IL-23 concentration-dependently regulates T24 cell proliferation, migration and invasion and is associated with prognosis in patients with bladder cancer

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Abstract. Interleukin-23 (IL-23, also known as IL23A), is an important proinflammatory cytokine whose role in the development and progression of tumors remains controversial. The present study on IL-23 focused on its impact on the tumor microenvironment. Existing studies on its direct role on tumor cells are limited. Previously, we reported that the expression level of IL-23 in human bladder urothelial carcinoma was significantly higher than that in adjacent tissues as determined by immunohistochemistry. In this study, we further validated the results of immunohistochemistry using the Oncomine database and we found that IL23A expression in non-muscle invasive bladder urothelial carcinoma (NMIBC) was significantly higher than that in muscle invasive bladder urothelial carcinoma (MIBC). Expression of IL23A was negatively correlated with the clinical stage of bladder urothelial carcinoma and had a positive correlation with prognosis. *In vitro* experiments revealed that different concentrations of IL-23 had different effects on T24 cells. A low concentration of 20 ng/ml IL-23 promoted T24 cell proliferation, migration, invasion and EMT transformation, while a high concentration of 40 ng/ml IL-23 inhibited these functions. These results indicated that IL-23 plays a dual role in the progression of bladder cancer. Low concentrations of IL-23 promote bladder tumor progression, while high concentrations of IL-23 have the opposite effect.

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Abbreviations: IL, interleukin; NMIBC, non-muscle invasive bladder urothelial carcinoma; MIBC, muscle invasive bladder urothelial carcinoma; EMT, epithelial mesenchymal transition; TCGA, The Cancer Genome Atlas; IL-23R, IL-23 receptor; DCs, dendritic cells; IFN-γ, interferon-γ; pH3, phospho-histone H3

Key words: interleukin-23, bladder cancer, urothelial cell carcinoma

Introduction

In China, bladder cancer is the urinary tract tumor with the highest incidence (1). Urothelial carcinoma is the main pathological type of bladder cancer, and ~70% of patients are initially diagnosed with non-muscle invasive bladder cancer (NMIBC), which has a recurrence rate of 50-70%. Of the patients who experience recurrence, 10-20% progress to muscle invasion of bladder cancer (MIBC) (2). Furthermore, ~30% of patients with an initial diagnosis of MIBC have distant metastases and a high risk of death (3). The pathogenesis of bladder cancer is not clear, but the hypothesis that chronic inflammation can promote tumor development has been recognized. It is reported that 50% of bladder cancers are related to chronic inflammation (4).

Interleukin 23 (IL-23) is an important proinflammatory cytokine (5). Our previous study found that IL-23 expression in bladder cancer tissue was associated with tumor-infiltrating dendritic cells (DCs) and that the IL-23 expression levels in cancer tissue were significantly increased compared to that in adjacent tissues, suggesting that IL-23 may be related to the progression of bladder cancer (6). It has been reported that in the human tumor microenvironment is mainly derived from inflammatory DCs (7), and that IL-23 is the key linker molecule between the tumor-induced proinflammatory process and adaptive tumor immunosuppression. IL-23 and IL-12 belong to the same family of proinflammatory heterodimer cytokines (8). The two cytokines co-possess the p40 subunit, which form IL-12 with p35 through a covalent bond, and form IL-23 with p19. IL-23 induces and amplifies Th17 cells (9) and IL-12 induces and amplifies Th1 cells (10). IL-23 is an essential cytokine for the maintenance of the normal number and function of Th17 cells and the secretion of IL-17 by Th17 cells (11). In addition to having similar functions with partial IL-12, IL-23 is also able to promote the autoimmune response and occurrence of immune-related inflammatory diseases through the IL-17 pathway. The balance between IL-12 and IL-23 is very important in the process of tumorigenesis. Mounting evidence suggests that IL-12 and IL-23 can independently play their respective roles without relying on interferon-γ (IFN-γ) and IL-17A, respectively (12). At present, there is little research on whether IL-23 can play a direct role in tumor cells; the

relationship between IL-23 and bladder cancer especially remains unclear.

In the present study, we further investigated the relationship between IL-23 and bladder cancer through Oncomine and TCGA database analysis, clinical specimen testing and *in vitro* cell experiments.

Materials and methods

Patients and samples. The tissue specimens were obtained from 10 MIBC patients who were treated at Daping Hospital of the Third Military Medical University (Chongqing, China) from March 2012 to December 2014. The study subjects included 2 females and 8 males, with an average age of 63 years (45-79 years). The pathological type of all specimens was independently determined by two pathologists. Tumor diagnosis and grading followed the International Union Against Cancer TNM Classification criteria (13); the staging criteria followed the WHO Classification of Tumors (14). The exclusion criteria were: i) non-urothelial tumors; ii) patients with an immune system disorder or receiving an immunosuppressive agent; and iii) patients who had undergone preoperative radiotherapy or immunotherapy. The tissue samples were rapidly frozen in liquid nitrogen immediately after dissection and stored in liquid nitrogen until further assessed by quantitative real-time PCR. The study was approved by the Ethics Committee on Human Experimentation of Daping Hospital of the Third Military Medical University and informed written consent was obtained from all patients. All collected samples were then eligible for experimental purposes.

Oncomine database and The Cancer Genome Atlas (TCGA) analysis. The expression level of the *IL23A* gene in bladder urothelial carcinoma was assessed using the Oncomine (www.oncomine.org) database (*IL-23A* is indicated in Oncomine database and The Cancer Genome Atlas to represent gene *IL-23*) (15). Hence, we compared the clinical specimens of cancer and normal bladder mucosa tissues (including paracancerous tissue). To reduce the false discovery rate, we selected $P<1x10^{-4}$ as the threshold and analyzed the results of P-values, fold change and cancer subtype. Correlation of IL23A expression with clinicopathological parameters of bladder urothelial carcinoma was analyzed using the TCGA BLCA-Bladder Urothelial Carcinoma (Nature 2014) data set (16).

Cells, reagents and transfection. The human bladder urothelial carcinoma cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). T24, SW-780, J82 and UM-UC-3 cell lines were treated with Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 10% fetal bovine serum (FBS). At 48 h after transfection, the cells were collected for qRT-PCR assays. Recombinant human IL-23 (IL-23) was purchased from R&D Systems (Minneapolis, MN, USA).

RNA isolation and quantitative RT-PCR. Total RNA was isolated from cultured cells or tissue using a Total RNA Isolation kit (Takara Biotechnology, Co., Ltd., Dalian, China), followed by reverse transcription and SYBR-Green-based RT-PCR analysis (Takara Biotechnology). IL-23p19, IL-23 receptor (IL-23R), IL-17 and IL-6 were assayed by qRT-PCR using PrimeScript RT Reagent kit, SYBR-Green Real-Time PCR Master Mix and Premix ExTaq (all from Takara Biotechnology) following the manufacturer's protocols.

These primers purchased from Sangon Biotech Co., Ltd. (Shanghai, China) are listed in Table I. IL-23p19, IL-23R, IL-17 and IL-6 levels were normalized to the β-actin expression level. The $2^{-\Delta\Delta Cq}$ method was conducted for analyzing gene expression (17). At least three independent experiments were conducted for each experimental condition.

Colony formation assay. T24 cells were plated in each well of a 6-well plate at a density of 500 cells/well and treated with IL-23 (0, 10, 20 or 40 ng/ml). When the plates appeared visibly cloned, the clones were fixed with methanol and stained by crystal violet for 20 min. The number of clones was over 50 cells in wells and was counted using a light microscope (Eclipse E800M; Nikon, Tokyo, Japan). At least three independent experiments were performed.

CCK-8 method for cell viability. T24 cells that were pre-treated with different concentrations of IL-23 (0, 10, 20 or 40 ng/ml) for 48 h were seeded at a density of $2x10^3$ cells/well in independent 96-well plates and the media were changed to media without IL-23. No vehicle control was used. The cell proliferation was then detected using Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Beijing, China) following the manufacturer's protocol; $2x10^3$ cells were plated in 96-well microplates, followed by the addition of 10 μl of CCK-8 (Dojindo Molecular Technologies) solution to each well and incubation of the samples for 1 h before measuring the absorbance at 450 nm in a microplate autoreader El309 (Bio-Tek Instruments, Inc., Woonoski, VT, USA). Experiments were performed in triplicate. No vehicle control was used.

Immunofluorescence. The cells were divided into the control group, the IL-23 10-ng/ml group, the IL-23 20-ng/ml group and the IL-23 40-ng/ml group, in triplicate in each group. The culture coverslips with a diameter of 18 mm were placed in 24-well plates and $1x10^4$ cells were inoculated in each well. When the cell density was increased to 40%, it was replaced with the serum-free DMEM/F12 and then starved for 2 h to make the cells' growth cycle consistent. After incubation for 48 h at 37˚C in a 5% CO_2 cell incubator, the cells were washed three times with phosphate-buffered saline (PBS) (0.1 mol/l), fixed in 4% paraformaldehyde for 15 min, washed three times with PBS solution, placed in 0.03% Triton X-100 for 20 min to improve cell membrane permeability, flushed with PBS 3 times, and then sealed with immunofluorescence blocking fluid for 20 min. Anti-Ki-67 (rabbit polyclonal to Ki-67, 1:100; cat. no. ab15580; Abcam, Cambridge, MA, USA) and Anti-Histone H3 (phospho-histone S28) (HTA28, 1:100; cat. no. ab10543; Abcam) were added dropwise and were uniformly covered on the slide and then placed in a refrigerator at 4˚C overnight. After washing with PBS solution 3 times (5 min/time), goat anti-rabbit IgG secondary antibody (cat. no. W10816; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added dropwise and incubated in a wet box at 37˚C for 1 h and washed 3 times (5 min/time) in PBS solution. DAPI staining was added dropwise and incubated at 37˚C for

Table I. Primer sequences.

Gene	Primer sequences
IL-6	5'-AAATGCCAGCCTGCTGACGAAG-3'
	3'-CATCGTACCCGTGAGTCTAACAACAA-5'
IL-17A	5'-AATACAACCGATCCACCTCAC-3'
	3'-GACACTAGACCCTCCGTTACT-5'
Il-23p19	5'-TGTGAATGACTTGGTCCCTGAA-3'
	3'-GACACTAGACCCTCCGTTACT-5'
Il-23R	5'-GCAAACGCACTAGGCATGGAAG-3'
	3'-CTCTATGTTCCGATGTTGTTTGGTT-5'
β-actin	5'-CTCTTCCAGCCTTCCTTCCT-3'
	3'-GACATGCGGTTGTGTCACGA-5'

IL-23R, IL-23 receptor.

3 min. After washing 3 times (5 min/time) in PBS solution, a 10 μl anti-fluorescent decaying tablet was added dropwise to coat cells, followed by sealing with clean coverslips and images were acquired with Leica Application Suite (Version 4.2.0; Leica Microsystems, Oberkochen, Germany) under a Leica DM 4000B microscope.

Wound-healing and Transwell assays. Before wound-healing or Transwell assays were conducted, the cells of each group were treated with IL-23 as aforementioned. The wound-healing assay was performed by scratching the single cell layer with tips. The images of the scratch area were recorded at three random spots at 0 and 24 h. A standard size field was used to measure the migrating distance of the wound edge for each image. An inverted microscope (Carl Zeiss Axiovert 25C; Carl Zeiss, Göttingen, Germany) was used to examine the scratch wounds. The mean migrating distances of the three spots were calculated according to the scale plate. Wound-healing assays were performed in triplicate and all data were statistically processed.

The Transwell assay was used to test cell migration and invasion abilities. For the migration assay, T24 cells were placed into the upper chamber of each insert (Corning Inc., Corning, NY, USA). For the invasion assay, the cells were placed into the upper chamber of inserts coated with 45 μg of diluted Matrigel (2 μg/μl); 600 μl medium (containing 10% FBS) was added to the lower chambers. After 24 h of incubation, a cotton tip was used to wipe the upper surface of the membrane and cells that were attached to the lower surface were stained for 20 min with crystal violet and then rinsed in PBS prior to light microscopic inspection (Eclipse E800M; Nikon). Invasion values were obtained by counting eight fields per membrane, which represent the average of three independent experiments.

Western blot analysis. Protein was collected from 2x10^6 cells after IL-23 treatment for 48 h with lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cell lysates were centrifuged at 12,000 x g at 4˚C for 15 min. Concentration was measured using BCA methods and 30 ng was loaded per lane and run to separate on 10% sodium dodecyl sulfate-polyacrylamide gels. The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA) after 90 min of electrophoresis. The membranes were blocked with 5% non-fat milk in 0.1% TBS-Tween-20 at room temperature for 1 h and were then incubated with anti-GAPDH (1:1,000 dilution; cat. no. ab8245; Abcam) at 4˚C overnight, followed by incubation with LI-COR IRDye 680-labeled secondary antibodies (1:10,000 dilution; cat. no. KFC200; Rockland Immunochemicals, Gilbertsville, PA, USA) for 1 h at room temperature. Signals were detected with an Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA) and quantified using the FluorChem 8900 system (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis. All data were analyzed with SPSS 19.0 software (IBM Corp., Armonk, NY, USA). Data were expressed as the mean values ± standard deviation (SD). Comparisons between two groups were carried out using Student's t-test. Comparisons of multiple groups were analyzed using one way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) post hoc test. The Chi-square test was performed to determine the association between the *IL23A* expression and the clinicopathological parameters of bladder cancer. Differences were considered statistically significant at $P<0.05$.

Results

Expression of IL-23, IL-23R, IL-6 and IL-17 in bladder urothelial carcinoma tissues. To study the effect of IL-23 on bladder urothelial carcinoma, we first detected the expression levels of IL-23 mRNA and IL-23R mRNA in human MIBC and adjacent tissues, and found that both expression levels were significantly increased in cancer tissues, which was consistent with our previously reported immunization results (Fig. 1A-D). In recent years, a number of studies about breast, prostate and gastric cancer revealed that the number of Th17 cells was significantly increased in the tumor microenvironment and its secreted IL-17 promoted the development of tumors through the induction of chronic inflammation (18). The initial differentiation of Th17 cells is dependent on IL-6, but IL-23 is a key cytokine in the process of survival, proliferation and secretion of IL-17 (11). Therefore, we also detected the expression of IL-6 and IL-17, and determined that IL-6 mRNA and IL-17 mRNA were also significantly increased in MIBC tissues, indicating that IL-23, IL-6, IL-17 and IL-23R were increased in the bladder tumor microenvironment (Fig. 1D-G).

Using Oncomine Cancer Microarray Database and The Cancer Genome Atlas (TCGA) to analyze the expression of IL23A in bladder urothelial carcinoma. To further validate the accuracy of the results, we used the Oncomine database to carry out bioinformatics analysis of IL23A gene expression in bladder urothelial carcinoma, and used normal bladder mucosa tissues and/or adjacent tissues as a control. To reduce the error, we set the filter keywords before the meta-analysis (Fig. 1A), with threshold parameters for gene expression analysis at

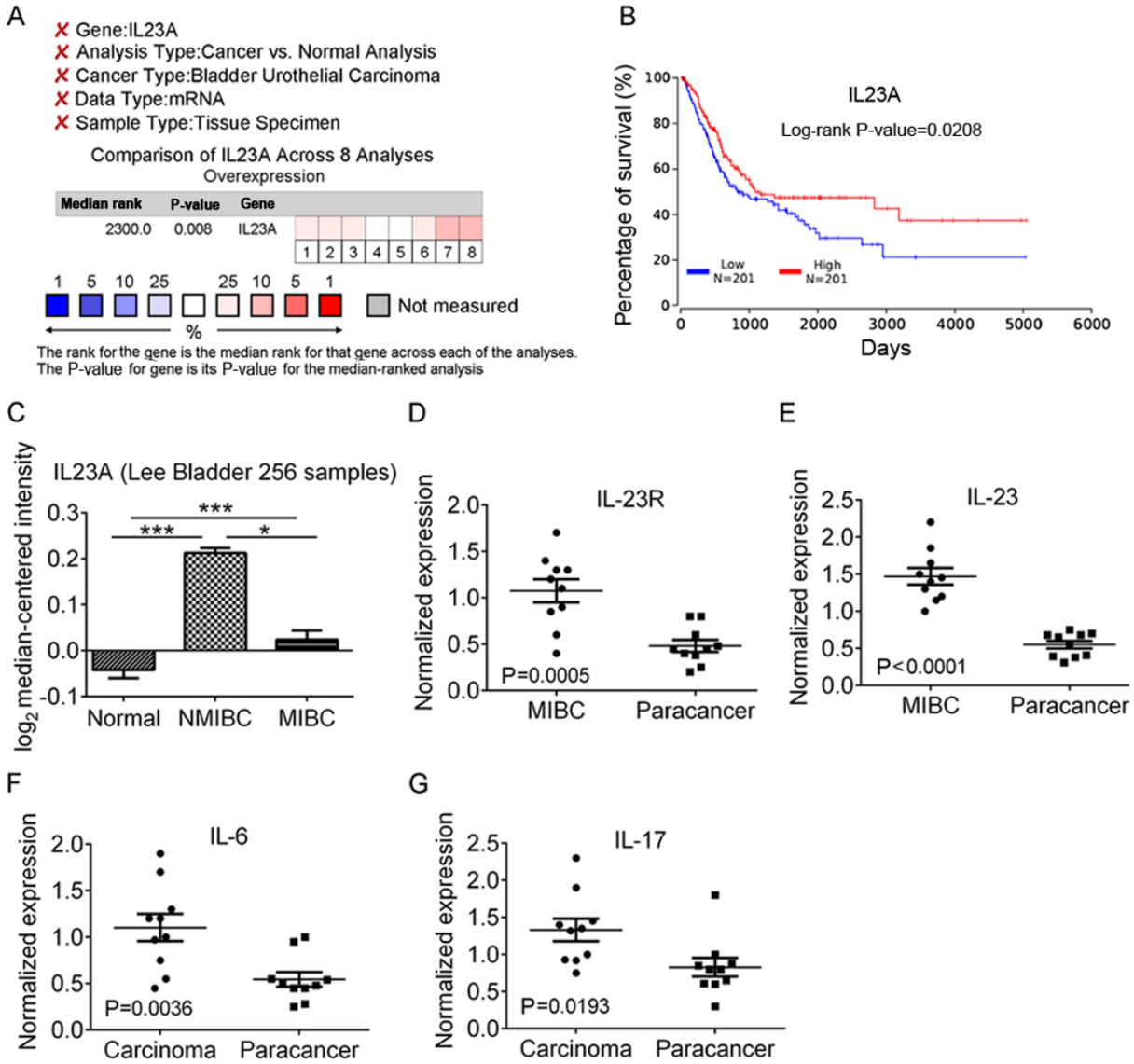

Figure 1. mRNA expression analysis of IL23A and related inflammatory cytokine. (A) Eight data sets of bladder urothelial carcinoma were obtained by searching in the Oncomine database with the key words including IL23A, Cancer vs. Normal Analysis, Bladder Urothelial Carcinoma, mRNA and Tissue Specimen and over-expression analysis revealed that the expression of IL23A in cancer tissue was significantly increased in comparison with normal bladder urothelial tissue. (B) TCGA BLCA-Bladder Urothelial Carcinoma (Nature 2014) (16) Data Center Kaplan-Meier plots revaled overall survival in bladder urothelial carcinoma. In red: Patients with expression above the median; in blue, patients with expression below the median. (C) The expression level of IL23A was significantly higher in NMIBC and MIBC than that in normal bladder tissue, and the expression of IL23A in MIBC was significantly lower than that in NMIBC. (D-G) The expression levels of IL-23R, IL-23, IL-6 and IL-17 mRNA in the MIBC were significantly higher than those in the adjacent tissues. Lee, represents the Lee Bladder dataset in Oncomine database (19); NMIBC, non-muscle invasive bladder urothelial carcinoma; MIBC, muscle invasive bladder urothelial carcinoma. *P<0.05, ***P<0.001.

P<1x10^{-4}, fold change >2, and median gene rank in the top 10%. We found IL23A gene expression in tissue samples of 424 patients in 8 data sets of 4 research projects, and the over-expression analysis results indicated P=0.008 (Fig. 1A). The two most significant data sets were infiltrating bladder urothelial carcinoma vs. normal (P=4.79x10^{-4}) (19) and infiltrating bladder urothelial carcinoma vs. normal (P=1.78x10^{-7}) (20). We selected data with the largest sample, which included 256 cases, to analyze the expression difference, and the results revealed that IL23A in NMIBC (also known as superficial tumor) and MIBC was significantly increased and was higher in NMIBC than in MIBC (Fig. 1C).

To further analyze the role of IL23A in bladder cancer, we used TCGA data to analyze the relationship between IL23A expression and clinicopathological characteristics (Table II) (21), since we were unable to obtain all of the Oncomine data. The results indicated that a high expression level of IL23A was correlated with a low clinical stage and good overall survival, while a low expression level of IL23A was correlated with a high clinical stage and poor overall survival. However, the expression level of IL23A was not significantly correlated with age, sex, tumor stage, grade or metastasis.

Low concentration of IL-23 promotes T24 cell proliferation, migration, and invasion, and high concentration of IL-23 inhibits T24 cell proliferation, migration and invasion. After IL-23 treatment (0, 10, 20 and 40 ng/ml) on bladder urothelial

Table II. Clinical association between the IL23A expression and the clinicopathological variables in bladder cancer patients.

Variable	No. of patients	IL23A		χ^2 test P-value
		High expression	Low expression	
Age (years)				0.112
>60	300	65	235	
≤60	107	32	75	
Sex				0.597
Male	300	69	231	
Female	107	28	79	
Smoking status				0.596
Smoking	394	95	299	
Non-smoking	14	2	12	
T classification				0.483
T_{1+2}	128	34	94	
T_{3+4}	41	8	33	
Lymph node stage				0.83
N_{0+1}	282	70	212	
N_{2+3}	83	19	64	
M classification				0.185
M_0	195	52	143	
M_1	209	43	166	
Metastasis				0.794
No	147	31	116	
Yes	55	10	45	
Clinical stage				0.017
I-II	132	41	91	
III-IV	273	54	219	

carcinoma for 48 h, a CCK-8 assay revealed that a low concentration of IL-23 (20 ng/ml) significantly promoted the proliferation of T24 cells (P<0.05), while IL-23 (40 ng/ml) inhibited its proliferation (P<0.05); 10-20 ng/ml IL-23 promoted the proliferation of SW780 cells, which had no concentration dependence; IL-23 had no significant proliferative effect on J82 cells and UM-UC-3 cells (Fig. 2A). Colony formation assay (Fig. 2B and E) and immunofluorescence (Fig. 3) were used to detect the number of Ki-67 and pH3 positive cells to verify the proliferation effect of IL-23 on T24 cells, which produced similar results with the CCK-8 assay. Concurrently, we observed the effect of different concentrations of IL-23 on Ki-67 and PCNA protein expression in T24 cells, revealing that 10 and 20 ng/ml of IL-23 promoted the upregulation of Ki-67 and PCNA protein, while a high concentration of IL-23 (40 ng/ml) played an inhibitory role (Fig. 2C, D and F). The relationship between IL-23 and migration and invasion of bladder urothelial carcinoma cells has been studied *in vitro*. In a wound-healing assay, low concentrations of IL-23 (10 and 20 ng/ml) promoted T24 cell migration (P<0.05) and a high concentration of IL-23 inhibited T24 cell migration (P <0.05) (Fig. 4A and C). A Transwell assay further demonstrated that low concentrations of IL-23 (10 and 20 ng/ml) promoted T24 migration and invasion, whereas a high concentration of IL-23 (40 ng/ml) played an inhibitory role (Fig. 4B, D and E).

Low concentration of IL-23 upregulates the expression of EMT-related proteins in T24 cells, but high concentration of IL-23 downregulates the expression of EMT-related proteins in T24 cells. Epithelial-mesenchymal transition (EMT) refers to the transformation of epithelial cells into interstitial cells after stimulation by external factors. In this process, epithelial cells lose polarity and the adhesion connection between cells, increase the migration and athletic ability of cells, and obtain the interstitial phenotype (22). It is characterized by spindle-shaped morphology, decreased cell adhesion, and increased migration and motor capacity, which is closely related to tumor metastasis. It is also characterized by downregulation of the expression of E-cadherin, and upregulation of the expression of N-cadherin, vimentin and OB-cadherin (cadherin-11) (23). Studies have reported that inflammatory factors can activate the EMT pathway. To determine whether IL-23 can promote T24 cell EMT, we detected the expression of EMT-related proteins in T24 cells under different concentrations of IL-23. The expression of E-cadherin was downregulated after treatment with IL-23 (10 and 20 ng/ml) for 48 h, and

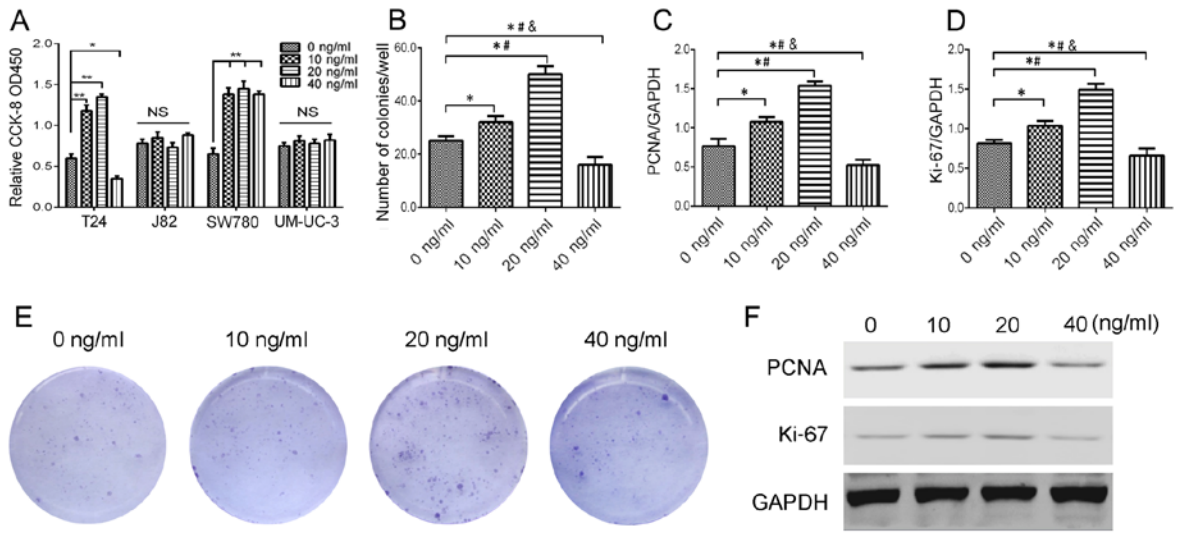

Figure 2. Low concentrations of IL-23 promote T24 cell proliferation and high concentrations of IL-23 inhibit T24 cell proliferation. Different concentrations of IL-23 (0, 10, 20 and 40 ng/ml) were applied to bladder urothelial carcinoma cells. (A) The CCK-8 experiment revealed that 10 and 20 ng/ml IL-23 promoted the proliferation of T24 cells and SW780 cells and that 40 ng/ml IL-23 inhibited T24 cell proliferation. (B-E) Cloning experiments indicated that 10 and 20 ng/ml IL-23 promoted T24 cell proliferation and 40 ng/ml IL-23 played an inhibitory role. (F) Western blot analysis revealed that IL-23 (10 and 20 ng/ml) promoted the expression of Ki-67 and PCNA in T24 cells and 40 ng/ml IL-23 played an inhibitory role. *P<0.05 vs. 0 ng/ml, **P<0.01 vs. 0 ng/ml, $^{\#}$P<0.05 vs. 10 ng/ml, $^{\&}$P<0.05 vs. 20 ng/ml. NS, non-significant difference. Each column and bar represent the mean ± SD of three independent experiments. The results are one representation of three independent experiments.

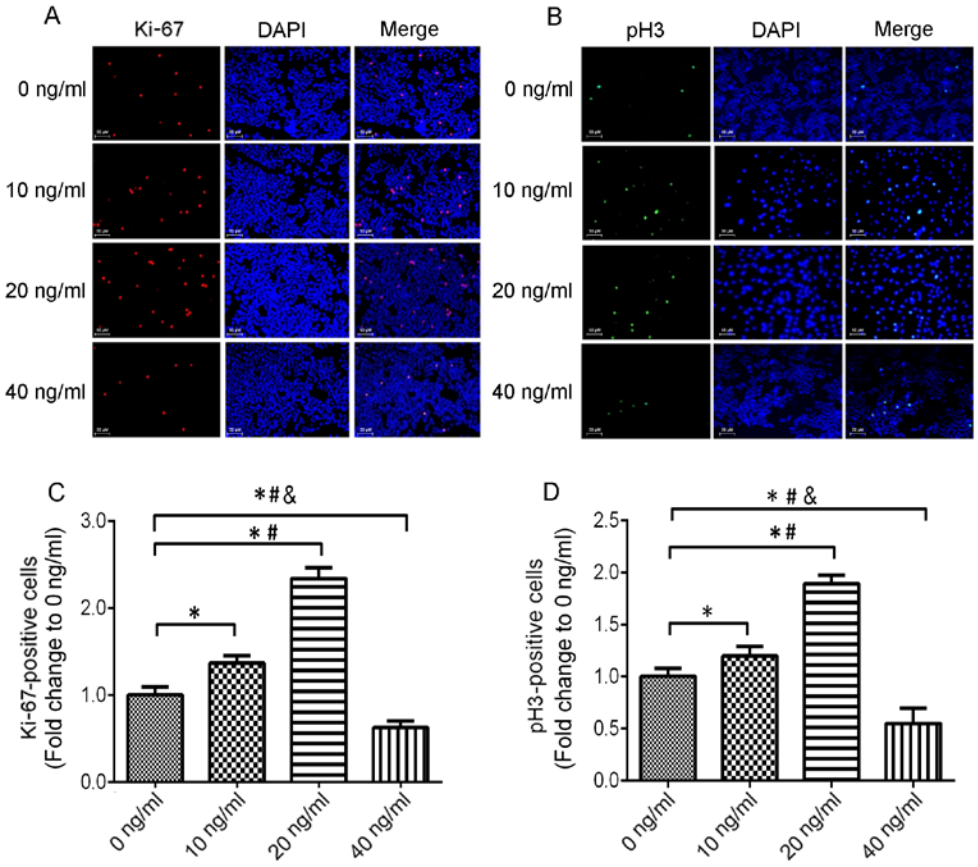

Figure 3. Immunofluorescence assay is used to detect expression of (A and C) Ki-67 and (B and D) pH3 in T24 cells treated with IL-23 (0, 10, 20 and 40 ng/ml). The results indicated that low concentration of IL-23 upregulated and high concentration of IL-23 downregulated the number of Ki-67- and pH3-positive cells. Magnification, x200; *P<0.05 vs. 0 ng/ml, $^{\#}$P<0.05 vs. 10 ng/ml, $^{\&}$P<0.05 vs. 20 ng/ml.

the expression of N-cadherin, OB-cadherin and vimentin was upregulated; however, IL-23 (40 ng/ml) increased the expression of E-cadherin, and decreased the expression of N-cadherin, OB-cadherin and vimentin (Fig. 4F).

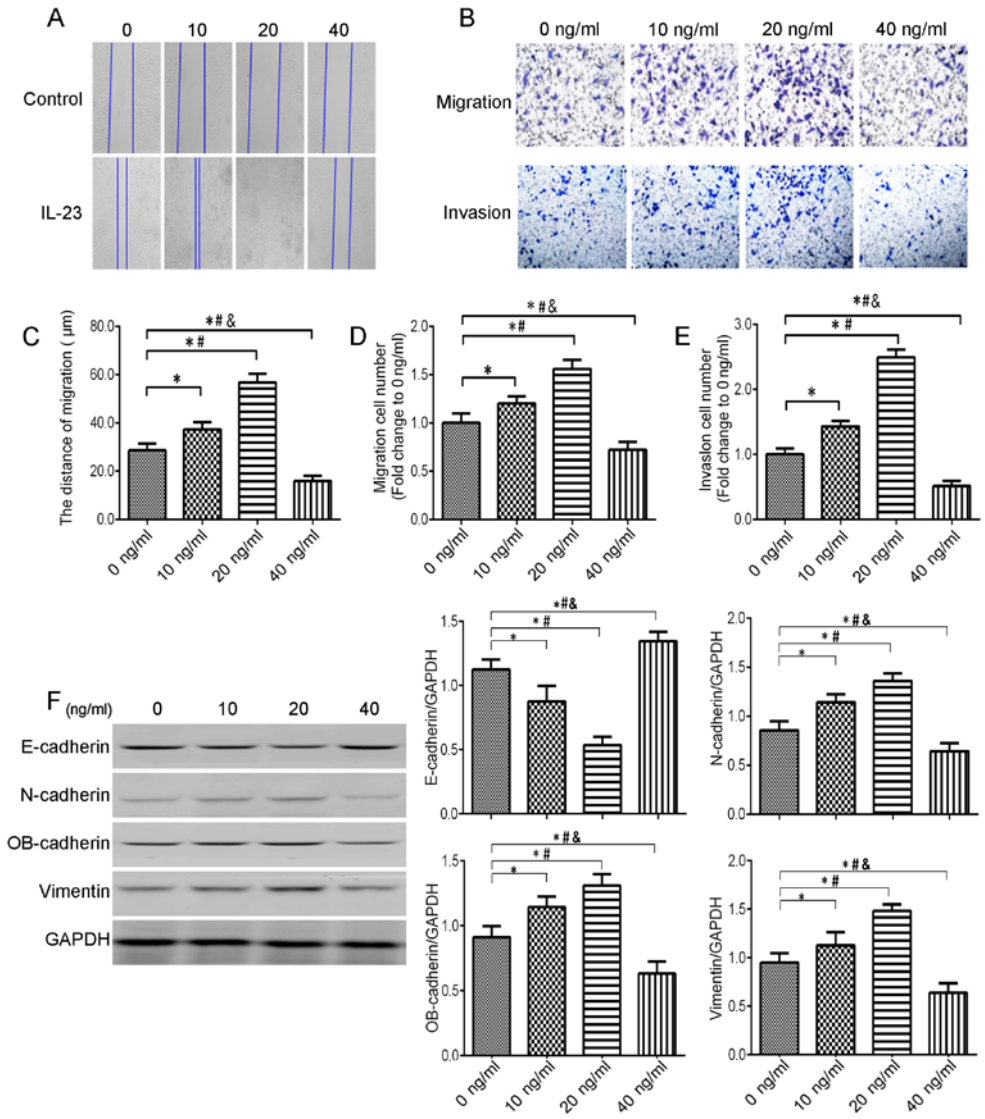

Figure 4. Low concentration of IL-23 promotes and high concentration of IL-23 inhibits T24 migration, invasion and EMT transformation. (A and C) Wound healing, (B, D and E) Transwell assays revealed that 10 and 20 ng/ml IL-23 promoted T24 cell migration and invasion, and 40 ng/ml IL-23 had the opposite effect. (F) Western blotting indicated that 10 and 20 ng/ml inhibited the expression of E-cadherin as well as promoted the expression of N-cadherin, OB-cadherin and vimentin and that 40 ng/ml IL-23 promoted the expression of E-cadherin as well as inhibited the expression of N-cadherin, OB-cadherin and vimentin. *P<0.05 vs. 0 ng/ml, $^{\#}$P<0.05 vs. 10 ng/ml, $^{\&}$P<0.05 vs. 20 ng/ml. Each column and bar represent the mean ± SD of three independent experiments. The results are one representation of three independent experiments.

Discussion

Chronic inflammation is an important factor in promoting tumor development. Due to the heterogeneity of the tumor microenvironment, the role of chronic inflammation in different tumor tissues is still widely controversial (24). In recent years, the role of the IL-12 cytokine family in chronic inflammation and tumor progression has been widely reported. The IL-12/STAT4 signaling pathway activates antitumor immune response, which mainly secretes IFN-γ (8). The IL-23/STAT3 signaling pathway activates the tumor-associated inflammatory response, which mainly secretes IL-17 and promotes tumor cell immune escape (11). It was revealed that exogenously overexpressed IL-23 inhibited tumorigenesis and development, whereas endogenously low levels of IL-23 promoted tumorigenesis and progression, while anti-IL-23 monoclonal antibody can inhibit tumor growth and metastasis (25). IL-23 appears to play a double opposing role in tumor development and progression, but there are few studies on the direct effect of IL-23 on tumor cells.

In the present study, we reported that the level of IL-23 expression in human bladder urothelial carcinoma was significantly higher than that in adjacent tissues as determined by immunohistochemistry. We further demonstrated these results by detecting mRNA expression levels and using the Oncomine database. IL-23 was significantly increased in most tumor tissues from different organs and was not expressed in the same specimen of normal tissues, which suggested that IL-23 was tumor-specifically upregulated and may not be the result of mutations in susceptible genes (26). The effect of IL-23 on tumorigenesis was first demonstrated in IL-23p19-deficient mouse models (27), which showed a significant inhibitory effect on 7,12-dimethylbenz[a]anthracene (DMBA)/12-*O*-tetradecanoyl-phorbol acetate (TPA)-induced

dermal papilloma. Another study also demonstrated that IL-23p19-deficient mice were resistant to DMBA/TPA-induced skin papilloma and methylcholanthrene (MCA)-induced murine fibrosarcoma (12). IL-23, IL-17A and IL-6 in peripheral blood of patients with bladder cancer were significantly higher than those in normal controls, and Th17 cells were significantly increased in bladder cancer tissues (28). It has also been reported that IL-23 can enhance the motility of tumor cells and activate the NF-κB/p65 signaling pathway to upregulate the level of MMP9 in tumor cells (29). In addition, the expression of IL-23 in patients with primary hepatocellular carcinoma was strongly correlated with IL-17A and MMP9. Similarly, IL-23 promoted the proliferation of human oral squamous cell carcinoma in patients with positive IL-23R by activating the NF-κB/p65 signaling pathway (30). This study also indicated that different concentrations of IL-23 play different roles in T24 cells. A relatively low concentration of IL-23 promoted T24 cell proliferation, migration, invasion and EMT transformation, while a relatively high concentration of IL-23 played the opposite roles. The TCGA data analysis also revealed that low expression levels of IL-23 were associated with higher clinical staging and poor clinical outcomes, while relatively high levels of IL-23 had the opposite effects.

In contrast to the results of this study, some results indicated that IL-23 only has a role in inhibiting tumor development (25). For example, mouse tumor cell lines expressing IL-23 can inhibit tumor growth *in vivo* (31); the use of adenovirus expressing IL-23 in established tumor models exhibited antitumor effects (32). It has also been reported that IL-23 can significantly enhance its antitumor effect only after adoptive transfer of peptide vaccine or antigen-specific T cells (33). The drawback of the aforementioned *in vivo* experiment is that it cannot accurately explain whether IL-23 acts directly on tumor cells and how to regulate its biological processes. Some studies are consistent with our findings. It has been reported that endogenously low concentrations of IL-23 promote a tumor microenvironment characterized by immunosuppression and promote tumor progression, whereas anti-IL-23 monoclonal antibody inhibits tumor growth and metastasis (34); exogenous overexpression of IL-23 has been reported to manifest antitumor effects (32). However, studies about the direct role IL-23 on tumor cells are rare, and the results of different studies have been contradictory, which may be related to tumor cell heterogeneity, IL-23 dose and different tumor microenvironments.

The evidence concerning IL-23 in the promotion of tumorigenesis appears to be contradictory, but it is important to note that it is difficult to assess whether the IL-23 used in the study is fully compliant with the physiologic dose, and these studies may not actually reflect the tumor regulation role of the host endogenous IL-23 in the natural state. Furthermore, the experimental results of different types of tumors are also different, and it is worth noting whether IL-23R is expressed. Studies on IL-23R-positive human lung cancer cell lines have also reported that low concentrations of IL-23 promote tumor cell proliferation while high concentrations of IL-23 have the opposite effect (35). Hence, we should carefully evaluate the study results that suggest that IL-23 can inhibit tumorigenesis.

It is clear that the expression level of IL-23 in the tumor microenvironment determines the cancer-promoting or tumor-suppressing characteristics of IL-23. Functional IL-23R is composed of IL-23R and IL-12Rβ1, the IL-23R downstream signaling molecule STAT3 promotes the secretion of IL-17-based immune response, while IL-12Rβ1 downstream signaling molecule STAT4 promotes Th1-type immune response. IL-12 and IL-23 belong to IL-12 family of cytokines, STAT4 is a key transcription factor of the IL-12-specific signaling pathway, and STAT3 is a key transcription factor of the IL-23-related signaling pathway. We found the following phenomenon through this study: Low concentrations of IL-23 promoted the progression of bladder tumors, while high concentrations of IL-23 played an inhibitory role. The possible mechanisms are as follows: When IL-23 concentration is low, it is integrated with IL-23r which has stronger affinity, activates the STAT3 signaling pathway and promotes tumor progression. Transcription factor STAT3 activation has been reported in multiple tumor studies and its activation is associated with cell proliferation, survival, pro-angiogenesis and immune escape (27,36,37). When the concentration of IL-23 is high, it not only binds to IL-23r, but also to IL-12Rβ1, activating the STAT4 signaling pathway and exerting a similar effect as IL-12. The STAT4 signaling pathway promotes the secretion of γ-interferon by activating NK cells and Th1-type immune responses, and activates cytotoxic T lymphocytes and promotes their proliferation, thereby exerting antitumor immune effects (8,25,36). From this point of view, the antitumor effect of high concentrations of IL-23 on the IL-12Rβ1/STAT4 signaling pathway is worthy of further study.

In general, the mechanism of IL-23 in regulating tumorigenesis and tumor immunity remains unclear. Related studies are still controversial. We believe that IL-23 plays a dual role in the progression of bladder cancer. In a future study, the properties of IL-23 in the microenvironment of bladder tumor, the mechanism of its secretion and the functional mechanism of IL-23 in tumor cells should be further elucidated.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

PW and ZS conceived and designed the study. PW participated in every step of the specific experiment, and was also the writer of this manuscript. ZS provided the overall idea of the study and controlled the quality of all the work throughout the entire process. YZ and JZ performed the experiments, analyzed the results, conducted statistical analysis of data and also wrote the manuscript. JJ provided practical advice and guidance,

and proposed many feasible solutions. FJ reviewed, edited the manuscript and provided the assistance with clinical case selection and statistical analyses. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Institutional Review Board of the Deparment of Laboratory Animal Science of Daping Hospital (Chongqing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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