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CXCL5 knockdown expression inhibits human bladder cancer T24 cells proliferation and migration

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ABSTRACT

CXCL5 (epithelial neutrophil activating peptide-78) which acts as a potent chemoattractant and activator of neutrophil function was reported to play a multifaceted role in tumorigenesis. To investigate the role of CXCL5 in bladder cancer progression, we examined the CXCL5 expression in bladder cancer tissues by real-time PCR and Western blot, additionally, we used shRNA-mediated silencing to generate stable CXCL5 silenced bladder cancer T24 cells and defined its biological functions. Our results demonstrated that mRNA and protein of CXCL5 is increased in human bladder tumor tissues and cell lines, down-regulation of CXCL5 in T24 cells resulted in significantly decreased cell proliferation, migration and increased cell apoptosis in vitro through Snail, PI3K-AKT and ERK1/2 signaling pathways. These data suggest that CXCL5 is critical for bladder tumor growth and progression, it may represent a potential application in cancer diagnosis and therapy.

1. Introduction

Bladder cancer is the fifth most common cause of malignancy and one of the highest costing disease from diagnosis to death in the United States, with an estimated 72,570 new cases and 15,210 deaths in 2013 according to the latest data [1]. More than 70% of bladder tumors are non-muscle invasive papillary tumors (stage Tis, Ta, T1) that rarely become lethal but almost always recur despite conservative measures such as transurethral and intravesical therapy [2–4]. Another 30% muscle-invasive (stage T2, T3, T4) tumors can rapidly progress to become metastatic and lead to death [2,5]. Despite the recent progress of diagnosis and treatment, the molecular mechanisms of bladder carcinogenesis remain poorly understood, and specific tumor biomarkers and therapeutic targets for bladder cancer are still limited.

Chemokines are emerging as critical mediators not only in the homing of cancer cells to metastatic sites but also in the recruitment of a number of different cell types to the tumor microenvironment. Dysregulated expression and activity of certain chemokines have been implicated in the initiation and progression of several cancers. Recently, CXCL5 (epithelial neutrophil activating peptide-78) has been the focus of studies examining the roles of chemokines in carcinogenesis and tumor progression. CXCL5 shares structural homologic qualities with and plays a similar role as IL-8, another member of the CXC chemokine family, in inflammation and angiogenesis [6]. It recognizes and binds the G-protein-coupled receptor CXCR2, acts as a proangiogenic chemokine, an inflammatory mediator and a powerful attractant for neutrophils [6–8]. Evidence is accumulating to suggest that CXCL5 is an important factor in cancer biology [9]. Recent studies demonstrated that CXCL5 directly stimulates cancer cells and endothelial cells proliferation and invasion [10–13] and promotes tumor angiogenesis in nonsmall cell lung carcinoma and pancreatic cancer to modulate tumor growth metastasis [9,14]. However, the expression of CXCL5 and mechanisms of how CXCL5 functions to bladder cancer progress remain elusive.

In our study, we determined the CXCL5 expression in human bladder cancer specimens and cultured human bladder cancer cell lines, we also investigated the effects of CXCL5 knockdown on the proliferation, migration and apoptosis in bladder cancer T24 cells and the underlying signaling pathways involved in. Results showed that CXCL5 mRNA and protein is highly expressed in human bladder tumor tissues and cell lines. Furthermore, CXCL5 knockdown effectively slowed bladder cancer T24 cells growth and migration in vitro by inhibiting Snail, PI3K-AKT and ERK1/2 signaling pathways, meanwhile the CXCL5 knockdown also promoted T24 cells apoptosis through modulating the apoptotic proteins expressions.

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2. Materials and methods

2.1. Patient samples and cell lines

12 pairs of matched primary human bladder carcinoma samples and adjacent tumor-free frozen tissue samples were obtained from patients at Beijing Friendship Hospital who underwent surgical resection between 2012 and 2013. All individuals who were enrolled were Han Chinese. Human bladder cancer cell line T24 cells and normal human bladder epithelial cell line SV-HUC-1cells were cultured in RPMI 1640 medium or F12K medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, Calif). This study was approved by the Ethics Committee of Beijing Friendship Hospital. Informed consent was obtained from each participant.

2.2. Construction and generation of T24 cells that knock down CXCL5

RNAi techniques were used to generate CXCL5 knockdown clones. A 19-nucleotide sequence (CAGTAATCTGCAAGTGTTC), separated by a 9-nucleotide noncomplementary spacer (TTCAAGAGA) from the reverse complement of the 19-nucleotide sequence, was cloned and sequenced after digestion with BamHI and HindIII and inserted into the pRNA-U6.1/Neo-siFluc backbone, using standard procedures. Stable cell lines were generated by transfecting pRNA-U6.1-shRNACXCL5 or empty vectors into T24 bladder cancer cells, and individual clones were selected in the presence of G418 (1000 μg/mL). At least three separate clones were picked up. Quantitative real-time PCR and Western blot were performed to detect whether CXCL5 expression was successfully inhibited.

2.3. Isolation of RNA and quantitative real-time PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, Calif) in accordance with the manufacturer's instructions. And cDNA was reverse transcribed from 3.0 μg of total RNA with random hexamer primers using an Maxima® First Strand cDNA Synthesis Kit (Fermentas, MBI) as recommended by the supplier. Real-time PCR was carried out using SYBR Green on a Lightcycler 480II Real-Time PCR Detection System (Roche, Indianapolis, Ind). The primers used for amplification of CXCL5 and GAPDH were as follows: CXCL5 (Genbank NM_002994) sense 5′-GAGAGCTGCGTTGCGTTTGTTTAC-3′ and antisense 5′-CCGTTCTTCAGGGAGGCTACCACT-3′ and GAPDH (Genbank NM_002046) sense 5′-TGTTCCAATATGATTCCACCC-3′ and antisense 5′-CTTCTCCATGGTGCGTGAAGA-3′. Relative mRNA levels were calculated based on the Ct values and normalized using GAPDH expression, according to the equation: $2^{-\Delta Ct}$ [ΔCt = Ct (CXCL5) -Ct (GAPDH)]. Each sample was analyzed in triplicate.

2.4. Western blot and antibodies

Total cell and tissue lysates were generated and proteins (30 lg each) were loaded onto 10% sodium dodecyl sulfate–polyacrylamide gels, electrophoresed, and transferred onto polyvinylidene difluoride membranes. Briefly, membranes were blocked for 2 h followed by incubation with primary antibody for 4 °C overnight. The primary antibodies and dilutions used were as follows: p-ERK1/2 (Thr202/Tyr204), ERK1/2,p-AKT,AKT,NF-κB1 p105/p50,Bcl2, Bax,Cleaved-caspase 3,E-cadherin, Snail (1:1000, Cell Signaling Technology, Beverly, MA), CXCL5 (1:500; clone 2A9, Abnova, Taipei, Taiwan), and GAPDH (1:2500; CWBIO, Beijing, China). After three washes with PBS containing 0.1% Tween-20, the membranes were incubated with secondary antibodies conjugated to LI-COR IRDye for 1 h at room temperature, and the antibodies were detected using the Odyssey Imager (LI-COR Biosciences, Lincoln, Neb).

2.5. Cell proliferation assay

Cells were seeded in 96-well plates at a density of 1000 cells per well. 3-(4,5-Di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added into the cell culture at a final concentration of 5 μg/mL and was allowed to remain in culture for 4–6 h before measurement. Cell proliferation was monitored every 24 h by measuring the absorbance at 490 nm in a microplate reader (Bio-Rad, Hercules, Calif).

2.6. Cell apoptosis assay

Cell apoptosis assay was detected using Annexin V-FITC kit-Apoptosis Detection Kit (Beckman Coulter, USA). Wash cell samples with ice-cold culture medium or PBS and centrifuge for 5 min at 500 × *g* at 4 °C. Discard supernatant, and resuspend the cell pellets in ice-cold 1× binding buffer to 5×10^5–10^6 cells/mL. Keep tubes on ice, add 1 μL of annexin V-FITC solution and 5 μl of dissolved PI to 100 μL of the cell suspensions. Mix gently, keep tubes on ice and incubate for 15 min in the dark. Add 400 μL of ice-cold 1× binding buffer and mix gently. Analyze cell preparations within 30 min by flow cytometry.

2.7. Crystal violet cell colony staining

Cells were transient transfected with pRNA-U6.1-shRNACXCL5 or empty vectors, then were added with G418 (1000 μg/mL) to select individual clones. After selection for 2 weeks, cells were washed with PBS for three times, remove PBS, fix the cells for 5 min with 4% PFA. Remove the PFA and wash cells, then add crystal violet staining solution to cover dish, stain for 20 min at room temperature. Remove stain solution, wash the dishes two times with tap water. Air dry dishes and then the dishes can be photographed.

2.8. Wound healing and invasion assay

Cells were cultured for 2 days to form a tight cell monolayer, the cell monolayer was wounded with a 10 μL plastic pipette tip. The remaining cells were washed twice with PBS to remove cell debris and incubated at 37 °C with serum free culture medium for serum starvation. At the indicated times, migrating cells at the wound front were photographed using an inverted microscope (Leica). A percentage of the cleared area at each time point compared with time zero was measured using Image-Pro Plus v6.2 software.

2.9. Data analysis

Data were analyzed with GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA), data were expressed as mean ± SD. Comparisons between tumors and peritumors were performed using paired Student's *t*-test and comparisons between groups were performed using Mann–Whitney U-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. CXCL5 expression in bladder cancer tissues and cell lines

CXCL5 expression profiles of 12 pairs of human bladder tumor tissues and corresponding peritumor tissues were examined by quantitative real-time PCR and Western blot. Overall, results showed that CXCL5 mRNA and protein were significant highly expressed in tumors, compared with peritumor tissues ($P < 0.0001$, $P = 0.0479$; Fig. 1A and B). Moreover, 75% (9/12) tumor tissues