Serum CXCL5 level is associated with tumor progression in penile cancer

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Running title: Serum CXCL5 is a cancer biomarker for PC
Abstract

Chemokine (C-X-C motif) ligand 5 is an important regulator of tumor progression in many cancers, and could serve as potential serum cancer biomarker. Our initial analysis identified CXCL5 as a cancer-related gene highly expressed in PC. Patients with PC exhibited markedly higher preoperative serum CXCL5 levels compared with that in healthy individuals (P<0.001). The area under the curve (AUC) was 0.880 with the sensitivity of 84.0%, and specificity of 80.4% to distinguish PC. Serum CXCL5 levels were also significantly decreased following tumor resection in patients with PC (P=0.001). Preoperative serum CXCL5 level was significantly associated with clinicopathological characteristics including T stage (P=0.001), nodal status (P<0.001), and pelvic lymph node metastasis (P=0.018). Cox regression analysis showed that serum CXCL5 level could serve as an independent prognostic factor for disease-free survival with a HR of 6.363 (95% CI 2.185-18.531, P=0.001). CXCL5 and its receptor CXCR2 exhibited correlated expression pattern in PC tissues. Differential CXCL5 expression was observed in normal penile tissues, PC cell lines and their culture supernatants. Furthermore, knockdown of CXCL5 or CXCR2 expression markedly suppressed malignant phenotypes (cell proliferation, clonogenesis, apoptosis escape, migration and invasion), attenuated STAT3 and AKT signaling and reduced MMP2/9 secretion in PC cell lines. In conclusion, our findings revealed that serum CXCL5 level might serve as a potential diagnostic and prognostic cancer biomarker for penile cancer. Autocrine CXCL5/CXCR2 signaling might activate multiple downstream oncogenic signaling pathways (STAT3, AKT, MMP2/9).
to promote malignant progression of PC, which may warrant further investigation in the future.

**Key words:** penile cancer; CXCL5; CXCR2; tumor progression, prognosis

**Introduction**

Penile cancer (PC) is a rare cancer in developed countries; however, its incidence rate is much higher in some regions of South America, Asia and Africa (1). Despite recent progress in multimodal therapies, the clinical outcome of PC remains unsatisfactory, as the survival of patients with PC has not improved during the last two decades (2). Serum cancer biomarkers, such as carcinoembryonic antigen (CEA), cancer antigen (CA)-125 and CA-15-3, have been beneficial in the diagnosis of cancer and disease monitoring. However, these serum cancer biomarkers are not beneficial in PC (3-5). Squamous cell carcinoma antigen (SCC) levels have been associated with PC tumor burden; although, it could not predict the clinical outcome (6). On the other hand, overexpression of p53 or Ki-67 has been associated with tumor progression of PC, yet these biomarkers are not used in a clinical setting (7-9).

Chemokines may play an important role in the development of tumorigenesis in numerous types of cancer (10, 11). Aberrant expression of some chemokines, such as CCL5, CXCL1, CXCL8 and CXCL13, has been detected in several types of cancer (12-15). C-X-C motif chemokine ligand 5 (CXCL5) is an important chemokine secreted by immune cells, such as monocytes and T lymphocytes (16). Recent studies
indicated that CXCL5 is aberrantly expressed in >14 different types of cancer, including hepatocellular carcinoma, prostate cancer, pancreatic cancer and gastric cancer (17). Moreover, CXCL5 expression has been found to be associated with the degree of malignancy, metastatic potential and degree of inflammatory infiltration in numerous types of cancer. In gastric cancer, CXCL5 is associated with late stages of the disease (18). The expression levels of CXCL5 in colorectal cancer tissues are also found to be associated with malignant phenotypes of prostate cancer (19). In hepatocellular carcinoma, CXCL5 was found to promote neutrophil infiltration and indicates poor prognosis (20). In pancreatic cancer, CXCL5 is overexpressed in cancer tissues and is significantly associated with poorer tumor differentiation, advanced clinical stage and shorter patient survival (21). Thus, the aim of the present study was to examine the expression of CXCL5 in PC and to evaluate the usefulness of serum CXCL5 levels as a potential cancer biomarker for PC.

**Materials and methods**

**Patient characteristics**

A total of 81 patients were included in the present retrospective study, and underwent surgery and were diagnosed with PC between 2016 and 2018 at Xiangya Hospital, Central South University (Hunan, China). Patients who received chemotherapy or brachytherapy previously were excluded from the study. The serum samples of 46 healthy male control were obtained from the Health Examination Center (Xiangya Hospital, Central South University, Hunan, China) and provided
informed consent. TNM staging was performed according to the American Joint Committee on Cancer guidelines, 8th edition (22). The clinical parameters of the patients with PC included age, T stage, nodal status, histological subtype, pathological grade and body mass index (BMI), as well as phimosis.

**Reagents and cell lines**

The primary antibodies against CXCL5, CXCR2, phosphorylated (p)-STAT3 (Tyr705), STAT3, p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-AKT (Ser473), AKT, and β-actin were purchased from Abcam. The human Penl1, Penl2, 149RCa and LM156 PC cell lines were kindly provided by Prof Hui Han (Department of Urology, Cancer Hospital, Sun Yat-Sen University) (23). The cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum as previously described (23). Lentiviral short hairpin (sh)RNAs targeting shCXCL5 or shCXCR2 were purchased from GeneCopoeia Inc, and were used as previously described (24, 25).

**ELISA assay**

All the blood samples were collected 1 day prior to (preoperative) or on day 28 following surgery (postoperative). Serum samples were separated and stored at -80°C for further analysis. Serum CXCL5 levels were measured using CXCL5 ELISA kit (RayBiotech, Inc.) according to the manufacturer’s protocol.

**Cell growth analysis**

Cell growth was measured using the Cell Counting Kit-8 (CCK-8) assay as previously described (24). The CCK-8 absorbance (optical density OD$_{450}$) was
measured using a MK3 microplate reader (Thermo Fisher Scientific, Inc.).

**Clonogenic assay**

The clonogenic assay was conducted to measure the clonogenic potential of PC cells as previously described (25). Briefly, PC cells were seeded in 6-cm culture dishes, and cultured for 12 days. The number of colonies (contains >50 cells) was counted.

**Wound healing assay.**

Cell migration ability was measured using a wound healing assay as previously described (26). Briefly, PC cells were cultured to confluency, and subsequently a uniform wound was created for each experiment group. The distance between the wound sides was measured immediately following the creation of the wound and after 24 h.

**Transwell invasion assay**

The cell invasion ability was measured using the transwell chamber as previously described (26). The invaded cells, on the bottom surface of the 8-µm pore membrane were stained using 0.2% crystal violet, eluted by acetic acid and measured with a MK3 microplate reader (Thermo Fisher Scientific, Inc.) at 570 nm.

**Western blot analysis**

The cell lysates were prepared using RIPA lysis buffer and the remaining western blot analysis used performed as previously described (26). The protein blots were visualized using an ECL kit (Abcam).

**Bromodeoxyuridine (BrdU) incorporation assay**
The BrdU incorporation assay was used to assess the proliferative potential of PC cells as previously described (27). Briefly, PC cells were plated (4×10^3 cells/well) into 96-well plate and incubated for 72 h. Following incubation, PC cells were labeled with 10 μM BrdU for 2 h and the incorporated BrdU was detected using a BrdU Assay kit (Abcam).

**Caspase-3 activity assay**

Cellular apoptosis was detected using caspase-3 colorimetric assay kit (Abcam) as previously described (27). Briefly, cells (5×10^5) were lysed on ice for 10 min and centrifuged at 10,000 × g for 1 min. Enzyme reactions were performed on the resulting supernatants in 96-well flat-bottom microplates, using 50 μl cell lysate (100 μg of total protein) for each reaction mixture. The OD was measured at 405 nm using a Multiskan MK3 microplate reader (Thermo Fisher Scientific, Inc.).

**Immunohistochemistry (IHC)**

Archived paraffin-embedded normal penile tissues (n=30) and PC tissues (n=40) were collected for IHC staining. These patients had undergone surgery and were diagnosed with PC between 2017 and 2018 at Xiangya Hospital, Central South University (Hunan, China). IHC was performed as previously described (28). Antigen-antibody reactions (dilution for CXCL5, 1:50; dilution for CXCR2, 1:100) were visualized by exposure to 3,3-diaminobenzidine and hydrogen peroxide chromogen substrate (DAKO; Agilent Technologies, Inc.). Positive CXCL5 staining (≥30%) was regarded as high expression (24).

**GEO dataset**
GEO dataset GSE57955 could be downloaded from NCBI GEO website (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57955). Gene expression data were analyzed as described previously (24). Genes with a mean log₂ signal ratio (penile cancer/normal tissue pool) of ≥ 1.0 and ≤ −1.0 within a 99% confidence interval were considered differentially expressed.

Statistical analysis

Statistical analyses were performed using SPSS v16.0 software. The serum CXCL5 levels between two groups were compared using Mann-Whitney tests. The pre- and post-operative serum CXCL5 levels were compared using a Wilcoxon rank sum test. The optimal cut-off value of preoperative serum CXCL5 was determined based on receiver-operating characteristic (ROC) analysis with reference to cancer recurrence. Kaplan-Meier curves of disease free survival (DFS) were plotted and survival in the groups was compared using a log-rank test. The prognostic factors that influence DFS were identified using univariable and multivariable Cox regression analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

CXCL5 is highly expressed in PC tissues

The mRNA expression of CXCL5 in PC was analyzed in public GEO dataset GSE57955 (n=39). CXCL5 was highly expressed in PC with reference to normal tissue pool (NT) (Mean Log₂(PC/NT) = 1.9). More than 50% of PC cases (20/39) exhibited high level of CXCL5 expression (Log₂ (PC/NT) ≥ 1), Fig. 1A). We also
examined the expression of CXCL5 in PC tissues (n=40) and normal penile tissues (n=30) using immunohistochemistry. The results showed that the CXCL5 expression was considerably higher in PC tissues than in normal penile tissues (P=0.005, Fig. 1B).

Preoperative serum CXCL5 levels are significantly elevated in patients with PC.

The finding that CXCL5 was highly expressed in PC tissues prompted us to further investigate the clinical significance of serum CXCL5 in PC. A total of 81 men diagnosed with PC were enrolled in the present study. The detailed summary of the patient and tumor characteristics, including treatment plan, TNM stage, histological subtype and pathological grade are shown in Table 1. Serum CXCL5 levels were measured in healthy male subjects and patients with PC. Preoperative serum CXCL5 levels were significantly higher in the PC cohort (357.9 ± 285.7 pg/ml) compared with that in healthy male control (98.7 ± 66.9 pg/ml; P<0.001; Fig. 2A). The area under the curve (AUC) was 0.880 with the sensitivity of 84.0%, and specificity of 80.4% to distinguish penile cancer (Cutoff =148.8 pg/ml, Fig. 2B). Moreover, serum CXCL5 levels were significantly decreased following PC surgery (P=0.001; Fig. 2C).

Preoperative serum CXCL5 levels are associated with tumor progression and unfavorable clinical outcome.

The association between preoperative serum CXCL5 levels and clinicopathological parameters (age, BMI, pathological grade, phimosis, histological subtype, tumor stage, and nodal status) was analyzed. As shown in Fig. 3, preoperative serum CXCL5 levels were significantly associated with oncological
parameters including T stage (P=0.001), nodal status (P<0.001) and pelvic lymph node metastasis (LNM) (P=0.018); however, it was not significantly associated with BMI (P=0.149), phimosis (P=0.935), age (P=0.182), histological subtype (P=0.909), and pathological grade (P=0.133). In addition, ROC analysis showed that CXCL5 had a sensitivity of 68.4% and a specificity of 90.3% to discriminate cancer recurrence (cutoff value: 442.75 pg/ml; AUC, 0.832; Fig. 4A). Survival analysis showed that patients with high serum CXCL5 levels exhibited shorter DFS (P<0.001) (Fig. 4B). Univariable Cox regression analysis revealed that nodal status (P<0.001), T stage (P=0.015), pelvic LNM (P<0.001), and higher preoperative serum CXCL5 levels (P<0.001) were associated with shorter disease free survival (DFS) in the PC cohort (Table 2). Meanwhile, multivariable Cox regression analysis indicated that nodal status (P=0.001; HR: 12.657), pelvic LNM (P=0.002; HR: 15.295), and higher preoperative serum CXCL5 levels (P=0.001; HR: 6.363) could serve as independent prognostic factors for DFS (Table 2).

**CXCL5 is differentially expressed in PC cell lines and culture supernatants.**

The clinical relevance of CXCL5 expression and its receptor CXCR2 in PC tissues was analyzed in GSE57955 dataset. As shown in Fig. 5A, PC cases with high CXCL5 expression also tended to exhibit high CXCR2 expression (spearman correlation r=0.341, P=0.033). We also observed differential expression of CXCL5 and its receptor CXCR2 in normal penile tissues (NPT1, NPT2) and a panel of PC cell lines (Penl1, Penl2, 149RCa, LM156) (Fig. 5B, Supplementary data Fig. S1A). Consistently, ELISA analysis showed that high CXCL5 level was detected in culture
supernatant from PC cell lines exhibiting high endogenous CXCL5 expression (Penl1, Penl2; Fig. 5C).

**Knockdown of CXCL5 attenuates malignant phenotype in PC cell lines.**

The oncogenic function of CXCL5/CXCR2 signaling in the PC Penl1 and Penl2 cell lines was further investigated. Endogenous CXCL5 or CXCR2 expression in Penl1 and Penl2 cells was considerably reduced by shRNAs compared to scramble (Scr) control (Fig. 6A, Supplementary data Fig. S1B). CCK-8 assay revealed that shCXCL5 (cell doubling time, 41.6 ± 1.8 and 48.0 ± 2.1 h for Penl1 and Penl2, respectively) or shCXCR2 (cell doubling time, 42.7 ± 1.2 and 52.2 ± 2.3 h for Penl1 and Penl2, respectively) transfected-PC cells grew slower compared with that in cells transfected with the Scr control (cell doubling time, 34.5 ± 2.2 and 36.0 ± 1.8 h for Penl1 and Penl2, respectively) (P<0.05; Fig. 6B). In addition, reduced BrdU incorporation was observed in CXCL5 or CXCR2 knockdown Penl1 and Penl2 cell lines, while caspase-3 activity was increased following CXCL5 or CXCR2 knockdown in Penl1 and Penl2 cell lines (P<0.05; Fig. 6C, 6D). Colony formation was decreased in the shCXCL5 or shCXCR2 groups compared with that in Scr control group in the Penl1 and Penl2 cell lines (P<0.05, Fig. 6E). We also over-expressed CXCL5 in CXCL5-low PC cell line LM156 and 149RCa. However, the expression of CXCR2 still remained low following CXCL5 over-expression (Supplementary data Fig. S2A). Consequently, we detected mild change of cell proliferation and clonogenesis in these two PC cell lines (Supplementary data Fig. S2B, S2C, S2D). Wound healing assay revealed that cell migration was reduced in
shCXCL5 or shCXCR2 groups compared with that in the Scr control group (P<0.05; Fig. 7A, Supplementary data Fig. S3A). Furthermore, transwell invasion assay revealed that knockdown of CXCL5 or CXCR2 expression inhibited the invasiveness of PC cells compared with that in the Scr control group (P<0.05; Fig. 7B, Supplementary data Fig. S3B).

CXCL5/CXCR2 regulates downstream STAT3 and AKT signaling and MMP2/9 secretion in PC cell lines.

The cancer-related signaling pathways such as PI3K/AKT, ERK1/2 and STAT3 were analyzed using Western blotting. Knockdown of CXCL5 or CXCR2 expression reduced p-STAT3 and p-AKT levels in Penl1 and Penl2 compared with that in the scramble (Scr) control (Fig. 8A, Supplementary data Fig. S4). The levels of p-ERK1/2 remained unchanged following knockdown of CXCL5 or CXCR2 in the Penl1 and Penl2 cell lines (Fig. 8A). Meanwhile, ELISA assay revealed that depletion of CXCL5 or CXCR2 reduced secretion of two invasion/metastasis-related molecules MMP2 and MMP9, as compared with Scr control (P<0.05) (Fig. 8B).

Discussion

CXCL5 has been found to be involved in tumor progression in numerous types of cancers, and could serve as a potential serum biomarker in various malignancies, including breast, nasopharyngeal, gastric, colorectal, and biliary tract cancer (29). Zhang et al (30) showed that serum CXCL5 levels could serve as a prognostic biomarker in nasopharyngeal carcinoma, while Lim et al (31) indicated that serum...
CXCL5 levels could serve as potential biomarkers to predict the distant metastasis of primary gastric cancer and Kawamura et al (32) identified serum CXCL5 levels as a potential prognostic biomarker for colorectal cancer. Recently, Lee et al (33) observed that serum CXCL5 levels could predict the unfavorable prognosis in advanced biliary tract cancer. The results of the present study found that the expression of tissue and serum CXCL5 levels were increased in PC compared with that in healthy control samples. Moreover, preoperative serum CXCL5 levels were also significantly associated with clinical parameters, including T stage, nodal status and pelvic LNM in PC; high preoperative serum CXCL5 levels were an independent prognostic factor for DFS in PC. These findings found that preoperative serum CXCL5 levels were associated with tumor progression and could serve as potential diagnostic and prognostic cancer biomarker for PC. However, the possible mechanisms leading to the upregulation of CXCL5 in PC still remains unknown, despite previous studies on other cancers revealing that CXCL5 expression might be driven by multiple cancer-associated pathways, including nuclear factor-κB and cyclooxygenase-2/prostaglandin E2 (34, 35). Due to the limitations of the present retrospective study (including from a single center, a small cohort, relatively short follow-up period and diversity of treatment), a multi-center prospective study would be required to further validate the potential value of serum CXCL5 as a biomarker for PC.

CXCL5 could regulate tumor progression in an autocrine or paracrine manner in a vast number of cancers. Gao et al (36) found that autocrine CXCL5/CXCR2 signaling
could promote the migration and invasion of bladder cancer cells, while Zhou et al (20) revealed that autocrine CXCL5/CXCR2 signaling could enhance epithelial-mesenchymal transition in hepatocellular carcinoma cells. Moreover, paracrine CXCL5 secreted by cancer-associated stromal cells (mesenchymal stem cells and macrophages) could also promote cancer cell invasion and dissemination (37, 38). The results from the present study revealed aberrant expression of CXCL5 in PC tissues, cell lines and their culture supernatants. Moreover, consistent expression of CXCL5 and CXCR2 was also observed in PC tissues and cell lines, suggesting CXCL5 could act in an autocrine manner in PC. Furthermore, knockdown of CXCL5 or CXCR2 attenuated cell proliferation, clonogenesis, migration/invasion and induced apoptosis in PC cell lines, suggesting autocrine CXCL5/CXCR2 signaling axis might be crucial to promote cell proliferation, tumorigenicity, migration/invasion and apoptosis escape in PC. Several CXCR2 inhibitors (AZD5069, SB225002, SCH-527123, and danirixin) are currently under development for cancer treatment (39). Experimental therapeutics on CXCL5/CXCR2 inhibitors would be the priority of future studies in penile cancer.

The CXCL5/CXCR2 axis could activate multiple downstream signaling pathways, including PI3K/AKT, ERK1/2 and STAT3 to promote tumor progression in cancers. CXCR2/CXCL5 axis could activate PI3K/AKT signaling in hepatocellular carcinoma cells (40) and also activate the STAT3 signaling pathway to promote the migration and invasion in gastric cancer (37). Hsu et al (41) demonstrated that CXCL5 could increase ERK1/2 activation during the tumor progression of breast cancer. In the
present study, knockdown of CXCL5 or CXCR2 attenuated downstream AKT and
STAT3 signaling pathways and reduced MMP2/9 secretion in PC cell lines. As AKT
and STAT3 pathways were proven to be important for PC tumorigenesis (42, 43), it
would be reasonable to propose that CXCL5/CXCR2 signaling might activate AKT
and STAT3 signaling pathways to promote tumor progression in PC. However, the
attenuation of ERK1/2 signaling following knockdown of CXCL5 or CXCR2 in the
PC cell lines was not observed, suggesting that CXCL5/CXCR2 signaling might
activate differential downstream signaling dependent in specific cancer types.

In conclusion, high preoperative serum CXCL5 levels were associated with PC
progression and could serve as a potential prognostic biomarker for PC. Furthermore,
the CXCL5/CXCR2 axis might be required for PC progression through activating
AKT and STAT3 signaling pathway and inducing MMP2/9 secretion. New
discoveries in the CXCL5/CXCR2 signaling would also aid clinical decision-making
for PC patients, bringing us closer to the promise of translational precision medicine.

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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

HXH designed the study; MM performed the experiments; YLL examined and scored the IHC staining. MM and HXH analyzed the data; HXH wrote the manuscript. All authors have read and approved the final version of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of Xiangya Hospital Central South University. Written informed consent was obtained from the patients.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1. CXCL5 is highly expressed in PC tissues. A. Waterfall plot of CXCL5 expression in GSE57955 dataset (n=39). B. Immunohistochemistry on CXCL5 expression in normal penile tissues (n=30) and PC (n=40) tissues. Representing micrographs showed high or low CXCL5 expression in two PC cases, respectively. Bars: 100 μm.

Figure 2. Serum CXCL5 level is highly elevated in PC cohort compared to that in normal male control. A. Serum CXCL5 level in preoperative PC cohort (n=81) and healthy male control (n=46). B. ROC curve analysis of the diagnostic value of serum CXCL5 level in PC patients. C. Serum CXCL5 level in the matched preoperative/postoperative PC cohort (n=18).

Figure 3. Association of preoperative serum CXCL5 levels with clinical parameters. A. Age; B. Phimosis; C. BMI index; D. Pathological grade; E Histological subtype; F. T stage. G. Nodal status. H. Pelvic LNM.

Figure 4. Serum CXCL5 level is associated with unfavorable prognosis in PC. A. ROC curves for preoperative serum CXCL5 levels with reference to cancer
recurrence. **B.** PC patients with high preoperative serum CXCL5 levels exhibited shorter DFS.

**Figure 5.** Correlation of gene expression between CXCL5 and CXCR2 in PC tissues. **A.** Correlated expression between CXCL5 and CXCR2 in GSE57955 dataset. **B.** Expression of CXCL5 and CXCR2 in normal penile tissues (NPT1, NPT2) and PC cell lines. **C.** CXCL5 secretion in culture supernatant of PC cell lines.

**Figure 6.** Knockdown of CXCL5 or CXCR2 suppresses cell proliferation, impairs clonogenesis and induces caspase-3 activity in PC cells. **A.** Western blotting analysis on CXCL5 or CXCR2 expression following shRNA-mediated knockdown in Pen1 and Pen2 cells. **B.** Depletion of CXCL5 or CXCR2 expression suppressed cell growth of PC cells. **C.** Knockdown of CXCL5 or CXCR2 reduced BrdU incorporation in PC cell lines. The BrdU incorporation in Scr control was regards as 100%. n=3, *P<0.05. **D.** Knockdown of CXCL5 induced caspase-3 activity in Pen1 and Pen2 cells. The caspase-3 activity in Scr control was regards as 100%. n=3, *P<0.05. **E.** Depletion of CXCL5 expression reduced clonogenesis of PC cells. The colony formed in Scr control was regards as 100%. n=3, *P<0.05.

**Figure 7.** Knockdown of CXCL5 or CXCR2 inhibits cell migration and invasion in PC cells. **A.** Knockdown of CXCL5 or CXCR2 expression inhibited cell migration of PC cells. Bars: 100 μm. The cell migration in Scr control was regards as 100%. n=3,
*P<0.05. **B.** Knockdown of CXCL5 or CXCR2 expression inhibited transwell invasion of PC cells. Bars: 50 μm. The cell invasion in Scr control was regards as 100%. n=3, *P<0.05.

Figure 8. Knockdown of CXCL5 or CXCR2 attenuates STAT3 and AKT signaling and reduces MMP2/9 secretion in PC cells. **A.** Effect of CXCL5 or CXCR2 depletion on cancer-related signaling pathways in PC cell lines. β-actin was used as a loading control. **B.** Knockdown of CXCL5 or CXCR2 reduced MMP2/9 secretion in PC cell lines. n=3, *P<0.05.
Figure 1

Relative CXCL5 expression
Log₂ (PC/NT)

GSE57955 (n=39)

Penile Cancer (n=40)
CXCL5 High (14/40)
CXCL5 Low (26/40)

Normal penile tissue (n=30)

Number of Cases

Normal

PC

CXCL5 High

CXCL5 Low

P=0.005
Figure 3

A. Age
   - P = 0.182

B. Phimosis
   - P = 0.935

C. Body mass index
   - P = 0.149

D. Pathological grade
   - P = 0.133

E. Histological subtype
   - P = 0.909

F. T stage
   - P = 0.001

G. Nodal status
   - P < 0.001

H. Pelvic LNM
   - P = 0.018
Figure 4

AUC=0.832
Cutoff=42.75 pg/ml
Sensitivity=48.4%
Specificity=80.3%

Cum Survival

Disease free survival (months)

High serum CXCL5 (n=19)
Low serum CXCL5 (n=62)

P<0.001
Figure 8

A

|       | Penl1 | Penl2 |
|-------|-------|-------|
| shCXCL5 | –     | +     |
| shCXCR2 | –     | –     |

B

Supernatant MMP2

- Scr
- shCXCL5
- shCXCR2

p-STAT3
STAT3
p-AKT
AKT
p-ERK1/2
ERK1/2
β-Actin

Supernatant MMP9
Table 1: Clinicopathologic characteristics of PC patient cohort

| Parameters                                | Cases (%)       |
|-------------------------------------------|-----------------|
| Age (year)                                |                 |
| >52                                       | 40 (49.4%)      |
| ≤52                                       | 41 (50.6%)      |
| Body mass index (kg/m²)                   |                 |
| <24                                       | 55 (67.9%)      |
| ≥24                                       | 26 (32.1%)      |
| Phimosis                                  |                 |
| Yes                                       | 64 (79.0%)      |
| No                                        | 17 (21.0%)      |
| Penile Surgery                            |                 |
| Penile preservation                       | 20 (24.7%)      |
| Partial penectomy                         | 56 (69.1%)      |
| Radical penectomy                         | 5 (6.2%)        |
| Pathological Grade                        |                 |
| G1                                        | 52 (64.2%)      |
| G2                                        | 24 (29.6%)      |
| G3                                        | 5 (6.2%)        |
| Histological subtype                      |                 |
| Usual                                     | 52 (64.2%)      |
| Papillary                                 | 6 (7.4%)        |
| Warty                                     | 13 (16.0%)      |
| Verrucous                                 | 10 (12.3%)      |
| T stage                                   |                 |
| T1                                        | 45 (55.6%)      |
| T2                                        | 31 (38.3%)      |
| T3                                        | 5 (6.2%)        |
| Nodal status                              |                 |
| Negative                                  | 48 (59.3%)      |
| Positive                                  | 33 (40.7%)      |
| Pelvic LNM                                |                 |
| M0                                        | 76 (93.8%)      |
| M1                                        | 5 (6.2%)        |
| Inguinal lymphadenectomy                   |                 |
| No                                        | 46 (56.8%)      |
| Yes                                       | 35 (43.2%)      |
Table 2: Cox univariate and multivariate proportional hazard model for factors affecting disease-free survival in PC cases

| Clinical parameters                          | Univariate analysis | Multivariate analysis |
|----------------------------------------------|---------------------|-----------------------|
|                                              | P value             | HR(95%CI)             | P value |
| **T stage**                                  | 0.015               | 0.320                 |
| (T1 vs. T2+T3)                               |                     |                       |
| **Pathological grade**                       | 0.268               |                       |
| (G1 vs. G2+G3)                               |                     |                       |
| **Histological subtype**                     | 0.318               |                       |
| (Usual vs. Others)                           |                     |                       |
| **Nodal status**                             | <0.001              | 12.657 (2.782-57.587) | 0.001   |
| (Negative vs. Positive)                      |                     |                       |
| **Pelvic LNM**                               | <0.001              | 15.295 (2.655-88.113) | 0.002   |
| (No vs. Yes)                                 |                     |                       |
| **High serum CXCL5**                         | <0.001              | 6.363 (2.185-18.531)  | 0.001   |
| (≥442.75 pg/ml vs. <442.75 pg/ml)            |                     |                       |
**Supplementary Figures**

**Figure S1.** Densitometric analysis and subsequent statistical analysis for **Fig 5B and Fig 6A.**  
**A.** Densitometric analysis and subsequent statistical analysis for Fig. 5B. \( *P<0.05, \) as compared with NPT1 and NPT2.  
**B.** Densitometric analysis and subsequent statistical analysis for Fig. 6A. \( *P<0.05, \) as compared with Scr control in Penl1 or Penl2, respectively.
Figure S2. Over-expression of CXCL5 mildly affected cell proliferation and clonogenesis in LM156 and 149RCa cells. **A.** Over-expression of CXCL5 in LM156 and 149RCa cells. **B.** Over-expression of CXCL5 mildly affected cell growth of PC cell lines. The cell viability in empty vector (EV) control was regards as 100%. **C.** Over-expression of CXCL5 mildly affected BrdU incorporation in PC cell lines. The BrdU incorporation in empty vector (EV) control was regards as 100%. **D.** Over-expression of CXCL5 mildly affected colony formation in LM156 and 149RCa cells. The colony formation in empty vector (EV) control was regards as 100%.
**Figure S3.** Knockdown of CXCL5 or CXCR2 expression inhibited cell migration and transwell invasion of Penl1 cells. **A.** Knockdown of CXCL5 or CXCR2 expression inhibited cell migration of Penl1 cells. Bars: 100 μm. **B.** Knockdown of CXCL5 or CXCR2 expression inhibited transwell invasion of Penl1 cells. Bars: 50 μm.

**Figure S4.** Densitometric analysis and subsequent statistical analysis for Fig. 8A.

*P<0.05, as compared with Scr control in Penl1 or Penl2, respectively.