

Original Article

Expression of COUP-TF2 in bladder carcinoma and effect of microenvironment of bladder cancer on endothelial cells

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Abstract: Vessel plays an important role in the occurrence and development of tumors. COUP-TF2 (Chicken Ovalbumin Promoter transcription factor 2) has been found associated with tumor development, metastasis and tumor angiogenesis in a number of tumor models, such as pancreatic cancer and breast cancer. The aim of this study was to analyze the expression of COUP-TF2 in human bladder cancer to evaluate the relationship between COUP-TF2 expression and clinicopathological features, and to study the effect of tumor microenvironment on vascular endothelial cells. We investigated the expression of COUP-TF2 and microvessel density (MVD) of 35 bladder tissue specimens with immunohistochemical assay and assessed the score using the prescribed scoring method. Furthermore, HUVEC was cultured with supernatant of bladder carcinoma cells and normal cells, respectively. The effect of microenvironment on HUVEC cells were evaluated using Real Time-PCR, Western blot and CCK-8. COUP-TF2 was strongly expressed in 74% of tumor tissues investigated and weakly expressed in 25% of normal bladder tissues investigated. The pathological grade and clinical stage of bladder cancer were closely related to COUP-TF2 protein expression and MVD ($P < 0.05$). In addition, the expression of COUP-TF2 protein was closely positively correlated to MVD ($P < 0.05$). The expression of COUP-TF2 in the bladder cancer supernatant was higher than that in normal bladder supernatant at both RNA and protein level, and promoted the proliferation of HUVEC. This indicates that COUP-TF2 may promote the progression of bladder cancer and therefore, may serve as a molecular marker for bladder cancer progression and used a new target for medical treatment.

Keywords: Bladder neoplasms, COUP-TF2, clinicopathological data, immunohistochemical assay, vascular endothelial cells

Introduction

Bladder cancer is the most common malignant tumor of the urinary system, it has multi-center, easy to relapse, and invasive growth characteristics. The incidence rate of malignancy in Chinese male genitourinary system is the first, and the incidence rate of malignant tumor is the eighth. Due to the aging population, increased smoking population, increased environmental pollution and other aspects of occupational protection imperfections, China has an upward trend in its incidence [1]. At present, the standard treatment for invasive bladder cancer is radical surgery with chemotherapy

and/or radiotherapy, but the effect is not ideal. If the bladder cancer patients have bladder metastasis, the 5-year survival rate was only 6%, treatment and prognosis will be poor [2]. A better understanding of the molecular mechanisms underlying the development of bladder cancer may help to improve the prognosis of patients with bladder cancer. Thus, there is a strong need for new noninvasive biomarker identification for early tumor detection.

Angiogenesis plays an important role in the development of tumor, it provides oxygen and nutrition for tumor cells. Tumor microvessel density (MVD) can quantitatively reflect the

growth of tumor blood vessels, and thus can predict tumor growth, metastasis and recurrence trend. The COUP-TF2 is a protein containing 414 amino acids with a molecular weight of 47 kDa. Its gene is located on human chromosome 15, 15q26, containing six exons, a total of 14336 bp base pairs. The COUP-TF is a member of the family of orphan receptors in the steroid hormone/thyroid hormone receptor superfamily. There are two height homologous subtypes, COUP-TF1 and COUP-TF2, possessing a highly conserved modular structure consisted of an amino-terminal DNA-binding domain and a putative C-terminal ligand binding domain [3].

COUP-TF2 has been shown to stimulate proliferation and migration and induce angiogenesis in a variety of mouse tumor models including breast cancer, pancreatic cancer, and so on, and play a key role in tumor progression as a promoter of oncogenes in a variety of cancers. There is a wealth of evidence for its involvement in tumor infiltration and metastasis. COUP-TF2 is strongly expressed in the nuclei of neovascularization and various cancers (breast, ovarian, prostate, colon, pancreas, etc.), and can participate in tumor and vascular lymphatic vessels through multiple pathways. It is closely related to the clinical stage, pathological grade, lymph node metastasis and prognosis of cancer [4-6]. In a number of inactivated COUP-TF2 spontaneous tumor models also showed inhibition of tumor progression and metastasis through inhibition of angiogenesis [7].

It was found that COUP-TF2 can regulate the two angiogenic signals, Ang-1/Tie2 and VEGF/VEGFR-2. First, COUP-TF2 regulates the expression of Ang-1 in the outer membrane, and Ang-1 is a paracrine ligand for the specific endothelial cell tyrosine kinase receptor Tie2 [8].

Ang-1/Tie2 signaling plays a prominent role in the maintenance of vascular stasis and the promotion of angiogenesis [9]. COUP-TF2 directly binds to the Ang-1 promoter and enhances Ang-1 expression, thereby activating the Ang-1/Tie-2 pathway to promote vascular remodeling [8]. Second, COUP-TF2 inhibits the expression of VEGFR-1 in endothelial cells. VEGFR-1 acts as a decoy receptor, isolating VEGF from the binding of VEGFR-2 [10]. Thus, COUP-TF2 can enhance VEGF/VEGFR-2 signaling by inhibiting

VEGFR-1 and promote endothelial cell proliferation and germination. It was found that COUP-TF2 up-regulates E2F1 signaling and down-regulates Notch signaling [11, 12], both of which have been shown to modulate angiogenesis through the VEGF/VEGFR axis [13, 14]. In the tumor microenvironment, there may be complex interaction networks between COUP-TF2 and E2F1, Notch signaling and VEGF/VEGFR signaling to coordinate angiogenesis. However, the detailed mechanism by which COUP-TF2 performs these signals remains to be elucidated.

Although the expression of COUP-TF2 protein has been found in many tumors and its potential clinical relevance has been studied, no studies have been done in bladder cancer. Therefore, our aim in this study is to elucidate the expression of COUP-TF2 protein in bladder cancer and its association with the clinicopathological features of bladder cancer and to investigate the effect of tumor microenvironment on endothelial cells.

Materials and methods

Bladder tissue specimens

27 cases of primary bladder transitional cell carcinoma tissues and 8 cases of normal bladder tissue paraffin specimens as control group were collected from January 2000 to August 2016 in our hospital. All of them were fixed in formalin, Conventional paraffin embedding, conventional section thickness 3 um. The paraffin specimens come from the department of Pathology, affiliated Zhongshan Hospital, Xiamen University. Among them, 30 males and 5 females, aged between 33 to 87 years old, mean 61.6 years. According to WHO tumor grade: 1 to 2 grade, 13 cases; 3 grade, 14 cases. Clinical stage according to UICC standards: Tis~T1, 12 cases; T2~T3, 15 cases, including lymphatic metastasis in 2 cases.

Immunohistochemistry

Immunohistochemical method: We analyzed the expression of COUP-TF2 protein in transitional cell carcinoma of urinary bladder and normal bladder tissue. COUP-TF2 monoclonal antibody was purchased from R&D Systems, mouse anti-human, monoclonal antibody CD34 and SP kit were purchased from Fuzhou Maixin

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Biotech. Co., Ltd. PBS buffer instead of primary antibody as a negative control group, the known COUP-TF2 and CD34 positive bladder tissue sections as a positive control. Samples were excised from a paraffin specimen (approximately 3 μ m) and placed on a poly-L-lysine coated slide. Dewaxed for 2 h in a thermostat at 60°C and then hydrated in xylene, xylene, 100%, 100%, 90%, 80%, 70% alcohol. PBS wash 3 times, 3 min/times. The antigen solution of citrate antigen was used for autoclave antigen retrieval and boiled for 1-2 minutes. Then, according to the experimental method of UltraSensitive™ SP hypersensitization kit (mouse/rabbit) of Fuzhou Maixin Biotech. Co., Ltd, incubation of A liquid blocking (peroxidase blocking solution) 10 min, PBS washed 3 times, 3 min/time, incubation of B liquid (normal non-immune animal serum) 10 min, remove B solution, COUP-TF2 antibody (R&D Systems, 1:200) incubated overnight at 4°C, washed with PBS, incubated with C (Streptomycin antibiotic-peroxidase solution) for 10 min, rinsed with PBS, then incubated with D solution (streptomycin antibiotic-peroxidase solution) for 10 min, washed with PBS, then observed under DAB staining microscope for 1-3 min, tap water rinse, Hematoxylin stained 45 s, PBS washed, and finally through the reverse concentration gradient of alcohol dehydration, xylene transparent, neutral gum sealing. After two days, the neutral gums were dried and the sections were fixed under microscope to observe the photographs, and the results were graded.

Immunohistochemical results to judgment: COUP-TF2 staining score calculation: COUP-TF2 was uniformly distributed in the nucleus of vascular endothelial cells in transitional cell carcinoma of bladder, which was brown and was also expressed in vascular smooth muscle cells of pericancerous stroma. The total expression of COUP-TF2 was determined by the sum of the staining intensity and the percentage of positive cells. Scoring criteria: (1) staining intensity: negative for 0; weak positive for 1; positive for 2; strong positive for 3. (2) the percentage of positive cells: positive cells 0% to 0; 25% to 1; 25% to 2; positive cells >50% to 3. (1)+(2) is 0~3 points is low expression, 4~6 for the high expression.

MVD calculation method: According to Weidner correction method, the slice was placed under a 40-fold microscope to determine the maxi-

um vascular density region, and then placed under 200 times the microscope, Microvessel counts were selected for the three highest vascular density regions, and the mean value was taken as the MVD value of the patient. Any stained individual cell or cell mass, whether or not it forms a bureaucratic cavity, is considered to be a countable microvessel, as long as it is clearly separated from surrounding microvessels, tumor cells and other connective tissue components. Intratumoral sclerosis and tumor at the junction of soft tissue microvessels are not counted, with thick smooth muscle wall and bureaucratic diameter greater than 8 red blood cell diameter of the blood vessels are excluded.

Cell culture

Bladder cancer cells J82, 5637 and bladder normal epithelial cells SV-HUC-1 were purchased from the Chinese Academy of Sciences, Department of Culture Collection Committee cell library. Human umbilical vein endothelial cells (HUVEC) is from the Affiliated Zhongshan Hospital, Xiamen University, Gastroenterology Laboratory gift. The culture medium of each cell was composed of 10% fetal bovine serum (Bioind company), 1% double antibiotic (penicillin and streptomycin, Hyclone) and 500 ml of basal medium. The basic medium of J82, 5637, SV-HUC-1 and HUVEC were MEM, RPMI1640, F-12K and DMEM of Gibco respectively. The cells were cultured at 37°C in an incubator containing 5% CO₂.

Real Time-PCR fluorescence quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from the cells by using TIANGEN TRNzol total RNA extraction reagent, with 1 μ g of total RNA reverse transcription in the Takara reagent kit reverse transcription into total cDNA. The COUP-TF2 expression level was amplified by using the specific COUP-TF2 primers on an ABI 7500 instrument using a Takara kit. Human GAPDH internal controls were used as controls.

COUP-TF2: Forward primer sequence [5'-3']: CACAGGCATCTGAGGTGAACAGG; COUP-TF2: Reverse primer sequence [5'-3']: CGCCTTTATGG-ACCACATACGG. GAPDH: Forward primer sequence [5'-3']: CAGGAGGCATTGCTGATGAT; GAPDH: Reverse primer sequence [5'-3']: GAAG-GCTGGGGCTCATT.

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Table 1. 27 cases of bladder cancer COUP-TF2 expression and clinicopathological factors

| Clinicopathological features | Classification | Total number of samples | COUP-TF2 | | P-value |
|------------------------------|--------------------|-------------------------|----------|----------|---------|
| | | | Positive | Negative | |
| Age (years) | ≥60 | 17 | 12 | 5 | 0.678 |
| | <60 | 10 | 8 | 2 | |
| Tumor size (cm) | ≥3 | 10 | 9 | 1 | 0.204 |
| | <3 | 17 | 11 | 6 | |
| Pathological grade | 1-2 grade | 13 | 7 | 6 | 0.033* |
| | 3 grade | 14 | 13 | 1 | |
| Clinical stage | Tis-T1 | 12 | 6 | 6 | 0.024* |
| | T2-T4 | 15 | 14 | 1 | |
| Lymph node metastasis | without metastasis | 24 | 18 | 6 | 0.610 |
| | with metastasis | 3 | 2 | 1 | |

*P<0.05.

Table 2. 35 cases of bladder cancer and normal tissue COUP-TF2 expression comparison

| Classification | COUP-TF2 Positive | COUP-TF2 Negative | Total | P-value |
|-----------------------|-------------------|-------------------|-------|---------|
| Bladder cancer | 20 | 7 | 27 | 0.032* |
| Normal bladder tissue | 2 | 6 | 8 | |
| Total | 22 | 13 | 35 | |

*P<0.05.

Table 3. 35 cases of bladder tissue COUP-TF2 expression and the relationship between tumor microvessel count statistics

| COUP-TF2 expression | The number of cases | MVD* (X±S) | P value |
|---------------------|---------------------|-------------|---------|
| Positive | 22 | 67.95±19.87 | 0.000 |
| Negative | 13 | 40.92±11.77 | |

MVD*: Microvascular density.

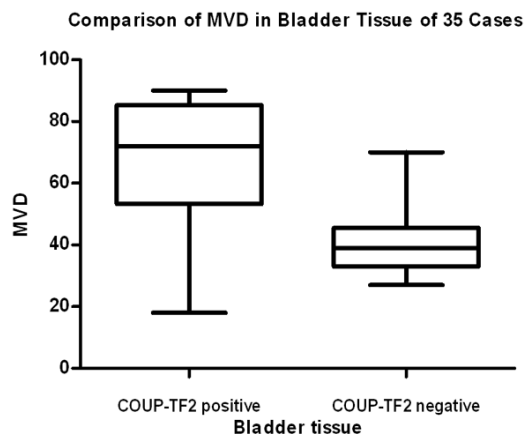


Figure 1. Relationship between COUP-TF2 expression and MVD in bladder tissues.

Western blot

The cells were lysed on RIPA lysate and protease inhibitor (100:1) on ice followed by shaking with ultrasonic instrument (12,000 rpm, 15 min). The supernatant was collected and boiled at 100°C for 10 min. The extracted protein was then separated by electrophoresis in 10% SDS-PAGE and transferred to

PVDF membrane by electrotransfer, blocked with 5% skim milk for 1 hour, washed 3 times with TBST, 10 min/time. And incubated overnight at 4°C in COUP-TF2 antibody (1:1000). The next day, the membrane was exposed to horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 hour at room temperature, washed 3 times with TBST, 10 min/time, and the protein was visualized by the enhanced chemiluminescence reagent ECL kit under the FluorChem HD2 multifunctional imaging system.

CCK8

HUVEC were plated into 96-well plates at 3000 cells per well. HUVEC was cultured with 200 µl supernatant of J82, 5637 and SV-HUC-1 respectively. After 24, 48, 72, 96 and 120 hours of culture, the supernatant fluid was removed, 200 µl of the mixture of 180 µl DMEM medium and 20 µl of CCK-8 reagent solution (Kyushu, Japan) was incubated in an incubator for 2 hours. The number of viable cells was measured and the number of HUVEC cells was reflected by absorbance at 450 nm using a microplate reader.

Expression of COUP-TF2 in bladder cancer

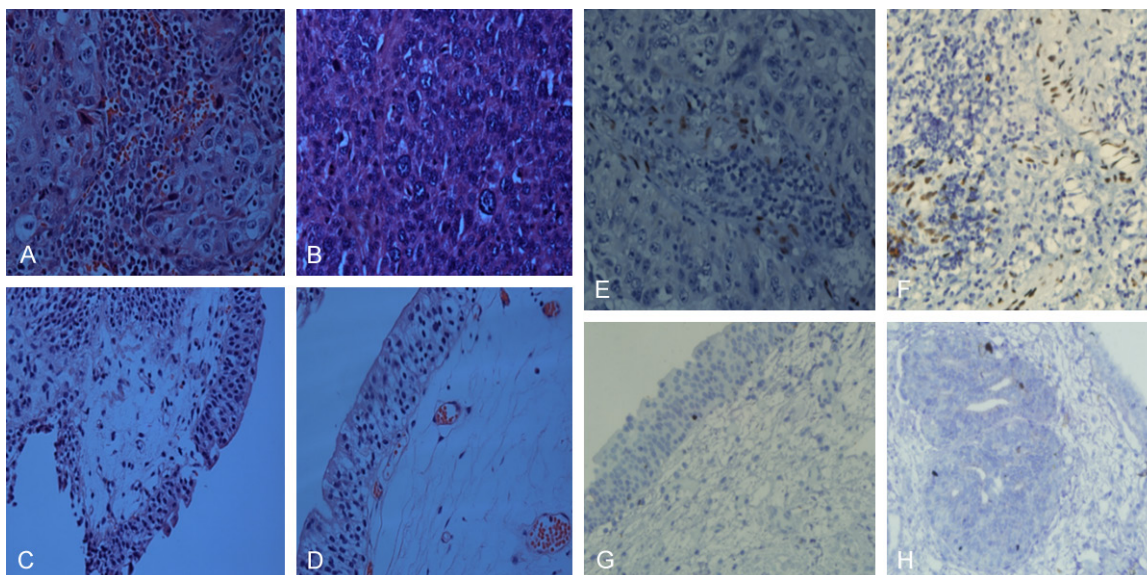


Figure 2. 1. Hematoxylin-eosin staining in Bladder patient groups. (A and B) Hematoxylin-eosin staining in Bladder normal tissue (C and D) (original magnification $\times 400$). 2. Immunohistochemical staining of COUP-TF2 protein in Bladder patient groups. (E and F) Negative staining in Bladder normal tissue. (G and H) Strong staining in Bladder cancer blood vessels (original magnification $\times 400$).

Statistical analysis

Each experiment is repeated at least three times. We used SPSS 19.0 software for statistical analysis. In **Table 1**, the relationship between the expression of COUP-TF2 and clinicopathological variables in bladder cancer was analyzed by chi-square test. In **Table 2**, the expression of COUP-TF2 in bladder cancer and bladder normal tissues was analyzed by chi-square test. In **Table 3**, the rank sum test was used to treat the association between MVD and clinicopathological variables in bladder cancer. The correlation analysis of two variables using rank correlation analysis; Real Time-PCR and CCK-8 were analyzed by GraphPad 5.0 software. The Real Time-PCR data were analyzed by multi-group independent rank analysis ANOVA. CCK-8 data were grouped by t-test; $P < 0.05$ was considered statistically significant when the data were statistically analyzed.

Results

Expression of COUP-TF2 in bladder tissue and its relationship with clinicopathological factors

COUP-TF2 was expressed on the nucleus of endothelial cells and stained into brown granules. In 27 cases of bladder cancer, 20 cases of positive expression (74.1%), and all showed a positive, mainly in the surrounding cancer tis-

sue of vascular endothelial cells, vascular smooth muscle cells (**Figure 2**). But there were only 2 positive cases (25%) in 8 cases of normal bladder tissues and weakly positive expression. The distribution was mainly in the small vascular endothelial cells of stroma, and there was significant difference between the two groups ($P = 0.032 < 0.05$). COUP-TF2 and CD34 negative control group were not brown particles exist. The expression of COUP-TF2 was closely related to the histological grade and clinicopathological stage of bladder cancer ($P < 0.05$, **Table 1**), but not to the age, tumor size and lymph node metastasis ($P > 0.05$, **Table 1**). The expression of COUP-TF2 in bladder cancer was significantly higher than that in normal bladder tissues (92.9% vs 53.8%, $P < 0.05$). The expression of COUP-TF2 in bladder cancer was significantly higher than that in normal bladder tissues ($P < 0.05$, **Table 2**).

The expression of COUP-TF2 and MVD in bladder cancer

CD34, a marker of vascular endothelial cells, is used to label MVD, which is mainly expressed in the vascular endothelial cell membrane and cytoplasm. The mean MVD of COUP-TF2 positive group was 67.95 ± 19.87 , which was higher than COUP-TF2 negative group (40.92 ± 11.77). There was significant difference between the

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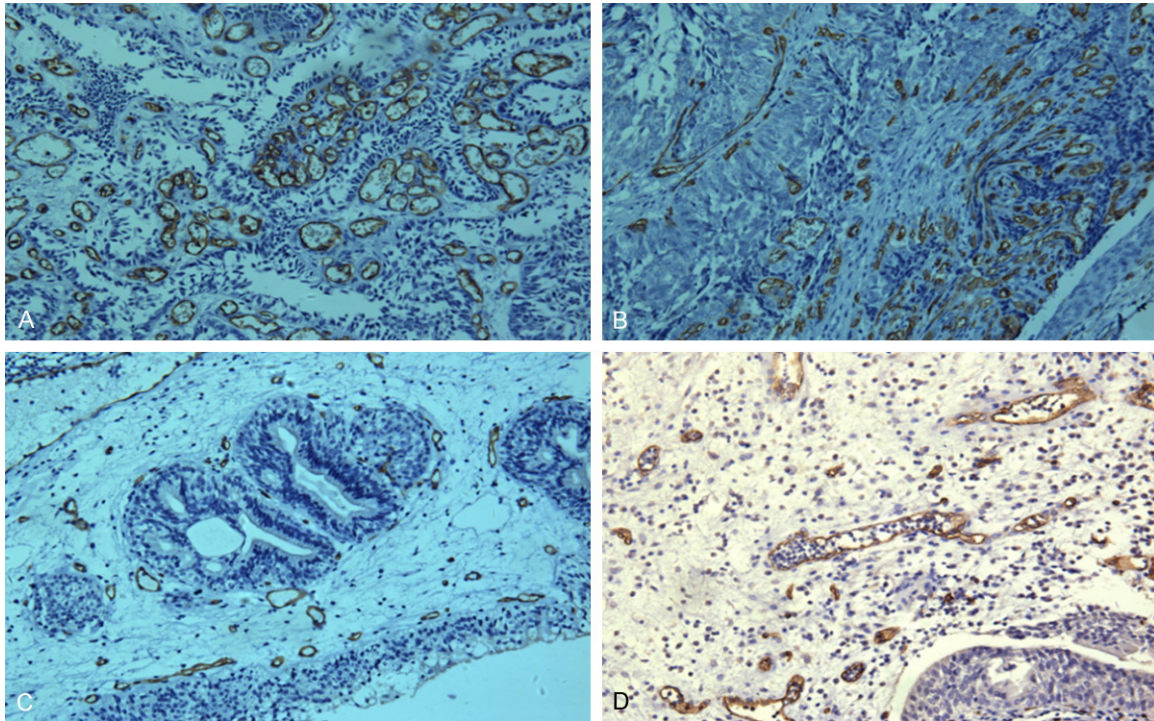


Figure 3. Immunohistochemical staining of CD34 protein in Bladder patient groups (A and B). Immunohistochemical staining of CD34 protein in in Bladder normal tissue (C and D). CD34 is vascular endothelial cell markers (original magnification $\times 400$).

two groups ($P < 0.05$, **Table 3**; **Figures 2, 3**). The correlation between MVD and COUP-TF2 was analyzed, and there was a positive correlation between the expression of COUP-TF2 and MVD in tumor tissue ($P < 0.05$, **Figure 1**). MVD increases as the expression of COUP-TF2 increased.

The expression of COUP-TF2 in HUVEC after different supernatant culture

The same number of cells was plated in 10 cm dishes, after waiting for the cells to grow over the dish, the supernatant of bladder cancer cells (J82 and 5637) and the supernatant of normal bladder epithelial cells (SV-HUC-1) were collected. HUVEC cells were cultured with these supernatant liquid for 12 h, 24 h, 48 h, respectively, the cells were collected and subjected to RT-PCR and Western blot. The results show that the expression level of COUP-TF2 in HUVEC cultured in supernatant of bladder cancer was significantly higher than that in normal cell culture supernatant HUVEC, especially in 48 h group, and had statistical significance ($P < 0.05$, **Figure 4A, 4B**).

The proliferation of HUVEC cells after different supernatant culture

The supernatants of bladder cancer cells and normal bladder epithelial cells were collected. The same amount of HUVEC cells were plated and cultured in 48-well plates for 24 h, 48 h, 72 h, 96 h and 120 h, respectively, the viable cells of HUVEC were detected by CCK-8. The results show that the number of HUVEC in the supernatant of bladder cancer group was more than that in normal bladder epithelium group on the fourth day and the fifth day, and further promote the proliferation of vascular endothelial cells, and the gap is statistically significant ($P < 0.05$, **Figure 4C**).

Discussion

Bladder cancer is one of the most common neoplasms in urology in China, and is a disease that directly threatens the survival of patients. Hematuria is the most common symptoms of bladder cancer. When bladder cancer has metastasized, the 5-year survival rate was only 6%, treatment and prognosis is poor. In animal tumor models, COUP-TF2 has been shown to

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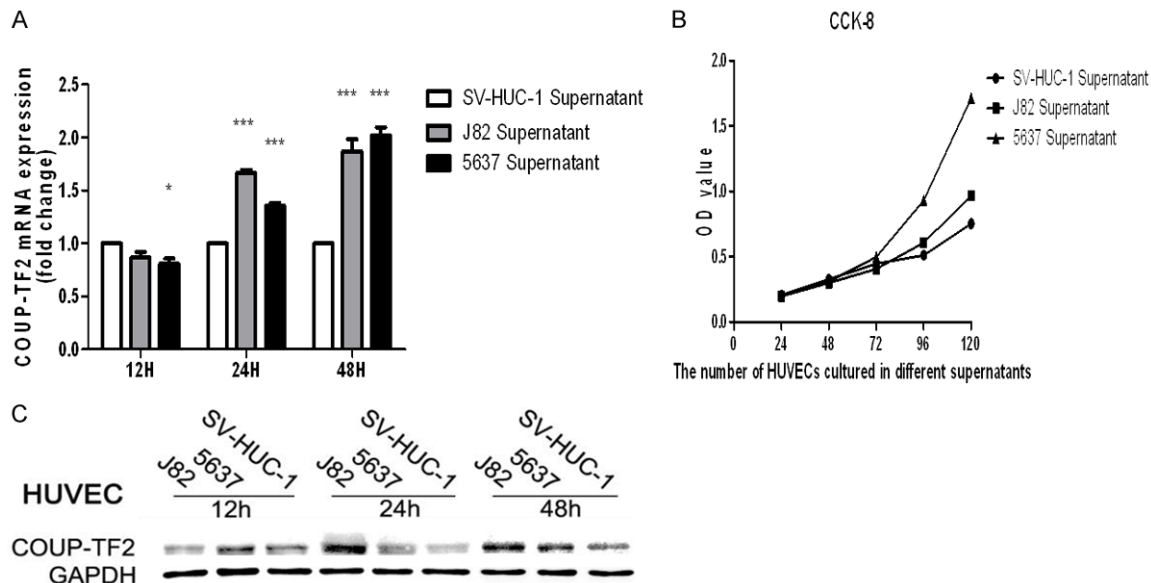


Figure 4. A. HUVEC cells cultured in the supernatant of bladder cancer cells (J82 and 5637) in supernatant and normal bladder cells (SV-HUC-1) were incubated at three different time points at 12 h, 24 h and 48 h at the RNA level COUP-TF2 expression differences. And the SV-HUC-1 supernatant group is the control group. B. HUVEC cells cultured in supernatant and supernatant of SV-HUC-1 supernatant and bladder normal cells (J82 and 5637) at different time points at 12 h, 24 h and 48 h, respectively, at the protein level COUP-TF2 expression differences. And the SV-HUC-1 supernatant group is the control group. C. Transwell assay of HUVEC cells in different medium, the results showed that the supernatant of bladder cancer group can promote the proliferation of HUVEC cells. And the SV-HUC-1 supernatant group is the control group.

modulate two angiogenic signals, Ang-1/Tie2 and VEGF/VEGFR-2, playing an important role in tumor progression in animal tumor models. But the role of blood vessels in human tumor progression is still not fully understood. We found that COUP-TF2 was strongly expressed in the nucleus of vascular endothelial cells in human bladder cancer tissues, but not in bladder cancer cells and normal bladder cells, and occasionally in vascular endothelial cells of normal bladder tissues. The expression of COUP-TF2 was closely correlated with the pathological grade and clinical stage of bladder cancer, especially in advanced bladder cancer. The positive rate of COUP-TF2 was positively correlated with MVD, and the number of MVD in advanced bladder cancer is the highest. It suggests that COUP-TF2 can promote the progression of bladder cancer through promoting the proliferation of endothelial cells. In a cell experiment, the tumor microenvironment can proliferate HUVEC and result in HUVEC cells expressing more COUP-TF2 protein, which all indicate that COUP-TF2 plays an important role in the progression of bladder cancer. These results suggest that the microenvironment of bladder cancer can lead to increased expression of

COUP-TF2, thereby promote the proliferation of vascular endothelial cells. This is consistent with the animal tumor model. But in the human body, the tumor microenvironment for COUP-TF2 regulation pathway is not yet known, the mechanism of COUP-TF2 to promote the proliferation of vascular endothelial cells also needs further study. For example, the relationship between COUP-TF2 and cell cycle, cell apoptosis has not been clearly reported. However, COUP-TF2 has not been studied in bladder cancer. The clinical significance of COUP-TF2 in bladder cancer remains to be elucidated. COUP-TF2 is expected to be a potential therapeutic target for bladder cancer.

Conclusion

In this study, we examined the expression of COUP-TF2 protein in bladder tissues and found that COUP-TF2 was closely related to the clinicopathological features of bladder cancer. COUP-TF2 is highly expressed in bladder cancer tissue, which indicates that COUP-TF2 plays an important role in the progression of bladder cancer, especially in the late stage of the disease. COUP-TF2 was positively correlated with

MVD, and the tumor microenvironment could also promote the expression of COUP-TF2 in endothelial cells, suggesting that tumor micro-environment could promote the expression of COUP-TF2 in endothelial cells, further promote the proliferation of endothelial cells. This indicates that COUP-TF2 plays an important role in tumor progression.

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Disclosure of conflict of interest

None.

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