Norepinephrine enhances cell viability and invasion, and inhibits apoptosis of pancreatic cancer cells in a Notch-1-dependent manner

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive types of cancer, which is associated with a poor prognosis due to complexities in prevention, early diagnosis and effective treatment. The lack of understanding regarding its induction and specific pro-cancer mechanisms may contribute to its poor prognosis. The Notch-1 pathway is widely considered to be a critical tumor-promoting factor in PDAC. Previous studies have indicated that chronic psychological stress may promote the development of PDAC partially via the main downstream stress hormone, norepinephrine (NE); however, to the best of our knowledge, the role of the Notch-1 pathway in this process has not been studied. Therefore, the present study aimed to explore this process. The expression levels of Notch-1 pathway-associated molecules were measured in response to NE using reverse transcription-quantitative polymerase chain reaction and western blotting. Alongside NE treatment, two Notch-1 pathway blockers, Notch-1-specific small interfering (si)RNA and DAPT (an inhibitor of the Notch-1 pathway), were used to explore the relationship between NE and the Notch-1 pathway in the development of pancreatic cell malignant biological behaviors, including cell viability, apoptosis and cell invasion. The results demonstrated that treatment with NE enhanced cell viability and invasion, and inhibited apoptosis of PDAC cells; however, these effects were suppressed following treatment with Notch-1-specific siRNA and DAPT. In conclusion, NE may enhance the malignant biological behaviors of PDAC via activating the Notch-1 pathway.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) has a 5-year survival rate of <8% (1), and is characterized by a highly aggressive nature and poor response to clinical treatment. Only a small percentage of patients are able to receive radical resection, which is the only curative treatment option, and/or adjuvant chemotherapy with agents such as gemcitabine and the oral fluoropyrimidine derivative S-1, or other advanced therapeutic strategies. Among patients who undergo radical resection, the 5-year survival rate is still only ~25% (2). At present, little is known about the factors that contribute to the initiation and progression of PDAC, and its specific underlying mechanisms.

Chronic psychological stress is considered to be a powerful tumor promoter in numerous types of cancer, including PDAC, via inducing activation of the hypothalamic pituitary adrenal (HPA) axis and/or the sympathetic nervous system (SNS) (3,4). Norepinephrine (NE) is a major stress hormone, which serves a vital role in the chronic psychological stress that may induce tumor progression. According to Lara et al (5), the concentration of NE can be ≤10 µM in the tumor microenvironment, and NE may promote the proliferation, invasion, migration and malignant biological behaviors of PDAC via activation of the β2-adrenergic receptor (β2-AR) in vitro and in vivo (6-9). However, these studies did not elucidate the possible downstream mechanisms; therefore, additional research is required.

The Notch-1 pathway is involved in several physical and pathological biological processes, including cancer (10-15). Notch is essential for embryonic development of the pancreas and is involved in the plasticity of adult exocrine cells (16-18); in addition, abnormal activation of the Notch-1 pathway is correlated with the initiation and progression of PDAC (10,11,19). Previous studies reported that chronic stress inhibits differentiation, and maintains the stem cell state of hematopoietic stem cells via activating the Notch-1 pathway (20,21). Furthermore, NE may promote angiogenesis.
in breast cancer via upregulation of the Notch pathway molecule Jagged-1 (22). These studies suggest that chronic stress, NE and the Notch-1 pathway may have an interactive relationship in PDAC. The present study hypothesized that the stress hormone NE may activate the Notch-1 pathway in PDAC, thus contributing to its malignant biological behaviors.

Materials and methods

Cell culture and reagents. Human PDAC cell lines (AsPc-1, BXPC-3, Panc-1, HPAC, Mia PaCa-2 and SW1990) were purchased from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (Shanghai, China). Panc-1 and Mia PaCa-2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), HPAC and BXPC-3 cells were cultured in RPMI-1640 (DMEM; HyClone; GE Healthcare Life Sciences), both media were supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and 1% penicillin-streptomycin. SW1990 cells were cultured in L-15 Leibovitz media (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were cultured under standard conditions in an atmosphere containing 5% CO2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using the Fastgen200 RNA isolation system (Fastgen, Shanghai, China), according to the manufacturer’s protocol. A Prime Script RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to reverse transcribe total RNA into cDNA, according to the manufacturer’s protocol. qPCR was conducted using an iQ5 Multicolor Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a SYBR Green PCR kit (Takara Biotechnology Co., Ltd.). The following PCR program was used: Denaturation at 94˚C for 5 min, followed by 35 cycles consisting of denaturation at 94˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 45 sec; finally, the samples were incubated at 72˚C for 5 min and then maintained at 4˚C. The specificity of the amplified PCR products was evaluated by melting curve analysis. The comparative Cq method (23), with β-actin as the normalization control, was used to assess the expression level of each target gene, as previously described (24). The PCR primer sequences used were as follows: Jagged-1, forward TTG TTAATGGTTATCGCTGTATC, and reverse GCA GTT CTTGCCCTCATAGTCC; Notch-1, forward GGC ACTTCTG TGTGAGGAGGA, and reverse GCGACTGGCCGTTGT GTTCT; RBP-Jk, forward GACTCAGACAAGCGAAG CA, and reverse GTGATTAAACAGAGACCAC; Hes-1, forward TAGCTCGCGATTCACAG, and reverse AAG CGGTCACCTCGTCCA; MMP-2, forward GATGATGCC TTTGCTGTCG, and reverse CAAAGGGTAGTACCTCG CA; MMP-9, forward TTCACCTTTGTGCTCTTCCCT, and reverse CTGCCACCGAGTGTAACCA; and β-actin forward GACCTAGTTGCGTATCCCTT, and reverse GACGGGTGAAGTGACAGCAT. The housekeeping gene β-actin was used as an internal control.

Western blot analysis. Whole-cell lysates of Panc-1 and BXPC-3 cells were prepared using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer’s protocol. The protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Thermo Fisher Scientific, Inc.). The protein lysates were resolved on a 10% polyacrylamide gel with a 5% stacking gel. The proteins were subsequently transferred to polyvinylidene difluoride membranes. The membranes were blocked for 2 h in Tris-buffered saline-0.1% (vol/vol) Tween-20 (TBST) containing 10% (wt/vol) nonfat dry milk powder at room temperature and were then incubated with primary antibodies overnight at 4˚C. Following incubation with goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP) (cat. no. sc-2004, 1:10,000) and goat anti-mouse IgG-HRP (cat. no. sc-2005, 1:10,000) secondary antibodies (Santa Cruz Biotechnology, Inc.), for 2 h at room temperature, the membranes were washed with TBST, and the immunocomplexes were detected using an enhanced chemiluminescence kit (EMD Millipore, Billerica, MA, USA) and the Molecular Imaging ChemiDoc XRS system (Bio-Rad Laboratories, Inc.). β-actin was used as the internal loading control.
Immunofluorescence analysis. After applying the aforementioned intervention strategies, the cancer cells were fixed in 4% formaldehyde diluted in PBS for 20 min at room temperature. After permeabilization with 0.3% Triton X-100, the cells were treated with blocking buffer (5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) in PBS) for 1 h, and then incubated with the primary antibodies at 4˚C overnight. The cells were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (green) secondary antibodies (cat. no. 111-545-003, 1:200; Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) at room temperature for 30 min, and the nuclei were stained with DAPI. Images were pseudo-colored using a Zeiss Instruments confocal microscope (Zeiss GmbH, Jena, Germany).

MTT assay. Cell viability was analyzed using an MTT assay according to a previously described method (25). Cancer cells were seeded in 96-well tissue culture plates at a density of 5,000-10,000 cells/well 24 h prior to serum starvation. After serum starvation for 24 h, cells were cultured in medium and were treated with the aforementioned intervention strategies. After 12, 24 or 48 h, the medium was removed, and MTT reagent was added to each well and incubated at 37˚C for 4 h. Subsequently, 150 µl dimethyl sulfoxide was added to each well and the cells were incubated in the dark for 10 min at room temperature. Optical density (OD) values were measured at 490 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Cell viability rate was defined as follows: OD (sample well)/OD (control well).

Apoptosis assay. Cell apoptosis was assessed by flow cytometry using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences, San Diego, CA, USA), according to the manufacturer’s protocol, as previously described (26). Briefly, cancer cells were seeded into 6-well plates at a density of 2x10⁵ cells/well, and after being serum-starved overnight, the cells were treated with the aforementioned intervention strategies for 48 h. Subsequently, the cells were trypsinized, washed with PBS and stained with Annexin V and PI. The percentage of apoptotic cells was quantified by flow cytometry using a FACSCalibur (BD Biosciences) instrument. Samples were analyzed and the percentage of apoptotic cells was evaluated.

Cell invasion assay. A Matrigel invasion assay was performed as previously described (27), in order to assess the invasive ability of PDAC cells. Briefly, the upper chambers of the wells were coated with Matrigel (BD Biosciences). Following treatment with the aforementioned intervention strategies for 48 h, the cancer cells (5x10⁵) were suspended in serum-free medium
and seeded into the upper chamber. Cells were allowed to migrate toward media (DMEM/RPMI-1640) supplemented with 10% FBS in the lower chamber at 37˚C for 24 h. The media were aspirated from the inside of the insert, and the non-invasive cells on the upper side were removed using a cotton swab. The membrane of the chamber was then fixed with 4% paraformaldehyde for 15 min at room temperature and stained with 0.1% crystal violet for 15 min at 37˚C. The number of invading cells was quantified by counting the stained cells under a light microscope (Nikon Corporation, Tokyo, Japan).

Statistical analysis. Each experiment was performed at least three times. Data are presented as the means ± standard deviation. Using GraphPad Prism version 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA), differences among groups were assessed by one-way analysis of variance followed by Dunnett's test for multiple comparisons. All tests were two sided. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression and location of Notch-1 pathway-associated molecules in PDAC cells. The present study detected the expression levels of Notch-1 pathway-associated molecules in the pancreatic cell lines. The results demonstrated that the expression levels of Notch-1 and its ligand Jagged-1 were different in all of the cell lines tested (Fig. 1A). β2-AR is a corresponding receptor of NE, which has been reported to be upregulated in pancreatic cancer tissue (28). The present study detected the protein expression levels of β2-AR in six pancreatic cancer cell lines; the results revealed that β2-AR expression was different in all cell lines analyzed (Fig. 1B).

Figure 2. NE promotes the expression of Notch-1 pathway-associated genes in pancreatic ductal adenocarcinoma cells. (A) Relative mRNA expression levels of Notch-1 pathway-associated molecules (Notch-1 and Jagged-1) were detected in BxPC-3 and Panc-1 cells following exposure to increasing doses of NE (0, 0.1, 1 or 10 µM) by RT-qPCR. β-actin used as an internal control. *P<0.05 vs. the control group. (B) Relative mRNA expression levels of Notch-1 pathway-associated molecules were detected in BxPC-3 and Panc-1 cells following treatment with 10 µM NE for various durations (0, 12, 24 or 48 h) by RT-qPCR. β-actin used as an internal control. *P<0.05 vs. the control group. (C) Protein expression levels of Notch-1 and Jagged-1 were detected in BxPC-3 and Panc-1 cells following treatment with 10 µM NE for various durations (0, 12, 24 or 48 h) by western blotting. NE, norepinephrine; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
through various ligand-receptor interactions, such as the interaction between receptor Notch-1 and its ligand Jagged-1 (10). In conclusion, the Notch-1 pathway may be involved in the development of PDAC in vitro.

**NE promotes the expression of Notch-1 pathway-associated genes in PDAC cells.** To explore the effects of NE on Notch-1 pathway-associated gene expression in PDAC cells, the cells were treated with NE at various concentrations (0, 0.1, 1 or 10 µM). After 24 h, Notch-1 pathway-associated gene expression was detected by RT-qPCR. The results demonstrated that as the concentration of NE increased, the expression levels of Notch-1 pathway-associated genes (Notch-1 and Jagged-1) were elevated in BxPC-3 and Panc-1 cells (Fig. 2A). Furthermore, PDAC cells were treated with 10 µM NE for various durations (0, 12, 24 or 48 h). Subsequently, RT-qPCR and western blotting indicated that the expression levels of Notch-1 and Jagged-1 were increased as treatment duration increased (Fig. 2B and C). These findings indicated that NE may activate the Notch-1 pathway in PDAC cells, which is consistent with the hypothesis that NE may promote the progression of PDAC via activating the Notch-1 pathway.

**NE enhances cell viability and inhibits apoptosis of PDAC cells via activation of the Notch-1 pathway.** To further explore whether the Notch-1 pathway mediated the tumor-promoting effects of NE on PDAC, si-Notch-1 and DAPT were used to suppress the Notch-1 pathway in PDAC cells. As shown in Fig. 3A and B, si-Notch-1 effectively inhibited Notch-1 expression at both the mRNA and protein levels. To explore the role of
Notch-1 in NE-mediated effects on cell viability and apoptosis of PDAC cells, cells were treated with DAPT or si-Notch-1. As shown in Fig. 3C, NE treatment alone significantly enhanced the viability of PDAC cells; however, this effect was abolished by si-Notch-1 and DAPT. Similarly, an apoptosis assay indicated that NE inhibited apoptosis of PDAC cells, whereas this effect was suppressed following inhibition of the Notch-1 pathway (Fig. 3D and E). These findings indicated that NE enhanced cell viability and inhibited apoptosis of PDAC cells via activation of the Notch-1 pathway.

NE enhances the invasive ability of PDAC cells via activation of the Notch-1 pathway. The present study confirmed that NE may promote PDAC cell viability and inhibit apoptosis; therefore, the effects of NE on the invasive ability of PDAC cells were subsequently investigated using a Transwell assay. The results demonstrated that NE promoted the invasive ability of BxPC-3 and Panc-1 cells, whereas these effects could be blocked by si-Notch-1 and DAPT (Fig. 4A and B). Furthermore, it was revealed that NE upregulated the mRNA and protein expression levels of critical Notch-1 pathway-associated and invasion-associated molecules, including Notch-1, Jagged-1, RBP-Jκ (a mammalian CSL protein) and Hes-1, and MMP-2 and MMP-9; however, these effects were reversed following inhibition of the Notch-1 pathway (Fig. 4C and D), which means NE enhanced the invasive ability of PDAC cells via activating the Notch-1 pathway.

Discussion

Complexities in the prevention, diagnosis and treatment of PDAC may be partly due to the fact that little is currently known regarding the factors involved in PDAC progression and its specific mechanisms. However, the cancer-promoting effects of chronic psychological stress, and its major downstream stress hormone NE, are widely known (3,29,30). Chronic psychological stress can influence various physical and pathological biological processes, and it has been reported to potentially initiate the progression of cancer.
NE promotes malignant biological behaviors of pancreatic cancer cells in a Notch-1-dependent manner. NE binds to its receptor β2-AR and activates downstream signaling, which elevates the expression of critical Notch-1 pathway-associated molecules, such as Jagged-1, Notch-1 and CSL (also known as recombination signal binding protein for immunoglobulinκ region in mammals). The Notch-1 pathway is abnormally activated in pancreatic cancer cells and its target genes promote several malignant biological behaviors; however, these effects may be suppressed by a Notch-1 inhibitor (si-Notch-1) and DAPT, an inhibitor of γ-secretase, which is a key molecule that can release NICD from Notch and activate the Notch-1 pathway. Consequently, NE promotes malignant biological behaviors of pancreatic cancer cells in a Notch-1-dependent manner. β2-AR, β2-adrenergic receptor; MAML, mastermind-like; MMP, matrix metalloproteinase; NICD, Notch intracellular cytoplasmic domain; si, small interfering RNA.

The present study initially measured the expression levels of Notch-1 pathway-associated molecules in six pancreatic cancer cell lines; after which one cell line with relatively high Notch-1 expression, BxPC-3, and one with relatively low Notch-1 expression, Panc-1, were selected for further experimentation. Notch-1 and Jagged-1 were revealed to be primarily located on the cytomembrane rather than in the cytoplasm or nucleus of BxPC-3 and Panc-1 cells. Subsequently, PDAC cells underwent a gradient dose/time intervention strategy with NE; the results indicated that NE activated the Notch-1 pathway by increasing the levels of two critical molecules, Notch-1 and Jagged-1. Moreover, in order to explore whether NE mediated the enhancement of the malignant biological behaviors of PDAC in a Notch-1 pathway-dependent manner, cells were treated with si-Notch-1 or DAPT (Notch-1 pathway inhibitor) to block the Notch-1 pathway during NE treatment. RT-qPCR and western blotting demonstrated that NE affected the expression of Notch-1 pathway-associated genes/proteins in BxPC-3 and Panc-1 cells, and MTT, Annexin V-FITC/PI and Transwell assays revealed that NE enhanced cell viability and invasiveness, and inhibited apoptosis of PDAC cells via activating the Notch-1 pathway. Notably, following inhibition of the Notch-1 pathway using DAPT and si-Notch-1, NE-induced upregulation of Notch-1 pathway target genes, such as Hes-1, MMP-2 and MMP-9, was inhibited. Furthermore, the upregulation of Notch-1 pathway-associated molecules, such as Notch-1, Jagged-1 and RBP-Jκ, was also inhibited. These findings suggested that NE may be an inducer and activator of the Notch-1 pathway, however, the specific mechanism requires further study.

Conversely, si-Notch-1 and DAPT could not completely block NE-mediated progression of PDAC, thus suggesting that other pathways or molecules may influence this process. In addition, the Notch pathway participates in various biological processes and has a complex network of interactions with
other pathways (42, 43, 47). Notably, it has been confirmed to serve dual roles in the initiation and progression of several types of cancers or the various stages of a single cancer type (36, 38, 48, 49). Furthermore, NE is just one of the chronic stress hormones, and its use in isolation cannot completely mimic the in vivo chronic psychological stress environment. This is a limitation of the present study, which aimed to explore how chronic stress affects the initiation and progression of PDAC. Therefore, additional studies are required to prove these findings in vivo.

In conclusion, the present study demonstrated that the stress hormone NE may activate the Notch-1 pathway in PDAC and promote its malignant biological behaviors (Fig. 5); this may be an important factor in its development and be associated with a specific underlying mechanism in the progression of PDAC. These findings may provide information regarding a novel approach for targeting NE and/or the Notch-1 pathway in the prevention and treatment of PDAC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

WQ, SL, QM and WD designed the experiments. JL performed the majority of the experiments. KC, ZJ and CZ analyzed the data. LC, BY and JC organized the figures and were involved in the conception of the study. WQ wrote the manuscript. QM, Duan W, Chen K, Jiang Z, Lei X, Xie K, and Xie K: Interaction of the sympathetic nerve with pancreatic cancer cells promotes perineural invasion through the activation of P38/MAPK pathway. Hepatogastroenterology 59: 889-893, 2012.


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