Expression of Notch receptors and their ligands in pancreatic ductal adenocarcinoma

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Abstract. Pancreatic cancer is the fourth leading cause of cancer-associated mortality in developed countries. Pancreatic ductal adenocarcinoma (PDAC) accounts for ~90% of all pancreatic cancer cases. The Notch signaling pathway serves a crucial role in embryonic development, as well as during the tumorigenesis of different types of cancer. However, Notch signaling serves either oncogenic or tumor suppressor roles depending on the tissue type. There are four Notch receptors (Notch1-4) and five ligands [Jagged1, Jagged2, δ-like ligand protein (DLL)1, DLL3 and DLL4]; therefore, it has been suggested that the different Notch receptors serve distinct roles in the same type of tissue. To determine whether this is the case, the present study measured the expression of all Notch receptors and their ligands in PDAC tissue samples and cells. Immunohistochemistry was performed to measure the expression of Notch receptors and their ligands in paraffin-embedded PDAC tissue samples. Immunofluorescence was used to detect the expression of Notch receptors in the pancreatic cancer cell lines human pancreatic adenocarcinoma (HPAC) and PANC-1. In addition, levels of Notch receptors and ligands in HPAC and PANC-1 cells were analyzed by western blot analysis. The results revealed that levels of Notch1 and Notch3 were increased in PDAC tissues, whereas levels of Notch2 and Notch3 were not. The expression of Notch receptors in the pancreatic cancer cell lines HPAC and PANC-1 was consistent with their expression in PDAC tissues. Additionally, levels of the ligands DLL1, DLL3 and DLL4 were increased in HPAC and PANC-1 cells, as well as PDAC tissue samples. However, the expression of Jagged1 and 2 remained low. These results indicate that Notch1, Notch3, DLL1, DLL3 and DLL4 are upregulated in PDAC, a positive correlation was observed between the expression of Notch1 and Notch3, and between Notch1 and the ligands DLL1, DLL3 and DLL4. whereas Notch2, Notch4, Jagged1 and Jagged2 are not. The interaction of Notch1 and Notch3 with Notch ligands DLL1, DLL3 and DLL4 may be important in maintaining the tumor phenotype of pancreatic cancer.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) accounts for >90% of all pancreatic cancer cases (1). It represents a severe health risk and is the fourth leading cause of cancer-associated mortality in developed countries (2). PDAC is a highly aggressive malignancy with a poor prognosis; the five-year survival rate is <5% (3). As PDAC is often diagnosed at an advanced stage with metastasis, it is often too late for patients to undergo curative surgery and traditional chemotherapy is not an effective treatment strategy (4). Therefore, it is critical to understand the molecular mechanisms underlying the development and progression of PDAC to enable the development of novel strategies to inhibit tumor development, impede tumor growth and reduce the recurrence rate of the disease.

In mammals, Notch is a highly conserved gene family that includes the Notch1-4 receptors and their ligands: δ-like ligand protein (DLL)1, DLL3, DLL4, Jagged1 and Jagged2 (5). The Notch signaling pathway consists of Notch receptors, their ligands and C-promoter binding factor 1, suppressor of hairless, Lag-1 (CSL), DNA binding proteins and downstream target genes that are involved in regulating cell functions, including proliferation, differentiation and apoptosis (6,7). Binding of the Notch receptor to its ligand in adjacent cells activates the Notch signaling pathway (8). Notch receptor proteins are sheared by proteolytic enzymes, releasing the C-terminal intracellular domain (NICD), which then translocates into the nucleus. In the nucleus, the NICD binds to CSL, changing CSL from a transcriptional repressor to an activator (9). This leads to the activation of downstream Notch target genes, including Hes and Hey family genes (5). The Notch signaling pathway regulates pancreatic cell differentiation in the developing pancreas (6) and participates in the development and progression of PDAC (10-13). Although previous studies have described the activation of Notch signaling components in PDAC (14-17), the link between elevated Notch expression and tumorigenesis

in PDAC is controversial as contradictory results have been reported by different studies. Mazur *et al* (11) demonstrated that Notch signaling has a tumor promoting effect, whereas Hanlon *et al* (18) demonstrated that it had an inhibitory tumor effect (11,18). In addition, the expression pattern of the Notch receptors and ligands in PDAC remains unclear.

The high expression of a potential oncogene means that it serves a significant role in cancer (17). Therefore, to elucidate the role of Notch signaling in PDAC, in the current study, immunohistochemical staining was performed on samples collected from 24 patients with the necessary associated clinical data. Immunofluorescence staining and western blot analysis were also performed to detect the expression of Notch receptors and their ligands in the pancreatic cancer human pancreatic adenocarcinoma (HPAC) and PANC-1 cell lines.

Materials and methods

Cell lines. The PDAC cell lines HPAC and PANC-1 and the 293 T cells and HeLa cell lines were all purchased from the cell bank of the Chinese Academy of Sciences (Beijing, China). PANC-1 and 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-high glucose (Hyclone™; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Zhejiang Tianhang Biotechnology Co., Ltd., Huzhou, China), 1% penicillin and 1% streptomycin (Beyotime Institute of Biotechnology, Haimen, China). HPAC cells and HeLa cells were maintained in RPMI-1640 (GE Healthcare Life Sciences) supplemented with 10% FBS, 1% penicillin and 1% streptomycin. All cells were maintained at 37˚C in 5% CO_2.

A total of 24 PDAC tissues were collected from patients who underwent surgery for pancreatic cancer at the Affiliated Center Hospital of Xinxiang Medical University (Xinxiang, China) from May 2010 to July 2015. PDAC tissues were then formalin-fixed (10% formalin for 24 h at room temperature) and paraffin-embedded. The study protocol adhered to The Code of Ethics of the World Medical Association (Declaration of Helsinki). The present study was approved by the Ethics Committee of the Affiliated Center Hospital of Xinxiang Medical University. Written informed consent was obtained from all patients prior to the procedure. Patient information is listed in Table I. PDAC tissues were confirmed using histopathological analysis.

Immunohistochemistry. For histological assessment, immunohistochemical analysis was performed using 5-μm-thick PDAC tissue sections. Xylene and graded alcohols were used for dewaxing and rehydration. Subsequently, sections were treated with citrate salt buffer (pH 6.0) in a microwave for 15 min for antigen retrieval (100˚C), followed by incubation with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity at room temperature. The samples were blocked with 5% donkey blood serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in PBS for 1 h at room temperature. The primary antibodies used in the experiments are listed in Table II. Samples were incubated with the primary antibodies at 4˚C overnight, followed by incubation with secondary horseradish peroxidase (HRP)-conjugated antibodies (cat. no. SP-9001; OriGene Technologies, Inc., Beijing, China) for 1 h at room temperature. Diaminobenzidine and hematoxylin were used for staining (20 sec) and counterstaining (10 sec), respectively at room temperature. Following dehydration with graded alcohols and xylene, slides were sealed with coverslips and neutral gum. The negative control group was incubated with PBS instead of the primary antibody. Staining intensities were quantified by two pathologists blinded to the sample group. The Video Pro32 color image analysis system was used, using the Grey value and optical density value to analyze the immunohistochemical positive expression strength. The intensity of Notch receptors and ligands staining was scored using the following scoring system: 0 (no appreciable staining; negative), 1 (barely detectable staining; weak positive), 2 (readily identifiable brown staining; positive) and 3 (dark brown staining; strong positive). The total score was calculated by multiplying the percentage of positive cells and the intensity score. A tumor sample was considered positive if the score was ≥4 and negative otherwise.

Immunofluorescence. Cell lines from adherent cultures were digested using 0.25% trypsin with EDTA at 37˚C for 8 min and centrifuged at 180 x g for 4 min at room temperature. The cell pellet was resuspended in complete DMEM-high glucose (10% FBS, 1% penicillin and 1% streptomycin). Following the preparation of 6-well plates with coverslips, cell suspensions were added to each well ($3x10^5$/well). Cells were cultured at 37˚C in 5% CO_2 for 48 h, washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were subsequently washed with 1% PBS with Triton-100 to penetrate the cell membrane. Following incubation with 10% donkey serum (cat. no. 017-000-121; Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 1 h, cells were incubated with primary antibodies against Notch1, Notch2, Notch3, and Notch4 and their ligands Jagged1, Jagged2, DLL1, DLL3 and DLL4 (Table II) at 4˚C overnight. The signals were generated following incubation with Alexa Fluor 594-conjugated donkey anti-rabbit immunoglobulin G (IgG) secondary antibodies, (dilution, 1:1,000; cat. no. R37119; Invitrogen™; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at room temperature for 1 h. Nuclear staining was performed with DAPI (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 5 min at room temperature. Stained coverslips were visualized using a laser scanning confocal microscope (Olympus Soft Imaging Solutions GmbH, Münster, Germany) at magnification x40 and x100. The negative control group was incubated with PBS instead of the primary antibodies.

Western blot analysis. The PDAC cell lines HPAC and PANC-1 were cultured in culture flasks and collected when they became confluent. Cells were subsequently homogenized in a radioimmunoprecipitation buffer for protein extraction [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 10 μl/ml protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride; cat. no. P0013B; Beyotime Institute of Biotechnology]. The protein samples were separated by either 8 or 10% SDS-PAGE and subsequently transferred onto nitrocellulose membranes (Merck KGaA). Following 3 washes for 10 min/wash with 20 mM Tris-Cl (pH 7.5), 0.15 M NaCl and 0.05% Tween-20

Table I. Patient information.

No.	Age	Sex	TNM	Grade	Stage
1	77	F	T2N0M0	1	I
2	46	F	T3N0M0	1	II
3	56	F	T2N0M0	1	I
4	47	F	T3N0M0	1	II
5	64	F	T2N0M0	1	I
6	77	M	T2N0M0	1	I
7	67	F	T3N0M0	1	III
8	50	M	T3N0M0	1	II
9	48	F	T3N0M0	1	II
10	77	M	T3N0M0	1	II
11	65	M	T2N0M0	1	I
12	47	M	T3N0M0	1	II
13	61	M	T2N0M0	1	I
14	65	M	T3N0M0	2	II
15	57	M	T3N0M0	2	II
16	38	M	T3N0M1	2	IV
17	39	M	T3N0M0	2	II
18	31	M	T3N0M0	2	II
19	42	M	T3N0M0	1	II
20	44	M	T3N0M0	2	II
21	57	M	T3N0M0	2	II
22	59	M	T3N0M0	2	II
23	75	F	T3N0M1	2	IV
24	52	M	T1N0M0	2	I

TNM, tumor, node and metastasis; M, male; F, female. T1, tumor invading the submucosa; T2, tumor invading the muscularis propria; T3, tumor invading through the muscularis propria into the subserosa or into the non-peritonealized pericolic or perirectal tissues; N0, no regional lymph node metastasis; N1, metastasis in 1-3 regional lymph nodes; M0, no distant metastasis; M1, distant metastasis.

Table II. Antibodies used within the study.

Antigen	Host species	Dilution		Supplier	Cat. no.
		IHC	WB		
Notch1	Rabbit	1:50	1:500	SC	sc-6014R
Notch2	Rabbit	1:500	1:2,000	LS	LS-B399
Notch3	Rabbit	1:50	1:500	SC	sc-5593
Notch4	Rabbit	1:50	1:500	SC	sc-5594
Jagged1	Rabbit	1:50	1:500	SC	sc-8303
Jagged2	Rabbit	1:50	1:500	SC	sc-5604
DLL1	Rabbit	1:50	1:500	Ab	ab76655
DLL3	Rabbit	1:100	1:1,000	CS	2483s
DLL4	Rabbit	1:50	1:1,000	BR	HP1274

SC, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; Ab, Abcam, Cambridge, MA, USA; CS, Cell Signaling Technology, Inc., Danvers, MA, USA; LS, LifeSpan Biosciences, Inc., Seattle, WA, USA; BR, Bio-Rad Laboratories, Inc., Hercules, CA, USA; IHC, immunohistochemistry; WB, western blot analysis.

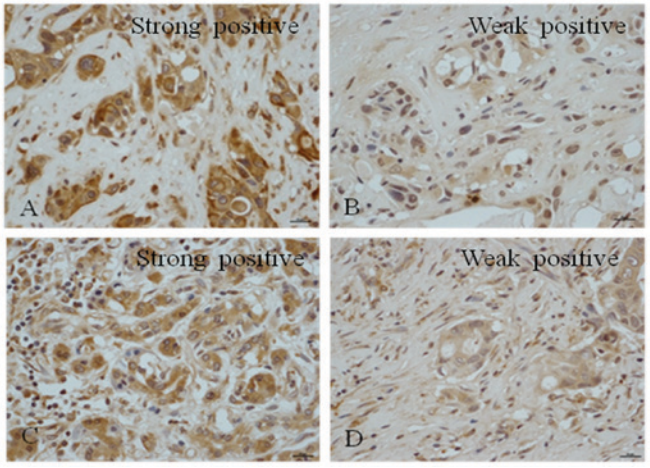

Figure 1. Representative examples of immunohistochemistical staining for Notch receptors in PDAC tissues, indicating that Notch1 and 3 expression levels are increased whereas Notch 2 and 4 levels are decreased in PDAC tissue. Rabbit polyclonal anti-Notch antibodies were used to determine the expression of (A) Notch1, (B) Notch2, (C) Notch3 and (D) Notch4 in PDAC tissues. Scale bar=20 μm; magnification x40. PDAC, pancreatic ductal adenocarcinoma.

(TBST), cells were blocked with 5% skimmed milk in TBST for 1 h at room temperature. Membranes were then incubated overnight with primary antibodies at 4˚C (Table II). The membranes were subsequently incubated with secondary goat anti-rabbit IgG HRP-conjugated antibodies (1:1,000; cat. no. 111-625-144; LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature. Protein bands were visualized using a chemiluminescence detection system (Odyssey® two-color infrared fluorescence imaging system; LI-COR Biosciences). Protein levels were normalized to GAPDH (1:10,000; cat. no. G9545; Sigma-Aldrich; Merck KGaA) levels and quantified using ImageJ software version 1.43b (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Statistical analysis was performed using SPSS software, version 19.0 (IBM Corp., Armonk, NY, USA). Pearson's correlation co-efficient was used to identify whether there were correlations between the expression of Notch receptors and their ligands. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Positive rate and intensity of Notch receptor expression. Rabbit polyclonal anti-Notch1-4 antibodies were used to detect the expression of Notch1-4 in human PDAC tissues (Fig. 1). It was observed that Notch1 was expressed in all PDAC samples; 91.7% of the tissues exhibited strong positive staining and 8.3% demonstrated weak positive staining; none of the samples were negative for Notch1 (Table III). A total of 41.7% of the samples were negative for Notch2, 37.5% exhibited weak positivity and 20.8% of the samples exhibited positive nuclear staining (Table III). It was observed that 41.7% of the samples had positive staining for Notch3, among which 12.5% were strongly positive (Table III). Among the samples, 45.8% were weakly positive for Notch3 and 12.5%

Table III. Positive rate and intensity of Notch receptors and ligands expression.

Antigen	0 (%)	1 (%)	2 (%)	3 (%)	Total positive rate (%)
Notch1	0.0	8.3	25.0	66.7	91.7
Notch2	41.7	37.5	20.8	0.0	20.8
Notch3	12.5	45.8	29.2	12.5	41.7[a]
Notch4	41.7	20.8	33.3	4.2	37.5[b]
JAGGED1	62.5	20.9	8.3	8.3	16.6[b]
JAGGED2	45.8	16.7	37.5	0.0	37.5[b]
DLL1	0.0	20.8	25.0	54.2	79.2[a]
DLL3	25.0	25.0	29.2	20.8	50[a]
DLL4	8.3	29.2	12.5	50.0	62.5[a]

[a]Statistically significant positive correlation with Notch1; [b]statistically significant negative correlation with Notch1. 0, no appreciable staining-negative; 1, barely detectable staining-weak positive; 2, readily identifiable brown staining-positive; 3, dark brown staining-strong positive. DLL, δ-like ligand.

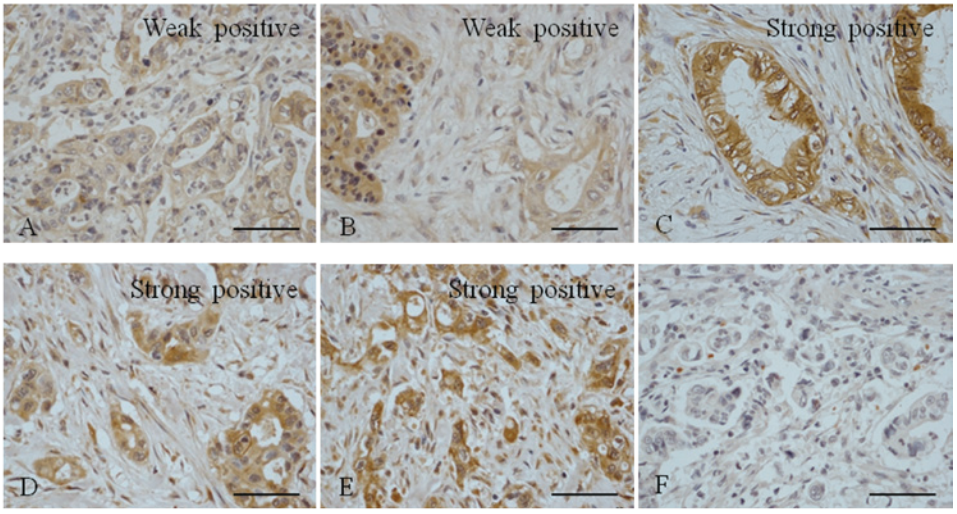

Figure 2. Representative examples of immunohistochemistry staining for Notch ligands in pancreatic ductal adenocarcinoma tissues, indicating that DLL1, DLL3 and DLL4 levels are increased whereas Jagged 1 and 2 levels remain low in PDAC. Rabbit polyclonal antibodies were used to measure the expression of (A) Jagged1, (B) Jagged2, (C) DLL1, (D) DLL3 and (E) DLL4 in PDAC tissues. (F) negative control. Scale bar=50 μm. PDAC, pancreatic ductal adenocarcinoma; DLL, δ-like ligand.

were negative (Table III). A total of 41.7% of the samples were negative for Notch4, 20.8% were weakly positive, 37.5% exhibited positive staining and 4.2% exhibited strong positive expression (Table III).

Positive rate and intensity of Notch ligand expression. Polyclonal Jagged1 and 2 antibodies were used to detect the expression of Jagged1 and 2 in PDAC tissues (Fig. 2). It was observed that 62.5% of the samples were negative for Jagged1 expression and 20.9% were weakly positive; 16.6% of samples were positive for Jagged1 but only 8.3% exhibited strong positive expression (Table II). A total of 45.8% of the samples were negative for Jagged2 expression, 16.7% were weakly positive and 37.5% exhibited positive staining.

It was observed that only 20.8% of the PDAC tissue samples exhibited weak positive staining for DLL1; 79.2% were positive for DLL1 and 54.2% exhibited strong expression (Table III). None of the samples were negative for DLL1. A total of 25% of the PDAC tissue samples were negative for DLL3 expression, 25% were weakly positive and 50% were positive, with 20.8% exhibiting strong positive expression (Table II). The results revealed that 8.3% of the PDAC tissue samples were negative for DLL4, 29.2% were weakly positive and 62.5% were positive, with 50% of the samples exhibiting strong positive expression (Table III). These results were similar to the results obtained regarding DLL1 expression.

Correlation between the expression of Notch receptors and their ligands. As demonstrated in Figs. 1 and 2 and Table III, the expression of Notch receptors and their ligands were examined. It was observed that the majority of PDAC tissue samples (91.7%) exhibited high Notch1 expression. A total of 41.7% of samples exhibited positive Notch3 expression. Among the ligands, the majority of PDAC tissues (79.2%) stained positive for DLL1; 62.5% stained positive for DLL4 and 50% stained positive for DLL3 (Table II). A significant positive correlation

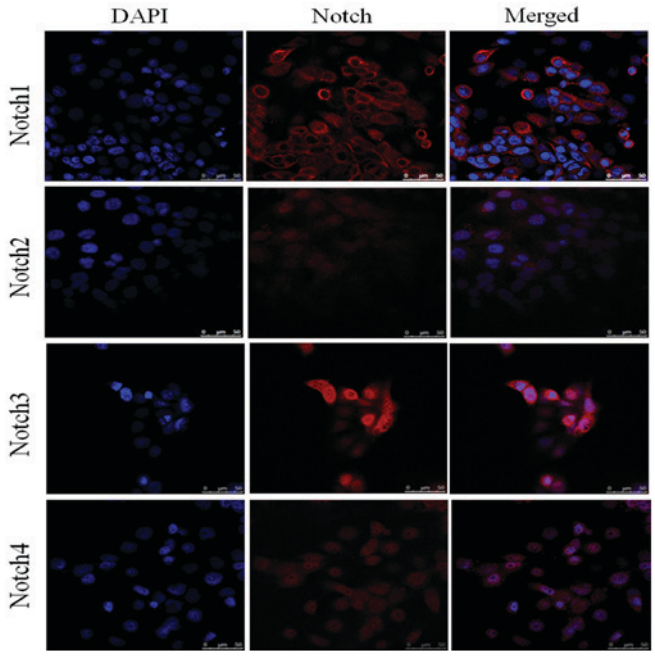

Figure 3. Representative examples of immunofluorescent staining for Notch receptors in HPAC pancreatic cancer cells. DAPI staining is indicated in blue and Notch staining is indicated in red. Scale bar=50 μm. HPAC, human pancreatic adenocarcinoma.

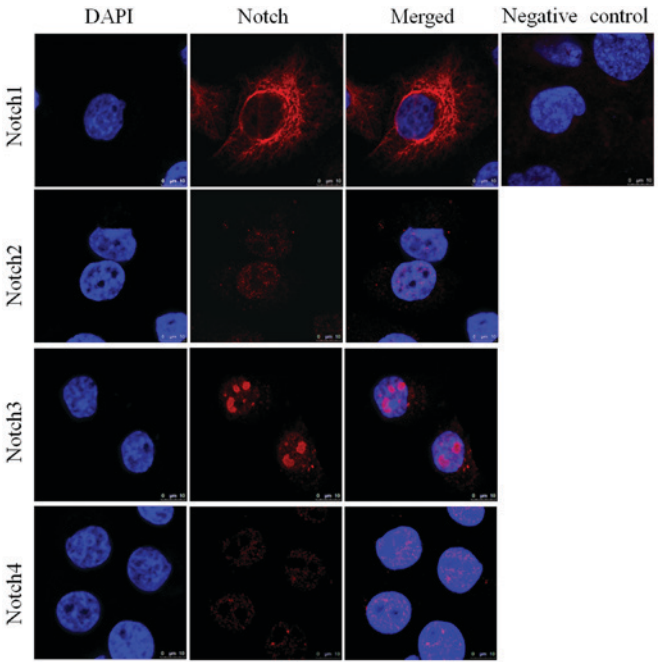

Figure 4. Representative examples of immunofluorescent staining for Notch receptors in PANC-1 pancreatic cancer cells. DAPI staining is indicated in blue and Notch is indicated in red. PBS was used for the negative control. Scale bar=10 μm. PDAC, pancreatic ductal adenocarcinoma.

between Notch1 and DLL1 was observed. Notch1 also exhibited a positive correlation with DLL4 and DLL3 (r=0.7; P=0.0020). However, Notch1 was negatively correlated with Notch4, Jagged1 and Jagged2. Notch2 expression was positively correlated with Notch4, Jagged1 and Jagged2 (r=0.5; P=0.0087).

Expression of Notch receptors in pancreatic cancer cell lines assessed by immunofluorescence analysis. Immunofluorescence analysis of HPAC (Fig. 3) and PANC-1 (Fig. 4) cell lines assessed the expression of Notch1, Notch2, Notch3 and Notch4 in each of these cell lines. DAPI (blue) was used to stain the nucleus. Notch1 exhibited positive expression in the cytoplasm and around the nucleus, whereas Notch3 had clear nuclear localization (Fig. 4). The expression of Notch2 and Notch4 was notably lower in each of the cell lines compared with Notch1 and Notch3. The elevated expression of Notch1 and Notch3 in HPAC and PANC-1 cell lines was in accordance with their pattern of expression in tissue samples from patients with PDAC. Similarly, the lower expression of Notch2 and Notch4 in the HPAC and PANC-1 cell lines was consistent with their lower expression in PDAC tissue samples.

Expression of Notch receptors in pancreatic cancer cell lines determined by western blot analysis. The expression of the Notch receptors in the pancreatic HPAC and PANC-1 cell lines was assessed (Fig. 5). The results revealed that the expression of Notch1 was notably increased in HPAC cells compared with PANC-1 cells, while Notch3 was highly expressed in the two cell lines. The expression of Notch2 and Notch4 was markedly lower than Notch1 and Notch3 in the two cell lines. The protein expression of the Notch ligands in HPAC and PANC-1 cells was also measured (Fig. 6). Levels of DLL1, DLL3 and DLL4 expression were higher than those of Jagged2 in each of the cell lines. The expression of Jagged1 was notably higher in HPAC cells compared with PANC-1 cells. The specificity of the antibodies was confirmed in HeLa and 293T cell lines (data not shown).

Discussion

In the present study, immunohistochemistry was performed to evaluate the expression of proteins in the Notch signaling pathway, including various receptors and ligands associated with PDAC. To the best of our knowledge, the current study is the first to evaluate the expression of Notch signaling pathway components and investigate the correlations among them.

The *Notch* gene was first identified in *Drosophila* in 1917 (19) and Notch1 was revealed to be associated with T-cell acute lymphoblastic leukemia in 1991 (20). The roles of the Notch signaling pathway in embryonic development (21,22), adult differentiation (23,24), and the development tumors (10-13) have been previously studied and positively confirmed. In eukaryotes, the Notch signaling pathway is highly conserved and regulates cell proliferation, differentiation and apoptosis through interactions between adjacent cells (25-27).

Notch signaling serves a central role in tumors and during the embryonic development of the pancreas, in which it controls cellular differentiation (26). However, the expression and functions of each of the Notch signaling pathway components differ in tumor development, including in PDAC. Miyamoto *et al* (14) revealed that the expression of the Notch1, Notch2, Notch3 and Notch4 receptors and their ligand Jagged1 was upregulated in resected pancreatic cancer samples. The expression of the Notch signaling target transcription factor Hes1 (Hes1) was also upregulated in pancreatic cancer cells (14). However, Vo *et al* (16) reported that among the Notch family members, Notch3 was primarily overexpressed in pancreatic cancer, followed by Notch4 and

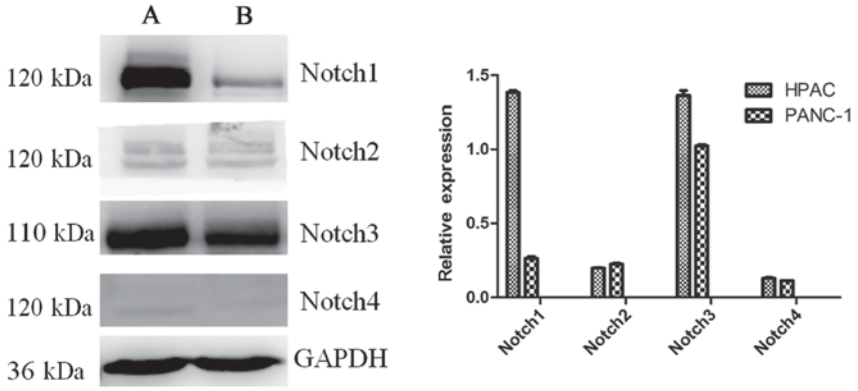

Figure 5. Western blot analysis measuring the expression of Notch receptors in pancreatic cancer cells. (A) HPAC cells; (B) PANC-1 cells. HPAC, human pancreatic adenocarcinoma; PDAC, pancreatic ductal adenocarcinoma.

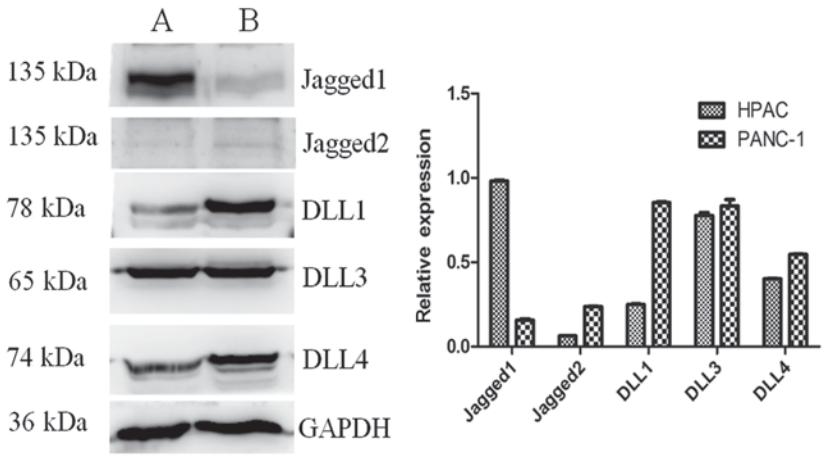

Figure 6. Western blot analysis measuring the expression of Notch ligands in pancreatic cancer cells. (A) HPAC cells; (B) PANC-1 cells. HPAC, human pancreatic adenocarcinoma; PDAC, pancreatic ductal adenocarcinoma; DLL, δ-like ligand.

Jagged1, whereas Notch1 was not expressed in malignant cells. Another previous study demonstrated that Notch3 was significantly overexpressed in the cytoplasm and nucleus in 43.5% of pancreatic adenocarcinoma tumors (28). Mann *et al* (29) demonstrated that the Notch signaling pathway components Notch1, Notch3, Notch4, Hes1 and hairy/enhancer-of-split related with YRPW motif protein 1 were significantly elevated in pancreatic adenocarcinoma. Therefore, it remains unclear how the expression of various Notch proteins changes during the progression of cancer, and to the best of our knowledge a complete investigation examining the expression of all Notch receptors and their ligands in PDAC has not yet been conducted.

The elevated expression of Notch1 in pancreatic cancer leads to the accumulation of undifferentiated precursor cells (30), whereas the downregulation of Notch1 decreases cyclin D1 and B-cell lymphoma 2 expression, which increases the apoptosis of pancreatic cancer cells (31). It has been demonstrated that inhibiting the Notch signaling pathway using Notch1 small interfering RNA triggers apoptosis in the pancreatic cancer cell lines BxPC-3, MIAPaCa-2 and PANC-1 (32). Blocking Notch2/3 inhibits tumor growth and tumor-initiating cells (33) and the inhibition of Notch1 and Notch4 expression inhibits tumor growth (34,35). Mazur *et al* (11) demonstrated that Notch2 is a central regulator of pancreatic intraepithelial neoplasia progression and malignant transformation. Notch1 has also been reported to function as a tumor suppressor gene in PDAC (18). A number of studies have reported conflicting results and the role of Notch signaling in PDAC remains highly controversial. In addition, there is little information available concerning the expression pattern of Notch receptors and their ligands in PDAC.

Therefore, the present study was conducted to estimate the expression and potential pathological significance of all Notch receptors and their ligands in human PDAC. In the present study, Notch1 exhibited increased expression in PDAC tissues, in which it may serve a role in the development of pancreatic cancer development by acting as an oncogene. Notch3 was also highly expressed, suggesting that it serves a similar role to Notch1 in PDAC. The DLL1, DLL3 and DLL4 ligands were upregulated. By contrast, levels of Notch2 and Notch4 were decreased in PDAC tissues. In the cohort of patients assessed in the current study, the expression of the ligands Jagged1 and Jagged2 were also decreased compared with DLL1, DLL3 and DLL4.

To determine the expression and potential functions of these molecules, HPAC and PANC-1 pancreatic cancer cell lines were selected and immunofluorescence staining and western blot analysis was performed. The results of immunofluorescence staining revealed that Notch1 was expressed in the

cytoplasm and around the nucleus. The expression of Notch3 was also positive, however, it was localized in the nucleus. Levels of Notch2 and Notch4 were decreased compared with Notch1 and Notch3. Therefore, the expression of Notch1 and Notch3 in the pancreatic cancer cell lines corresponded with their expression in PDAC cancer tissues, confirming that they are highly expressed in PDAC. The expression of Notch2 and Notch4 in the cancer cell lines was also consistent with their expression in cancer tissues; Notch2 and Notch4 expression were decreased compared with Notch1 and Notch3. Western blot analysis also revealed notably elevated expression of Notch1 and Notch3 compared with Notch2 and Notch4 in the pancreatic cancer cell lines HPAC and PANC-1. The Notch ligands DLL1, DLL3 and DLL4 exhibited markedly higher expression than that of Jagged2.

In the present study, a positive correlation was observed between the expression of Notch1 and Notch3, and between Notch1 and the ligands DLL1, DLL3 and DLL4. The results of the western blot analysis were consistent with those of immunohistochemistry, suggesting that the Notch1 and Notch3 pathways may be initiated by DLL1, DLL3 or DLL4. However, they do not seem to be initiated by Jagged2.

Due to the limited number of patients recruited and the lack of normal controls in the present study, these results may not be representative of the entire population. Future studies should be conducted to investigate a greater number of samples to confirm the results of the present study. Understanding the molecular characteristics of tumors may provide an important basis for the clinical diagnosis and treatment of patients with pancreatic cancer. The present study suggested that Notch1 and Notch3 may be potential targets for treatments against PC, and may provide the basis for a novel method of treatment and diagnosis of PC in the future.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YZ and HS conceived and designed the experiments. HS, YW and HL conducted the experiments.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Affiliated Center Hospital of Xinxiang Medical University and written informed consent was obtained from all patients prior to their inclusion within the study.

Consent for publication

All patients provided written consent for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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