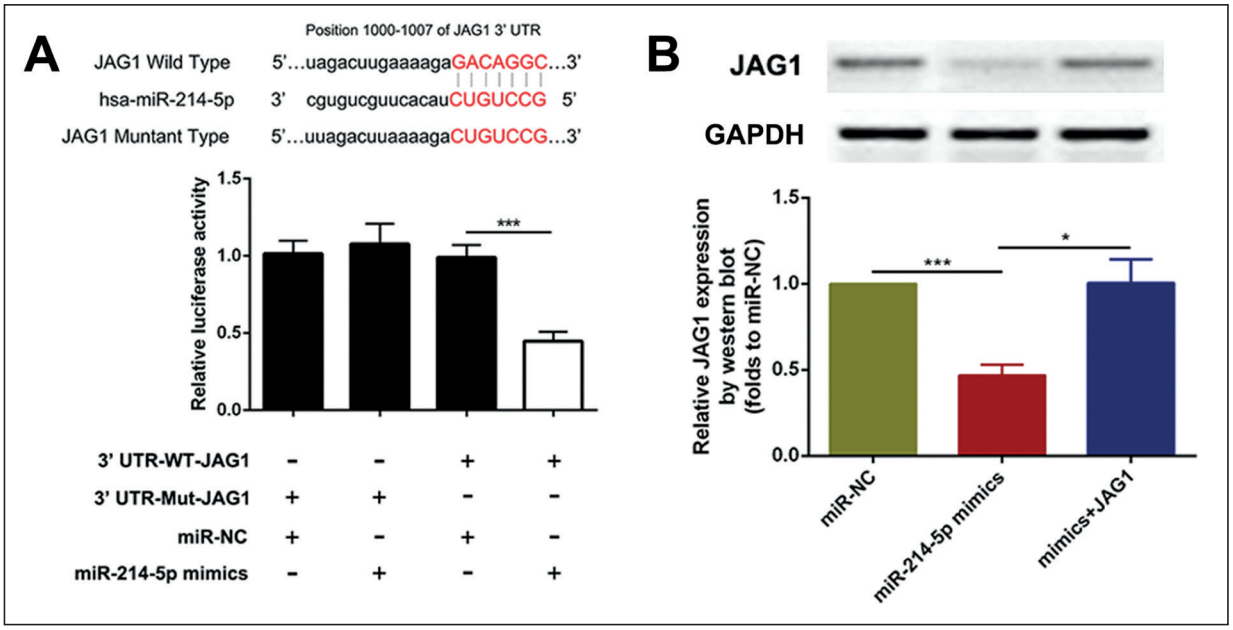

Figure 2. JAG1 was a direct and functional target of miR-214-5p. ***A,*** Diagram of putative miR-214-5p binding sites of JAG1 and relative activities of luciferase reporters. (***$p < 0.001$). ***B,*** MiR-214-5p decreased the expression level of JAG1 detected by Western blot experiment (*$p < 0.05$, ***$p < 0.001$).

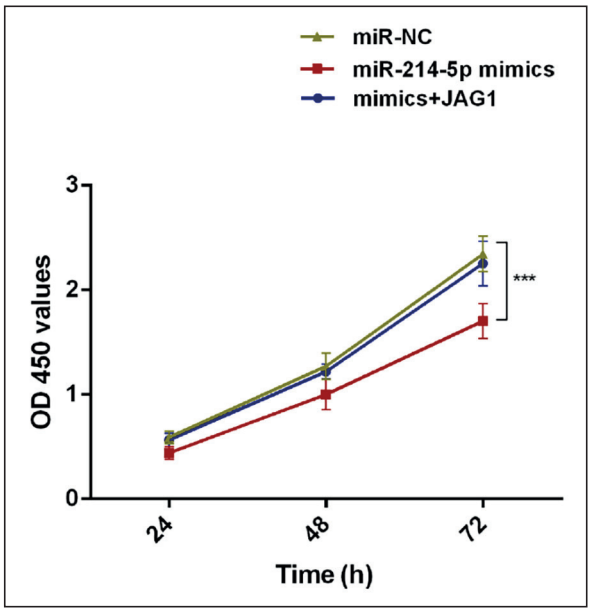

Figure 3. MiR-214-5p decreased the cell proliferation of PANC-1 cells. Cell proliferation was detected by CCK-8 assay (***$p < 0.001$).

chymal phenotype, thereby acquiring the invasion and migration capacities[16]. EMT is the first and critical step in the invasion and migration of tumor cells. The symbolic responses of EMT are the decreased expression of epithelial cell marker (E-cadherin) and the increased expression of mesenchymal cell marker (vimentin). Western blotting results showed that the E-cadherin expression increased while the vimentin expression decreased in miR-214-5p transfection group compared with that in NC group, indicating that EMT of PNCA-1 cells was inhibited. Interestingly, JAG1 could reverse the above effects of miR-214-5p (Figure 5).

Discussion

PC seriously threatens human life and health, so it is extremely important to explore its diagnostic molecular markers. In recent studies, various miRNAs, such as miR-124, miR-216a, miR-365, miR-744[17-20], have been found to be closely related to PC. Besides, they are expected to become important targets for the diagnosis and treatment of PC as biomarkers. Recent studies have reported that miR-214-5p is differentially expressed in osteosarcoma and hepatocellular carcinoma. In this experiment, it was found that the expression of miR-214-5p in PC tissues is significantly down-regulated compared with that in para-carcinoma normal tissues. Cellular level of miR-214-5p in PC cells exerted the same results. The above findings indicated that the down-regulation of miR-214-5p expression possibly possess certain correlations with the occurrence and de-

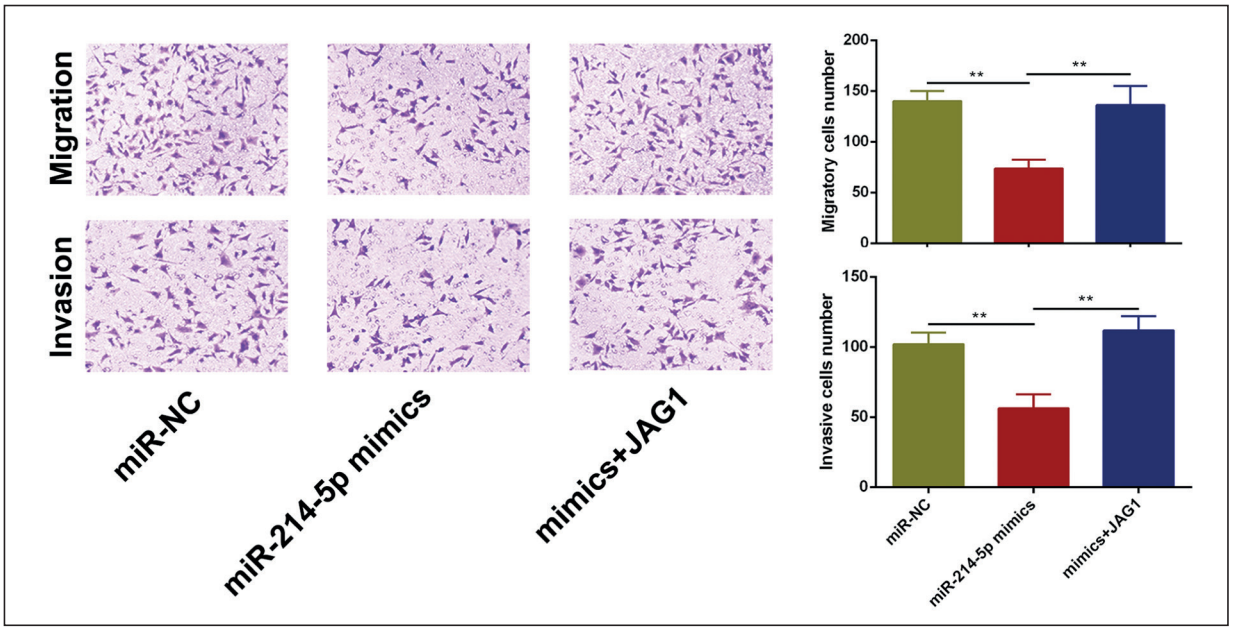

Figure 4. MiR-214-5p/JAG1 axis inhibited the invasion and migration of PANC-1 cell. The invasion and migration were tested by transwell assay. All data were presented as means ± standard deviations. (**$p < 0.01$).

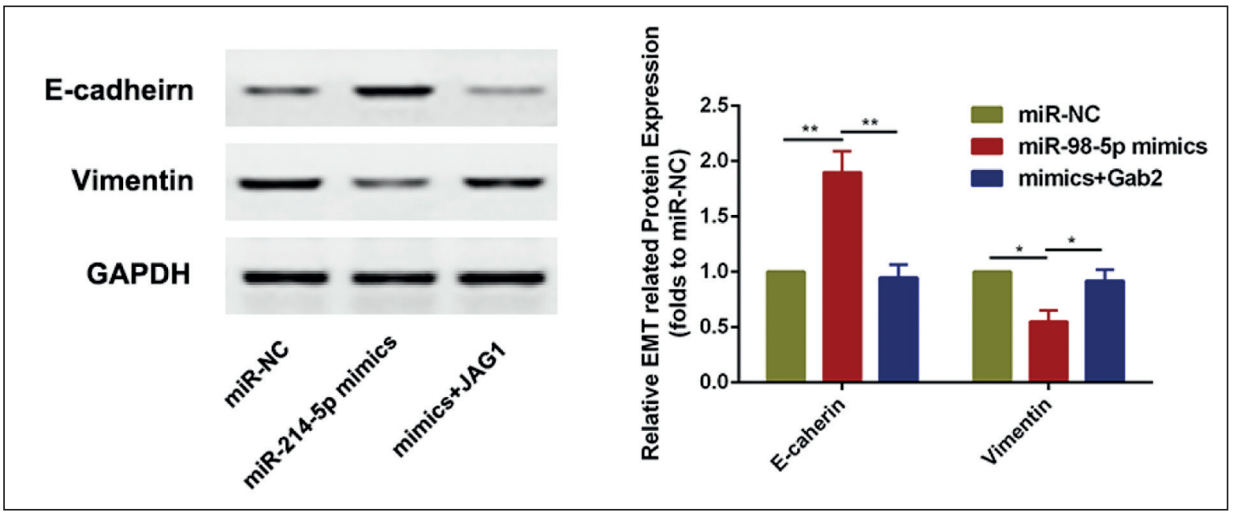

Figure 5. MiR-214-5p/JAG1 axis inhibited the EMT of PANC-1 cell. The expression of EMT markers after transfection with miR-214-5p mimics or si-JAG1 was detected by Western blotting. All data were presented as means ± standard deviations. (*$p < 0.05$, **$p < 0.01$).

velopment of PC. Corresponding functional targets of miRNA are important in the development of disease. Early previous studies have found the role of related pathogenic genes, including K-RAS, TP53, SMAD4 and DPC4, in the pathogenesis of PC[21-25]. Expression changes of these specific genes lead to the changes in downstream specific signaling pathways, which might play a certain role in the incidence of PC. Scholars[26,27] have demonstrated that several intracellular signaling pathways play key roles in the pathological progression of PC, such as phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (AKT), nuclear factor-κB (NF-κB) and S-phase kinase-associated protein 2 (Skp2). Notch signaling pathway exerts important roles in proliferation, differentiation, development and homeostasis of tumor cells. According to previous study, Notch1 could inhibit pancreatic intraepithelial neoplasia, namely the precursor lesion of PC. However, another previous work revealed that Notch exerts an extremely important carcinogenic effect in the pancreas[28]. JAG1 is the ligand of Notch receptor first confirmed in mammals, which distributes on the cell membrane of mammals and triggers the Notch signaling pathway through binding to the extracellular domain of Notch receptor on the surface of adjacent cells. After JAG1 interacts with the extracellular domain of Notch receptor, the intracellular domain of Notch receptor will be cleaved into soluble intracellular Notch (Notch IC). Subsequently, Notch IC enters the nucleus and binds to the transcription factor of CBF1/Suppressor of Hairless/Lag-1 (CSL) family members to activate HES and HRT (HEY) family members (target genes), thus regulating the transcription of target genes. It is found to participate in the normal cell development, differentiation, proliferation and apoptosis, as well as the occurrence and development of a variety of diseases, including the tumors[29-31]. Recent studies have shown that the activation of JAG1/Notch signaling pathway plays a key role in the EMT of tumor cells *via* regulating its downstream target genes. As a result, malignant tumor cells obtain stronger capacity of invasion and distant metastasis[32]. In this study, it was found that miR-214-5p could directly act on the 3'-UTR of JAG1, thus leading to the down-regulation of JAG1 expression. It was preliminarily proved in transwell assay that miR-214-5p could inhibit the proliferation, invasion and migration of PC cells. In addition, miR-214-5p could inhibit EMT of PC cells, manifesting as decreased expression of Vimentin and increased expression of E-cadherin. Moreover, it was found that overexpression of JAG1 could reverse the inhibitory effect of miR-214-5p on PC cells.

Conclusions

We found that miR-214-5p could affect invasive and migratory capacities of PC cells through inhibiting the expression of JAG1. Thus, miR-214-5p might serve as a therapeutic target for the treatment of PC.

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Conflict of Interest
The Authors declare that they have no conflict of interests.

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