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CXCL5 promotes mitomycin C resistance in non-muscle invasive bladder cancer by activating EMT and NF-κB pathway

Chanjuan Wang [a], Aiwei Li [a], Shuo Yang [a], Rui Qiao [a], Xi Zhu [b, *], Jie Zhang [a, **]

[a] Department of Laboratory Medicine, Peking University Third Hospital, Beijing, China
[b] Department of Urology, Beijing Friendship Hospital Affiliated to Capital Medical University, Beijing, China

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ABSTRACT

The emergence of chemoresistance greatly increases the recurrence risk for non-muscle invasive bladder cancer (NMIBC) patients, which is still a big concern of clinicians. Understanding the mechanisms of drug resistance is of great significance for preventing and reversing it. We showed here that CXC motif chemokine ligand 5 (CXCL5) was overexpressed in mitomycin C-resistant bladder cancer cell line M-RT4. Meanwhile, parental RT4 cell treated with recombinant human CXCL5 (rhCXCL5) reduced its sensitivity to mitomycin C. Conversely, knockdown CXCL5 sensitized M-RT4 cell. We further investigated the molecular mechanisms finding that epithelial mesenchymal transition (EMT) and NF-κB pathway were activated in M-RT4 cell, which could be attenuated by knockdown CXCL5. All these data indicated that CXCL5 may promote mitomycin resistance by activating EMT and NF-κB pathway. Thus, our study identifies CXCL5 as a novel chemoresistance-related marker in NMIBC, thereby providing new strategies to overcome chemoresistance for NMIBC patients.

1. Introduction

Bladder cancer is the most common carcinoma in urinary system, of which the incidence ranks fourth in western men [1]. Non-muscle invasive bladder cancer (NMIBC) accounts for roughly 75% of total cases. Although these patients receive complete resection of the tumor, the recurrence risk after 5 years still ranges as high as 50%–90%. Among all of them, 15%–20% will finally progress into muscle invasive bladder cancer (MIBC) [2]. Intravesical chemotherapy or immunotherapy is recommended after transurethral resection of bladder tumor (TURBT) to reduce the rate of NMIBC recurrence [3]. However, the appearance of chemoresistance counteracts its effects, which gives rise to poor prognosis of patients. Unfortunately, the molecular mechanisms of how resistance occurs remain poorly understood. Hence, finding proper chemoresistance-related molecules is of great significance to restore the sensitivity to chemotherapy.

CXC motif chemokine ligand 5 (CXCL5), also known as epithelial cell derived neutrophil activating peptide 78 (ENA-78), is a member of the Glu-Leu-Arg (ELR) positive CXC chemokines. By binding to its receptor CXCR2, CXCL5 is able to recruit neutrophils and promote angiogenesis [4]. Recently, it has been reported that CXCL5 participates in cancer-related inflammation, which may promote cancer metastasis and progression [5]. Our previous studies have demonstrated that CXCL5 was overexpressed in bladder cancer tissues and cells, which was associated with cancer relapse, progression and poor survival [6]. Also, the knockdown of CXCL5 in T24 cell inhibited its proliferation and migration [7]. In addition, Gao Y et al. found that CXCL5/CXCR2 axis promoted the migration and invasion of bladder cancer cells by activating PI3K/AKT-induced upregulation of MMP2/MMP9 [8].

Earlier studies have found that epithelial mesenchymal transition (EMT) is essential for the invasion and metastasis of cancers, including bladder cancer [9]. It features cadherin switching and the change of cell polarity and cytoskeleton, which promotes the motility of cells [10]. Recently, it has been reported that EMT has a close relationship with the emergence of chemoresistance in several cancers [11]. Other reports pointed out that intravesical recurrence is also related to EMT [12,13]. Our previous work found that downregulation of CXCL5 by shRNA in T24 cells enhanced E-cadherin and decreased Snail expression [6], suggesting that CXCL5 may affect the process of EMT. Therefore, whether CXCL5 is associated with chemoresistance of bladder cancer remains to be

* Corresponding author.
** Corresponding author.
E-mail addresses: alex_850107@hotmail.com (X. Zhu), zhangjiebjmu@163.com (J. Zhang).

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investigated.

2. Materials and methods

2.1. Cell lines

Human bladder cancer cell line RT4, purchased from ATCC, is a good representative of NMIBC. To generate corresponding mitomycin C (MMC)-resistant cell line, RT4 cells were exposed to MMC ranging in concentrations from 100 μg/ml serially diluted down to 0.78 μg/ml according to methods in Alan's study [14]. There were no viable cells left in flasks containing more than 1.56 μg/ml MMC in the end. Cells surviving the first cytotoxic insult were repopulated the flasks and further selected for three months. Finally, MMC-resistant cell line named M-RT4 was harvested. Both cell lines were cultured in DMEM/F12 (1:1) medium supplemented with 10% standard fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, penicillin: 100IU/ml and streptomycin: 100 mg/ml) at 37 °C in 5% CO_2 atmosphere.

2.2. Quantitative real-time PCR

The extraction of total RNA, reverse transcription to cDNA and quantitative real-time PCR were performed as described in our previous study [5]. The primers used for amplification of CXCL5 (GenbankNM_002994) were (sense)5′-GAGAGCTGCGTTGCGTTTGTTTAC-3′, (antisense) 5′-CCGTTCTTCAGGGAGGCTACCACT-3'. And primers used for GAPDH (GenbankNM_002046) were (sense) 5′-TGTTCCAATATGATTCCACCC-3′, (antisense) 5′-CTTCTCCATGGTGCGTGAAGA-3'. The relative mRNA expression of CXCL5 was normalized to GAPDH, which was calculated based on the Ct value according to the equation: 2^-ΔΔCt [ΔCt = Ct (CXCL5) -Ct (GAPDH)]. Each sample was analyzed in triplicate.

2.3. Western blot

RT4 cell and M-RT4 cell were harvested and then lysed by RIPA. Protein extraction and Western blot were performed as described previously [5]. The primary antibodies used are as follows: CXCL5 (1:500, ab126763, Abcam, US), CXCR2 (1:1000, ab65968, Abcam, US), EMT antibody sampler kit (1:1000, #9782, Cell Signaling Technology, Beverly, MA), NF-κB family member antibody sampler kit (1:1000, #4766, Cell Signaling Technology, Beverly, MA) and GAPDH (1:2500, MBL, Japan).

2.4. Cell proliferation assay

RT4 cell and M-RT4 cell were seeded into 96-well plates at a density of 2000 cells per well. 10 μl Cell Counting Kit-8 (CCK8) solution (Zoman Biotechnology Co, Ltd. Beijing, China) was added into each well and allowed to remain for 2 h before measurement. Changes in proliferation were evaluated every 24 h by measuring the absorbance at 450 nm in a microplate reader (Bio-Rad, Hercules, Calif).

2.5. CCK8 assay

Initially, RT4 cell and M-RT4 cell were seeded in triplicate into 96-well plates at a density of 4×10^4 cells and 1×10^4 cells per well, respectively. After incubation for 24 h, 10 μl of MMC at the concentration from 0.39 μg/ml to 100 μg/ml was added. 800 μg/ml stock solution of MMC (HZB0149, HARVEY, US) was gained by reconstructing in PBS according to the manufacturer's instructions, from which the working solutions were prepared. After another incubation for 24 h, 10 μl of CCK8 solution was added along with further incubation for 2 h. The optical density (OD) was measured at 450 nm. Cell viability was calculated as follows: (the OD value of experimental groups-the OD value of blank groups)/(the OD value of control groups-the OD value of blank groups).

2.6. Wound healing assay

1×10^4 cells were seeded in 60 mm dishes and cultured for 2 days to form a tight monolayer. Then wounds were made with a 10 μl plastic pipette tip. The remaining cells were washed with PBS for three times and incubated in serum free DMEM/F12 medium for 24 h. Microphotographs were taken at 0, 12 h and 24 h using an inverted microscope (Leica). The distance migrated was quantified and compared.

2.7. Recombinant human CXCL5

Recombinant human CXCL5 (rhCXCL5) was purchased from R&D Systems. 100 μg/ml stock solution of rhCXCL5 was achieved by dissolving 25 μg powder in 250 μl PBS, followed by adding 0.1% BSA in the final solution. Western blot and CCK8 assay were performed in triplicate using RT4 cell treated with 10 ng/ml rhCXCL5 for 24 h and 48 h, respectively.

2.8. Knockdown of CXCL5

Three siRNA of CXCL5 were synthesized by RiboBio company (Guangzhou, China) and resolved by PBS. M-RT4 cell was cultured in DMEM/F12 medium to a density of 50% when 100 nM siRNA was added using standard procedures. After incubation for another 48 h, cells were harvested to perform western blot and CCK8 assay.

2.9. Statistical analysis

Data were analyzed using the GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA) and SPSS Statistics 20 (IBM, US). Results were expressed as mean ± SD. Student's *t*-test was used for comparison of parental and MMC-resistant cells, considering $P < 0.05$ statistically significant.

3. Results

3.1. M-RT4 cell presented resistance to MMC compared with RT4 cell

The chemoresistant cell line M-RT4 was established by continuous exposure of RT4 cell to increasing concentrations of MMC. The morphological difference was assessed from the image (Fig. 1A). Unlike parental RT4 cell, M-RT4 cell presented a mesenchymal, spindle-like morphology in a dispersed growth pattern. According to CCK8 assay, M-RT4 had a comparably larger IC50 value than RT4 cell, indicating that M-RT4 cell acquired resistance to MMC (Fig. 1B and C). Apart from gaining chemoresistance, M-RT4 cell also presented stronger proliferation and migration capacity than RT4 cell, according to proliferation and wound healing assay. (Fig. 1D, E and F).

3.2. The expression of CXCL5 in M-RT4 cell was significantly upregulated

To identify the expression of CXCL5 in two cell lines, we used quantitative real time PCR to examine the mRNA level of CXCL5. Meanwhile, we performed western blot to quantify its protein level. Compared to parental RT4 cell, CXCL5 was significantly upregulated in M-RT4 cell in both mRNA and protein level ($P < 0.01$; Fig. 2A and B).

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