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β2-adrenergic receptor signaling promotes pancreatic ductal adenocarcinoma (PDAC) progression through activating PCBP2-dependent c-myc expression

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ABSTRACT

The β2-adrenergic receptor (β2-AR) plays a crucial role in pancreatic ductal adenocarcinoma (PDAC) progression. In this report, we identified poly(rC)-binding protein 2 (PCBP2) as a novel binding partner for β2-AR using immunoprecipitation-mass spectrometry (IP-MS) approach. The association between β2-AR and PCBP2 was verified using reciprocal immunoprecipitation. Importantly, we found significant interaction and co-localization of the two proteins in the presence of β2-AR agonist in Panc-1 and Bxpc3 PDAC cells. β2-AR-induced recruitment of PCBP2 led to augmented protein level of c-myc in PDAC cells, likely as a result of enhanced internal ribosome entry segment (IRES)-mediated translation of c-myc. The activation of β2-AR accelerated cell proliferation and colony formation, while knockdown of PCBP2 or c-myc restrained the effect. Furthermore, overexpression of PCBP2 was observed in human PDAC cell lines and tissue specimens compared to the normal pancreatic ductal epithelial cells and the non-cancerous tissues respectively. Overexpression of β2-AR and PCBP2 was associated with advanced tumor stage and significantly worsened prognosis in patients with PDAC. Our results elucidate a new molecular mechanism by which β2-AR signaling facilitates PDAC progression through triggering PCBP2-dependent c-myc expression.

Introduction

Pancreatic cancer is one of the five most frequent causes of tumor-associated deaths in the western world with a 5-year survival rate of only 5% [1]. Pancreatic ductal adenocarcinoma (PDAC) accounts for 90% of pancreatic cancer cases. Although our understanding of the pathological and molecular mechanisms of PDAC has advanced significantly over the past decades, we are still far away from a remarkable success in the treatment of this deadly disease. PDAC remains one of the most aggressive tumor entities [2]. Recently, clinical studies indicate that stress, chronic depression and other psychological factors might influence cancer onset and progression [3,4]. Evidence suggests that patients with pancreatic cancer produce the severest symptoms of anxiety and depression among 14 investigated cancer types [5]. In addition, several laboratory models show that chronic stress accelerates PDAC growth and invasion in mice [6]. These findings point to a critical relevance of psychological stress in the regulation of PDAC progression.

β-adrenergic signaling plays a crucial role in psychological stress-induced physiological responses. Numerous studies have proved that chronic psychological stress could significantly up-regulate the level of catecholamines, including norepinephrine (NE) and epinephrine (E), in human bodies [7]. In addition, β-adrenergic receptors (β-ARs), especially β2-AR, have been reported to be widely expressed in various cancer cells, including PDAC cells [8]. β2-AR is a member of the G protein-coupled receptor family, and emerging experimental and clinical data indicate that β2-AR has a vital role in PDAC progression and metastasis [9–11]. Previous researches suggest that β2-AR exerts the effect on carcinogenesis through activating signaling via adenylyl cyclase and its downstream effectors cAMP, PKA, p-CREB and STAT3 as well as transactivation of the EGFR

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pathway [12–14]. However, the molecular mechanism underlying β2-AR-mediated PDAC progression remains to be clarified.

To further elucidate the role of β2-AR in pancreatic tumorigenesis, we determined the protein-protein interactions of β2-AR using immunoprecipitation-mass spectrometry (IP-MS) analysis. In this way, we identified poly(rC)-binding protein 2 (PCBP2) as a novel binding partner for β2-AR. PCBP2 belongs to the family of poly(rC)-binding proteins, which are characterized by the high-affinity and sequence-specific interaction with polycytosine [15]. PCBP2 can directly bind to mRNA, and plays a vital role in mRNA stabilization, translational silencing and enhancement [16–18]. PCBP2 has been documented to be involved in leukemia, glioma, gastric carcinoma and oral cancer, and may contribute to uncontrolled proliferation of cancer cells [19–22]. However, the expression and significance of PCBP2 in PDAC remains obscure. In the present study, we demonstrated that PCBP2 was significantly overexpressed in human PDAC specimens and cell lines. We showed that β2-AR interacted with PCBP2 in the presence of β-AR agonist, and the interaction promoted the expression of c-myc protein in PDAC cells. Furthermore, the impact of β2-AR/PCBP2 interaction on cell proliferation was analyzed using PDAC cell cultures. Importantly, high expression levels of β2-AR and PCBP2 were associated with significantly worsened prognosis in patients with PDAC. On the basis of these data, we speculate that PCBP2 may play a crucial role in pancreatic carcinogenesis by linking β2-AR activation to enhanced c-myc expression and PDAC proliferation.

Materials and methods

Mass spectrometry analysis

The fresh-frozen PDAC tissue was extracted with immunoprecipitation lysis buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 1% NP-40, pH 7.8) and precleared with protein G sepharose (Sigma) for 2 h. Then, 100 mg of protein was immunoprecipitated overnight at 4 °C with 5 μg of anti-β2-AR antibody, or rabbit normal immunoglobulin G (IgG) (Bioworld Technology, MN). Immune complexes were recovered with protein G sepharose for 2 h. After three times of washes, the samples were loaded onto 10% polyacrylamide gel and stained with coomassie brilliant blue. The gel lanes were cut and the proteins were determined using an LTQ mass spectrometer (Thermo, San Jose, CA). The peptide maps belonging to one experiment were clustered and aligned using clustering parameters. The peptide clusters were aligned with Mascot identification files to assign sequence identity. Protein identifications were accepted if they could be established at 95.0% probability and contained at least two unique identified peptides.

Cell culture, expression vectors and transfection

The human PDAC cell lines Panc-1, AsPC-1, BxPC-3, CFPAC-1, Capan-1 and the normal pancreatic ductal epithelial cell line HPDE6-C7 were purchased from the Cell Bank of Type Culture Collection Committee, China Academy of Sciences (Shanghai, China). The cell lines were cultured in DMEM (for Panc-1, CFPAC-1 and HPDE6-C7 cells) or RPMI 1640 (for AsPC-1 and Capan-1 cells) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) at 37 °C in a humidified atmosphere of 5% CO_2.

The cDNA encoding β2-AR was amplified from a human embryonic brain cDNA library using PCR and subcloned into pcDNA3-Flag construct using appropriate restrictive enzymes. The pCMV-HA-PCBP2 plasmid was kindly provided by Prof. Zhengfan Jiang (College of Life Sciences, Peking University, China). The deletion mutants of PCBP2 were constructed by PCR amplification of the indicated regions and cloned into pCMV-HA vector. The short hairpin RNAs (shRNAs) were designed and synthesized by Genechem (Shanghai, China). The target sequences for β2-AR, PCBP2 and c-myc shRNAs were 5′-CATCAACTGCTATGCCAAT-3′, 5′-CATCACTATTGCTGGCATT-3′ and 5′-CAAGGTAGTTATCCTTAAA-3′, respectively. The sequence of control shRNA was 5′- TTCTCCGAACGTGTCACGT′, which was the random sequence that was not related to the three above mRNAs. Transfection was performed using lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol.

Western blot and immunoprecipitation

The tissues and harvested cells were promptly homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 5 mM EDTA, and a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland)), and then centrifuged to collect the supernatant. Equal amount of protein sample was separated on 8–12% SDS-PAGE, and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). After blocking with 5% nonfat milk in phosphate-buffered saline (PBS) for 2 h, the membrane was incubated with the primary antibodies against β2-AR (Santa Cruz Biotechnology, Santa Cruz, CA), PCBP2 (Santa Cruz), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz), Flag (Abcam, Cambridge, MA), HA (Abcam), c-myc (Santa Cruz) or PCNA (Santa Cruz) overnight at 4 °C. After three times of washes, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology) for 2 h at room temperature. Finally, the bands were detected by the ECL (enhanced chemiluminescence) detection systems (Pierce, Rockford, IL). Alternatively, secondary antibody incubation was performed using fluorescent secondary antibodies (LI-COR Biosciences, Lincoln, NE), and the protein bands were visualized using an Odyssey system (LI-COR Biosciences).

For immunoprecipitation, the supernatants of cell lysates or human PDAC tissues were precipitated with the primary antibodies or control IgG of rabbit or mouse in conjunction with Protein G Sepharose. The precipitates were collected for Western blot analysis.

Quantitative real-time PCR (qRT-PCR)

The mRNA levels of PCBP2, FHL3, MAP2K5, MAPK7, CSF1, c-myc, BRCA1 and GAPDH were analyzed by qRT-PCR using the SYBR Green Supermix kit (TIANGEN, China). The mRNA levels of target genes were normalized to GAPDH mRNA level, and the comparative cycle threshold (Ct) method was used to calculate relative amount of target mRNAs. The primer sequences for the genes are shown in Supplementary Table S1.

Immunofluorescence assay

After fixing in 4% paraformaldehyde in PBS for 1 h at room temperature, the cells were incubated with rabbit polyclonal anti-β2-AR and mouse monoclonal anti-PCBP2 antibodies overnight at 4 °C. After washing with PBS for three times, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse or Alexa Fluor 568-conjugated goat anti-rabbit antibodies (Invitrogen) for 1 h at room temperature. 10 mg/ml of DAPI was added to stain the nucleus. The slides were mounted and visualized using a Nikon confocal microscope (Nikon, NY).

Cell proliferation assay and plate colony formation assay

Cell proliferation was measured using Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) following the manufacturer's instructions. Briefly, the cells were seeded into 96-well plate with a density of 3×10^4 cells/well. For the measurement of cell proliferation, 10 μl of CCK-8 reagent was added to each well and incubated for 90 min at 37 °C. The absorbance of the cells was read in a microplate reader (Bio-Rad, CA) at 450 nm with a reference wavelength of 630 nm. The experiments were repeated at least three times.

For plate colony formation assay, the cells (500 cells/well) were plated in 6-well plate. After 14 days, the colonies were washed with PBS, fixed with paraformaldehyde for 30 min, and then stained with 0.5% crystal violet for 30 min. The cell colonies (>0.5 mm in diameter) were counted after staining.

Patients and tissue specimens

The tissue microarray containing 185 matched pairs of primary PDAC samples and adjacent normal tissues was obtained from the pathology department of the Affiliated Hospital of Nantong University. The study population consisted of 124 males and 61 females, and the age ranged from 37 to 78 years. The main clinical and pathologic variables were summarized in Table 1. 8 pairs of PDAC and adjacent normal fresh samples were frozen in the liquid nitrogen immediately after surgical removal and then maintained at –80 °C until using for Western blot analysis. Total cases of PDAC patients who underwent surgery from 2004 to 2013 were collected using protocols that had been approved by the Ethics Committee of Affiliated Hospital of Nantong University.

Immunohistochemical analysis

The tissue microarrays were deparaffinized using a graded ethanol series, processed in 10 mM citrate buffer (pH 6.0) and then heated to 120 °C for 20 min to retrieve the antigen. After rinsing in PBS and blocking the nonspecific reactions with 10% goat serum, the slides were incubated with β2-AR, PCBP2 or c-myc antibodies at 4 °C overnight. The negative control slides using a nonspecific immunoglobulin IgG (Sigma Chemical Co., St. Louis, MO) as primary antibody were included in all assays. All slides were processed using the peroxidase-antiperoxidase method (Dako, Hamburg, Germany). After washing with PBS, the sections were incubated with diaminobenzidine (DAB) solution and hematoxylin. Then, the sections were dehydrated with graded alcohol and cover slipped. All immunostained sections were randomly examined by two independent pathologists using a Leica fluorescence microscope (Germany). At least five high-power fields were randomly chosen and at least 500 cells were counted. The expression score was determined by immunoreactive cell percentage and staining intensity. The extent of staining was recorded: 0, <1%; 1, 1–25%; 2, 26–50%; 3, 51–75% and 4, >75%. For density evaluation, a score of 0 was for no staining, 1 was for weak staining, 2 was for moderate staining, and

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