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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

showed higher CXCL5 protein expressions than adjacent preitumor tissues (Fig. 1C). We then examined CXCL5 expressions in bladder cancer T24 cells and normal human bladder epithelial SV-HUC-1 cells, CXCL5 showed higher mRNA and protein expressions in T24 cells compared with SV-HUC-1 cells ($P = 0.0361$; Fig. 1D and E). In accordance with results observed in tumor tissues, these data support the view that CXCL5 expression is over-expressed in bladder cancer tissues and cells.

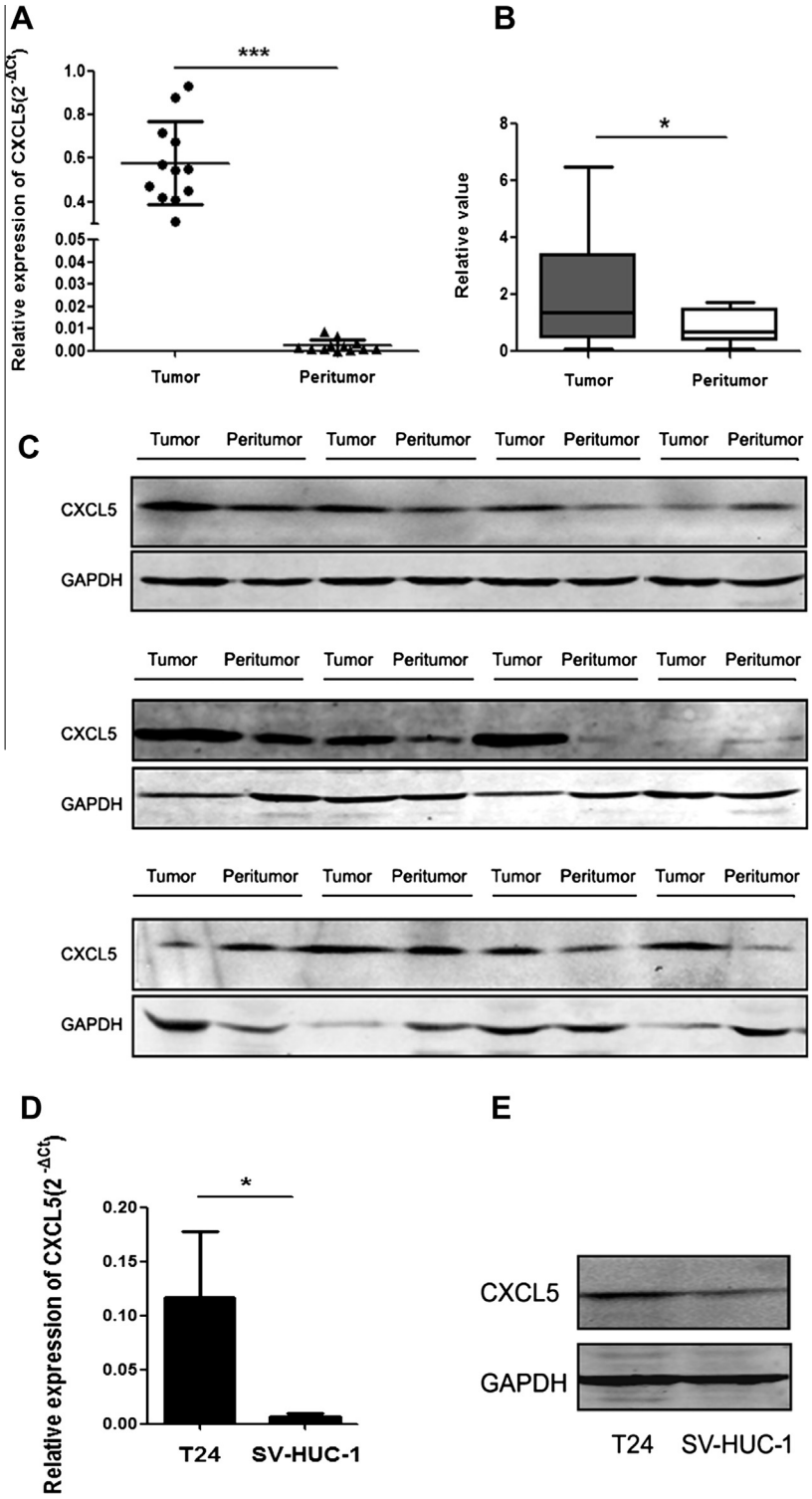

Fig. 1. CXCL5 expression is increased in bladder cancer tissues and cell lines. (A) Quantitative RT-PCR analysis of CXCL5 mRNA expression in 12 bladder cancer tissues and paired peritumor tissues. (B) Analysis of CXCL5 protein expression in total bladder cancer tissues and paired peritumor tissues. (C) Western blot analysis of CXCL5 in 12 bladder cancer tissues and paired peritumor tissues. (D) Quantitative real-time PCR analysis of CXCL5 mRNA expression in human bladder cancer T24 cells and normal human bladder epithelial SV-HUC-1 cells. (E) Western blot analysis of CXCL5 protein expression in T24 cells and SV-HUC-1 cells. Data were presented as mean ± SD (***$P < 0.001$, *$P < 0.05$).

3.2. CXCL5 knockdown inhibits T24 cells proliferation and migration

To determine the roles of CXCL5 in bladder cancer cells, T24 cells with stably down-regulated CXCL5 were generated. The down-regulation of CXCL5 by shRNA in T24 cells was confirmed by quantitative real-time PCR and Western blot ($P = 0.0002$; Fig. 2A and B). Then we examined the effect of silencing CXCL5 on cells proliferation using MTT assay. Significant suppression of cells proliferation was observed in CXCL5 silenced cells compared with control cells at 2–5 days (Day 2: $P < 0.0001$, Day 3: $P = 0.0011$, Day 4: $P < 0.0001$, Day 5: $P < 0.0001$; Fig 2C). The crystal violet cell colony assay also showed that the sizes and numbers of colonies in CXCL5 silenced cells were smaller and fewer than control group (Fig 2D). Next, we examined the cell motility in CXCL5 knockdown cells and control cells, we observed that the CXCL5 knockdown cells displayed significant reduced cell migration ability compared with control cells from 0 h to 24 h after scratch (12 h: $P = 0.0040$, 24 h: $P = 0.0008$; Fig. 2E and F).

3.3. CXCL5 knockdown promotes T24 cells apoptosis

Since the observed inhibition effect of CXCL5 knockdown on T24 cells proliferation and migration, we further detected whether CXCL5 knockdown could affect cells apoptosis. Flow cytometry showed that CXCL5 silenced cells displayed a significant increased apoptosis with the Annexin V+ PI– ratio 41.9% compared with control cells (Annexin V+ PI- ratio 18.5%) after 48 h transient transfection ($P = 0.0062$; Fig. 3A and B). To further confirmed the apoptosis effect, we detected the apoptosis related proteins expressions in CXCL5 silenced cells and control cells. Western blot

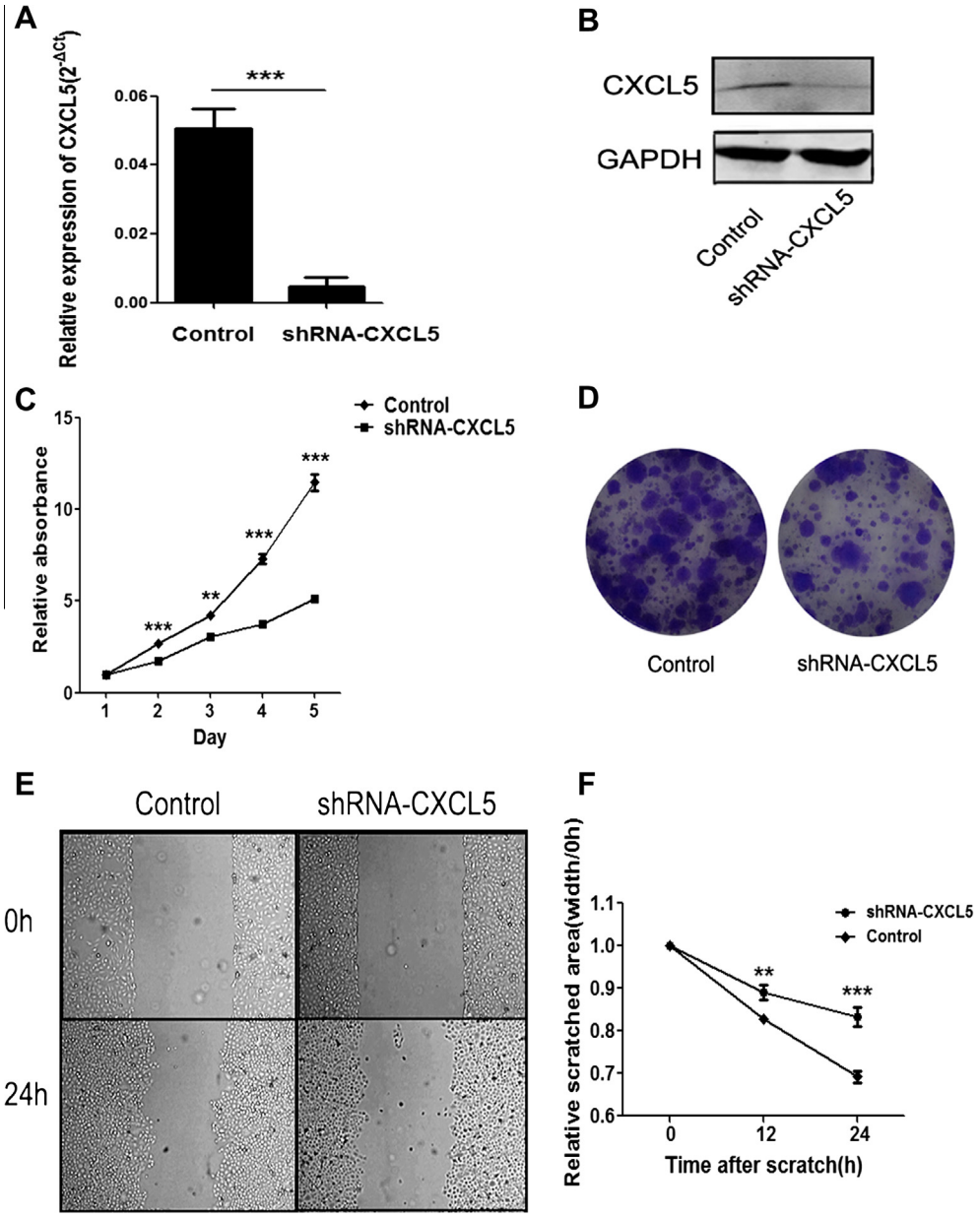

Fig. 2. CXCL5 knockdown decreases bladder cancer T24 cells proliferation and migration. (A) Quantitative RT-PCR confirmed CXCL5 mRNA expression in stably transfected and parent cells. (B) Western blot confirmed CXCL5 protein expression in stably transfected and parent cells. (C) Cell proliferation was detected by MTT assay. (D) Cell colony formation was detected by crystal violet staining. (E) Cell monolayers were wounded and then monitored at 0, 12, 24 h for wound channel. Images at 0, 24 h after wounded were shown. (F) The scratched area for wound healing assay was measured and plotted as the percentage of the original time points (0 h). Data were presented as mean ± SD from three independent experiments (***$P < 0.001$, **$P < 0.01$).

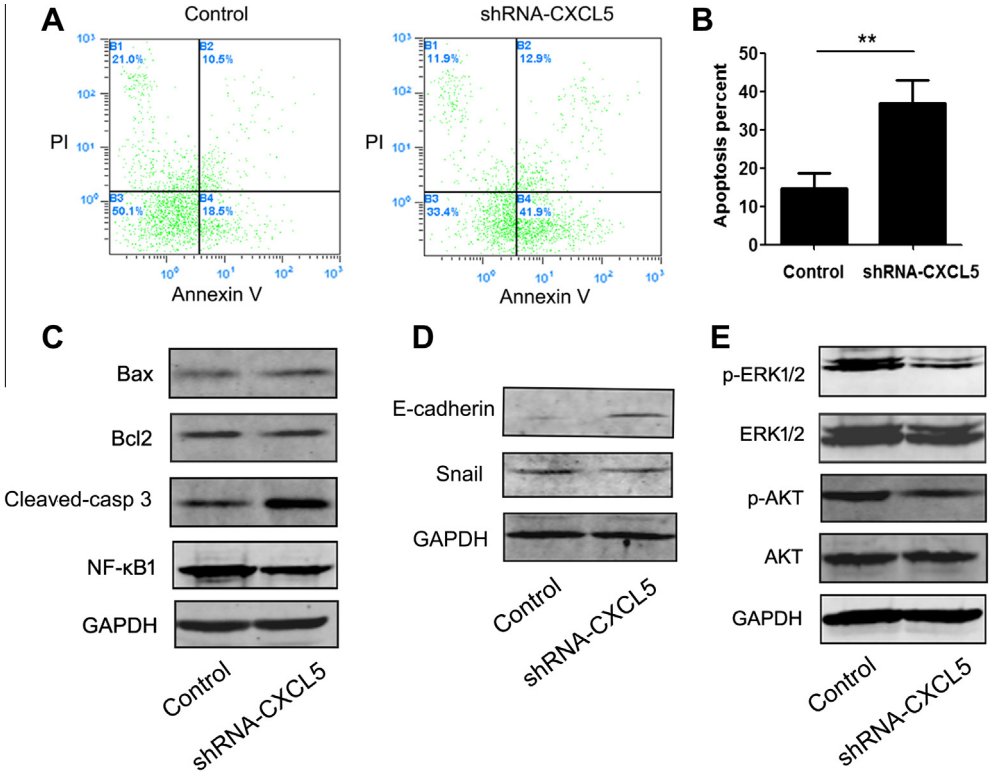

Fig. 3. CXCL5 knockdown enhances bladder cancer T24 cells apoptosis. (A) Apoptotic cells were measured by flow cytometry after 48 h transient transfection. The cell populations of Annexin-V+/PI− were used to assess apoptotic events. (B) Statistic apoptosis rate of different groups were from three independent experiments. (C) Western blot analysis of apoptotic proteins Bax, Bcl2, cleaved caspase-3 and NF-κB1 expressions in CXCL5 knockdown T24 cells and control. (D) Western blot analysis of E-cadherin and Snail in CXCL5 knockdown T24 cells and control. (E) Western blot analysis of phosphorylation of PI3 K-AKT and ERK1/2 in CXCL5 knockdown T24 cells and control. Data were presented as mean ± SD from three independent experiments (**$P < 0.01$).

showed the increased expressions of apoptosis proteins cleaved caspase-3 and Bax, and decreased anti-apoptosis protein Bcl2 in CXCL5 knockdown cells compared with that of in control cells (Fig. 3C). Previous study has reported that cleaved caspase3 is the direct target of NF-κB1 and it accumulates in cells when NF-κB1 levels are reduced [15], Our results also confirmed the decreases of NF-κB1 levels corresponded to the increase in cleaved caspase-3 levels.

3.4. CXCL5 knockdown inhibits Snail, PI3K-AKT and ERK1/2 signaling pathways in T24 cells

Finally, to analyze the signaling pathways involved in CXCL5-mediated bladder cancer cells proliferation and invasion, we performed Western blot analysis to detect E-cadherin and Snail, AKT and ERK phosphorylation in response to CXCL5 knockdown. Down-regulation of CXCL5 by shRNA in T24 cells enhanced E-cadherin and decreased Snail expression suggesting that CXCL5 knockdown could effectively inhibit cell invasion through modulating the E-cadherin and Snail levels (Fig. 3D). Additionally, CXCL5 knockdown also caused a significant decrease in the phosphorylation levels of AKT and ERK1/2 (Fig. 3E). These data suggest that CXCL5 activates Snail, PI3K-AKT and ERK1/2 signaling pathways in T24 bladder cancer cells.

4. Discussions

In this study, we demonstrated that CXCL5 mRNA and protein is remarkably increased in bladder cancer tissues compared with that in adjacent peritumor tissues, similar result was also observed in human bladder cancer T24 cells compared with normal human bladder epithelial SV-HUC-1 cells. Furthermore, knockdown of CXCL5 could inhibit bladder cancer T24 cells growth, migration and progression in vitro by inhibiting PI3K-AKT, ERK1/2 and Snail signaling pathways, meanwhile knockdown of CXCL5 also promoted T24 cells apoptosis through modulating the apoptotic proteins expressions.

Inflammation has emerged as hallmark of cancer, and recent studies have linked bladder cancer initiation to chronic inflammation [16,17]. Chemokines and their receptors have been identified as components of cancer-related inflammatory conditions [18–20], and the dysregulated expression and activity of certain chemokines in bladder cancers have been implicated in the potential progression from an inflammatory environment to cancer initiation [21,22]. Chemokines family members CXCL1 and CXCL8(IL-8) have been reported to highly expressed in human bladder cancer and associated with high tumor stage and poor prognosis [23,24]. As the homologue of CXCL8(IL-8), CXCL5 is another pivotal chemokine, it participates in many diverse biological cancer process including proliferation, metastasis, tumor tissues neutrophil infiltration and tumor angiogenesis. Much evidence has linked CXCL5 to tumorigenesis in various cancer cells. To the best of our knowledge, this is the first study to demonstrate the over-expression of CXCL5 in bladder cancers, which was in line with the studies that CXCL5 is overexpressed in gastric [25], prostate [10,26], endometrial [27], squamous cell [11], pancreatic [14], and colon cancer [28], hepatocellular carcinoma(HCC) [29] and intrahepatic cholangiocarcinoma (ICC) [30]. Additionally, we also tested the serum CXCL5 levels in 44 bladder cancer patients and 31 healthy controls, however, serum CXCL5 of bladder cancer patients did not show significant higher levels than healthy controls (data not shown). This similarity that the circulating and tissue concentrations of CXCL5 in cancer patients were not correlated with one another was also found in colorectal cancer pa-

tients [28]. A possible explanation is that CXCL5 derives from different sources such as leukocytes, epithelial and endothelial cells. The reduced plasma concentration of CXCL5 in bladder cancer patients relative to controls may indicate that cancer patients have an immunologic imbalance resulting in impaired production of CXCL5 from leukocyte and endothelial cells and/or restricts the secretion of CXCL5 from epithelial cells in bladder.

Certain studies on CXCL5 have confirmed its vital roles in cancer progression, CXCL5 could indirectly promotes HCC [29] and ICC [30] growth and metastasis by recruiting intratumoural neutrophils. Furthermore, CXCL5 could stimulate prostate epithelial cells proliferation [10,26] and mediate pancreatic cancer angiogenesis [14]. However, it is unclear as to the molecular mechanism of how CXCL5 influences bladder cancer development. We have demonstrated here, that by using shRNA to silence CXCL5 expression, bladder cancer cells proliferation, colony formation and migration were inhibited. Meanwhile, down-regulation of CXCL5 also promoted cells apoptosis through modulating apoptosis-associated proteins Bcl2, Bax and cleaved-caspase3. Additionally, Western blot confirmed increases of the cleaved caspase-3 levels corresponding to a decrease in NF-κB1 levels, which was in line with recent report that cleaved caspase-3 is a direct target of NF-κB1 and it accumulates in cells when NF-κB1 levels are reduced. NF-κB1 is also a robust marker of highly aggressive tumor cells and has been shown to be involved in cytoskeleton remodeling [15]. Furthermore, we also demonstrated down-regulation of CXCL5 mediated bladder cancer cells proliferation and migration inhibition were through PI3K-AKT and ERK1/2 signaling pathways. The result was coincidence with the previous reports that CXCL5 had a direct chemoattractant effect on neutrophils in vitro through PI3K-AKT and ERK1/2 signaling pathways in various cancers [10,14,29,30]. The activation of AKT and ERK regulates multiple aspects of cellular function [31,32] activation of AKT is crucial for the chemotaxis and migration of neutrophils induced by chemoattractants [33,34]. Nevertheless, further studies also need to clarify the striking correlation between CXCL5 production and neutrophil infiltration in bladder cancer. Generally, our results suggested multiple signaling pathways are involved in CXCL5 mediated bladder cancer progression. In lines with other studies of CXCL5 in ICC, HCC, pancreatic cancer and prostate cancer, our results were the first to confirm CXCL5 plays an important role in promoting and modulating bladder carcinogenesis. However, the precise molecular mechanism of CXCL5 in the tumorigenesis and proliferation of bladder cancer needs to be further explored.

Epithelial-to-mesenchymal transition is a process by which cancer cells change their epithelial phenotype into a mesenchymal phenotype and acquire metastatic ability, which is closely associated with the invasive and metastatic process of cancers [35]. Transcription factor Snail is a key regulatory factor of EMT and cell migration, and its expression is elevated in several cancer types [36,37]. Snail transcriptionally suppresses the adherent junction protein E-cadherin, resulting in epithelial-mesenchymal transition [15,38]. E-cadherin loss and EMT have been implicated in tumor progression, and are closely correlated with poor prognosis [39]. Several reports have addressed the association between CXCL5 and Snail signaling pathways, inducement of cancer progression by CXCL5 is associated with upregulation of Snail by ERK/MSK1/Elk-1/snail signaling pathway in breast cancer [40] and Erg-1/Snail signaling pathway in prostate cancer [10], and Snail over-expression leads to elevated levels of CXCL5 in non-small cell lung cancer [41], and they all suggested that inhibition of CXCL5-mediated ERK/Snail signaling is an attractive therapeutic target for treating metastases in cancers mentioned above. Our results also showed down-regulation of CXCL5 could inhibit bladder cancer cells migration and invasion by decreasing Snail expression and increasing E-cadherin expression to suppress epithelial-mesenchymal transition in T24 cells. Our findings further demonstrated CXCL5 mediated bladder cancer progression was partially contributed by Snail activation, and subsequent acquisition of EMT characteristics. However, the exact mechanism needs further investigation.

In conclusion, our data demonstrated that CXCL5 was over-expressed in human bladder tumor tissues and cell lines, CXCL5 knockdown by shRNA interference effectively inhibited bladder cancer cells proliferation, migration and promoted cell apoptosis in vitro though multiple signaling pathways. Our findings suggest that CXCL5 is critical for bladder tumor growth and progression, whether it could represent a novel diagnostic marker and/or a therapeutic target implicated in bladder cancer still requires further investigation.

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