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HMGN5 expression in bladder cancer tissue and its role on prognosis

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Abstract. – OBJECTIVE: High mobility group protein N5 subtype (HMGN5) is overexpressed in bladder cancer tissue, while its specific mechanism in bladder cancer oncogenesis has not been fully elucidated. This study intends to investigate the impact of HMGN5 on clinical staging and prognosis of bladder cancer.

PATIENTS AND METHODS: A total of 26 cases of patients with bladder transitional cell carcinoma (BTCC) received transurethral resection (TUR-BT) in our hospital between March 2015 and February 2016. Para-carcinoma tissue at 5 cm away from cancer tissue was selected as normal control. The expressions of HMGN5 mRNA and protein in different clinical stages were tested by Real-time PCR and Western blot. The relationship between HMGN5 expression and clinical stage along with prognosis was analyzed.

RESULTS: HMGN5 mRNA was significantly elevated in BTCC tissue compared with that in para-carcinoma tissue ($p < 0.05$). HMGN5 mRNA level was gradually upregulated following BTCC upstage according to UICC-TNM stage and WHO stage. The level of HMGN5 protein showed similar changes with mRNA. Follow-up results demonstrated that patients with high HMGN5 level have more tendency of occurrence.

CONCLUSIONS: HMGN5 protein level has an important influence on BTCC clinicopathological staging and prognosis. This investigation provides theoretical basis the future therapy of bladder cancer.

Key Words:
HMGN5, Bladder cancer, Urothelial cancer, Clinical stage, Pathological type, Prognosis.

Introduction

Bladder cancer represents the most common malignant tumor in genitourinary system. Urothelial cancer is the most common histological type which accounts for more than 90% of the bladder cancer[1,2]. Despite the great progress on diagnosis and treatment, there are still 30% of patients with urothelial cancer that have the risk of recurrence and progression[3,4]. Therefore, searching new molecular markers is of extreme importance for bladder cancer diagnosis and treatment.

High mobility group (HMG) protein belongs to the group of proteins with high migration rate in polyacrylamide gel electrophoresis, including HMGA, HMGB, and HMGN[5-7]. The study found that HMGN5 was overexpressed in prostate cancer, breast cancer, and bladder cancer. HMGN5 gene recombination and gene disruption are closely related to numerous oncogenesis, such as gastrointestinal tract tumor, glioma, lipoma, and female reproductive system tumor[8-10]. However, the specific role of HMGN5 protein in bladder cancer has not been clarified. This work, therefore, aims to explore the HMGN5 impact on bladder cancer clinicopathological staging and prognosis.

Patients and Methods

Patients

A total of 26 cases of patients with bladder transitional cell carcinoma (BTCC) received transurethral resection (TUR-BT) in West China Hospital, Sichuan University, between March 2015 and February 2016. Para-carcinoma tissue at 5 cm away from cancer tissue was selected as normal control. All the enrolled subjects were diagnosed by pathology. There were 18 males and 8 females, with a mean age 62 ± 15 and 48 ± 17 years old, respectively. According to the UICC-TNM stage, there were 14 cases in superficial type (T1-T2) and 12 cases in infiltrating type (T3-T4). According to WHO criteria, there were 11 cases in G1 stage, and 15 cases in G2-G3 stage. A total of 8 cases appeared lymph node metastasis (2 cases in internal iliac lymph node, 1 case in external iliac lymph node, 4 cases in obturator lymph node, and 1 case in common iliac lymph node), and 2 cases occurred distant metastasis (1 case in hepatic me-

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tastasis and 1 case in pulmonary metastasis). No patients showed severe cardiovascular disease or other tumors. A total of 20 cases of healthy volunteers received physical examination in our hospital were enrolled. There were 10 males and 10 females, with the average age at 28 ± 11 and 26 ± 14 years old. No statistical significance on age, gender, or BMI was observed between bladder cancer group and healthy control ($p > 0.05$). The study was approved by Ethics Committee of the West China Hospital, Sichuan University (Chendu, Sichuan Province, P.R. China) and all the subjects had signed informed consent.

PCR Primers Design and Synthesis

HMGN5 mRNA primers sequences were designed based on NCBI database. Forward, 5'-ATAGCACCGCGAGATCTGTT-3', reverse, 5'-TGGCACAAGCATAGCAGACA-3'. GAPDH, forward, 5'-TGACTTCAACAGCGA-3', reverse, 5'-TTTAATGTCACGCACGATTTC-3'. The primers were synthetized by Invitrogen (Carlsbad, CA, USA).

Main Materials

TRIzol and RNA reverse transcription kit were bought from Invitrogen (Carlsbad, CA, USA). SYBR Green PCR kit was obtained from TaKaRa (Otsu Shiga, Japan). Anti-HMGN5 polyclonal antibody and goat anti-rabbit HRP antibody were from Abcam (Cambridge, MA, USA).

Main Instruments

Horizontal electrophoresis apparatus (Bio-Rad, Hercules, CA, USA), ultraviolet spectrophotometer (Shanghai Aoxi Scientific Instruments Co., Ltd, Taiyuan, Shanxi, China), ABI7500 fluorescence quantitative PCR (Thermo Fisher Scientific, Waltham, MA, USA), Kubota 3700 high-speed low temperature centrifuge (Kubota, Osaka, Japan), gel imaging system (Jiangsu Kirin Medical Instrument Factory, Suzhou, Jiangsu, China), super clean bench (BAKER, Sanford, ME, USA), thermostatic water bath.

RNA Extraction and Reverse Rranscription

Tissue RNA extraction: the tissue was grinded in liquid nitrogen and treated with TRIzol (100 mg tissue: 1 ml TRIzol). Next, the solution was moved to an Eppendorf (EP) tube and added with 200 µl chloroform. After vibrated for 15 s, the upper aqueous phase was added with 500 µl isopropanol for 10 min. After centrifuged at 12000 g for 10 min, the precipitation was added with 1 ml ethanol (75%). After centrifuged at 4°C and 7500 g for 5 min, the supernatant was removed and the tube was dried for 10 min. Next, the RNA was solved in diethyl pyrocarbonate (DEPC) water and qualified by 0.8% agarose gel electrophoresis (Figure 1). RNA content and purity were determined by ultraviolet spectrophotometer.

Reverse transcription: reaction solution was prepared according to the instruction, including 2 µg total RNA, 1 µl oligo primer (50 µM), 1 µl dNTP mix (10 µM), and ddH_2O. The solution was pre-degenerated at 65°C for 5 min. Next, cDNA first chain synthesis reaction system was prepared, including 2 µl 10×RT buffer, 4 µl $MgCl_2$ (25 µM), 2 µl DTT (0.1 M), 1 µl RNAase OUT (40 U/µl), 1 µl SuperScrip III RT (200 U/µl), and ddH_2O. The reaction condition was composed by 50°C for 50 min and 85°C for 5 min.

Real-time PCR

Real-time PCR reaction system consisted of 2 µl cDNA, 0.5 µl forward primer (20 µM), 0.5 µl reverse primer (20 µM), 9 µl ddH_2O, 0.5 µl ROX, and 12.5 µl 2×SYBR Premix EX Taq. The PCR reaction condition contained 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 31 s, and one cycle of 60°C for 1 min and 95°C for 15 s for extension. Relative gene expression was calculated by 2-ΔCt method [ΔCt=Ct(HMGN5)-Ct(GAPDH)].

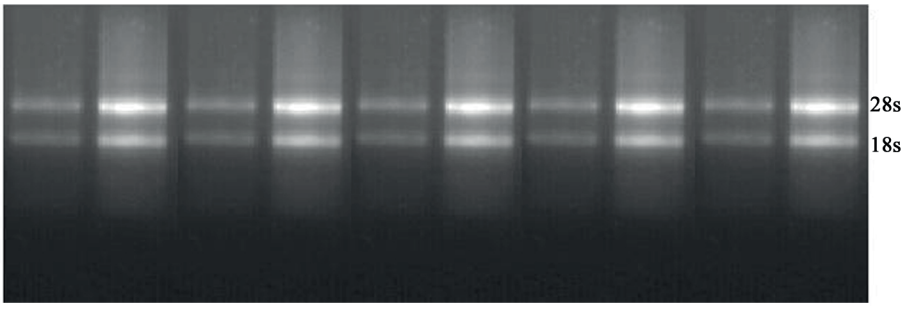

Figure 1. RNA qualified by 0.8% AGE.

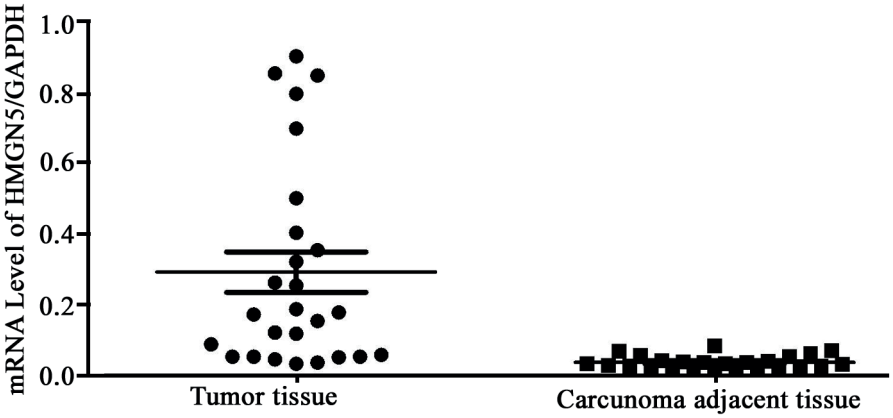

Figure 2. HMGN5 mRNA expression in bladder cancer tissue and para-carcinoma tissue.

Western Blot

The tissue was cracked in liquid nitrogen and added with tissue extraction solution (4 ml 100% glycerin, 4 ml 20% SDS, 2 ml Trisil, and 10 ml ddH_2O) on ice for 3-4 h to extract protein. After centrifuged at 8000 g and 4ºC for 40 min, the supernatant was collected. A total of 40 μg protein were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis as 80 V for 30 min and 150 V for 90 min. Next, the protein was transferred to polyvinylidene difluoride (PVDF) membrane at 230 mA for 50 min. After blocked by 10% skim milk for 2 h, the membrane was incubated in HMGN5 primary antibody (1:2000) at 4ºC overnight. The membrane was incubated with secondary antibody at 1:5000 for 1 h and washed by phosphate-buffered saline and tween (PBST). At last, the membrane was treated with enhanced chemiluminescence (ECL) and scanned.

Follow-up

All of the 26 patients received follow-up after surgery. Bladder perfusion was performed every one or two weeks (a total of 4 times), followed by once a month to two years. Cystoscope examination was performed every three months for three years and followed by every half a month. If tumor recurrence was found, follow-up was stopped and treatment towards the patient was performed according to recurrence therapy.

Statistical Analysis

All data analyses were performed on SPSS 19.0 (IBM, Armonk, NY, USA). Paired data was compared by *t*-test. Univariate survival analysis was applied by Kaplan-Meier method. Diversity was evaluated by log-rank test. $p < 0.05$ was considered as statistical significance.

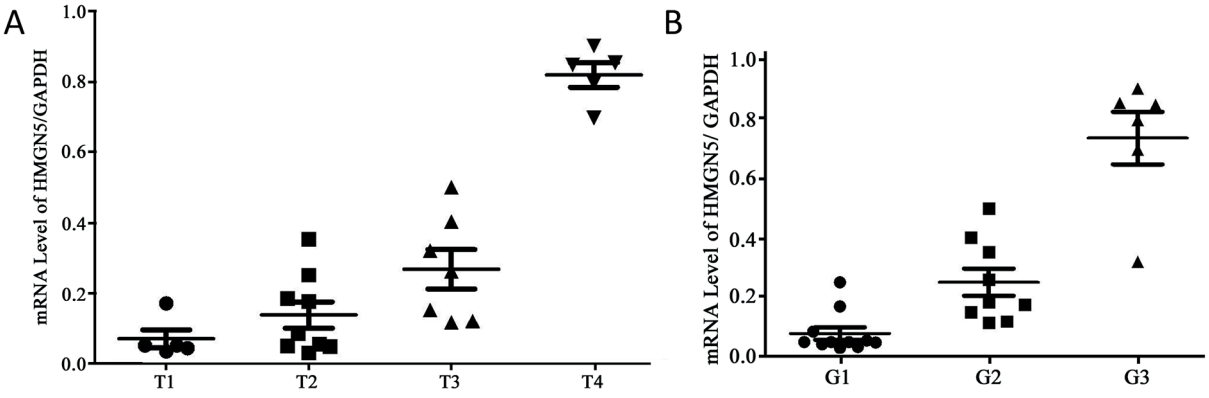

Figure 3. HMGN5 expression according to UICC-TNM stage and WHO stage.

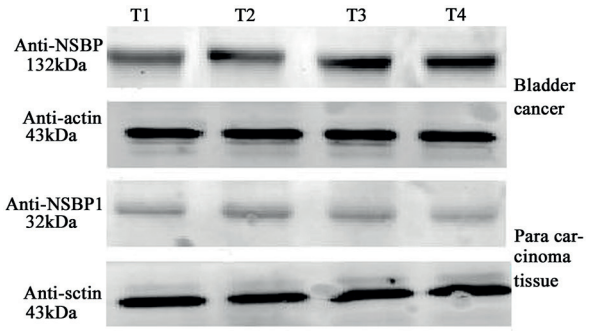

Figure 4. HMGN5 protein expression in bladder cancer tissue and para-carcinoma tissue according to UICC-TNM stage.

Results

HMGN5 mRNA Expression in Bladder Cancer Tissue and Para-Carcinoma Tissue

Compared with para-carcinoma tissue, the level of HMGN5 mRNA was significantly elevated in bladder cancer tissue ($p < 0.01$) (Figure 2). According to UICC-TNM stage, higher level of HMGN5 was found in patients at T3-T4 stage than that at T1-T2 stage (Figure 3A). Similarly, according to WHO stage, higher expression of HMGN5 mRNA in patients at G2 stage was observed than that at G1 stage, manifesting that HMGN5 mRNA level was gradually upregulated in a UICC-TNM/WHO stage-dependent manner (Figure 3B).

HMGN5 Protein Expression in Bladder Cancer Tissue and Para-Carcinoma Tissue

Total protein was extracted from bladder cancer tissue and para-carcinoma tissue. According to UICC-TNM stage, HMGN5 protein level in patients at T3-T4 stage was increased compared with that at T1-T2 stage (Figure 5). According to WHO stage, markedly growing expression of HMGN5 protein was shown in patients at G3 stage compared with that at G1-G2 stage. HMGN5 protein level gradually increased following upstage, whereas the expression showed no changes in para-carcinoma tissue (Figure 5).

The Relationship Between HMGN5 Expression and Bladder Cancer Recurrence

All of 26 patients received postoperative follow-up. According to UICC-TNM stage, no recurrence in patients at T1-T2 stage appeared, while cancer reoccurred in 5 patients at T3-T4 stage and 3 patients at T4 stage. Kaplan-Meier survival curve analysis showed that the 5-year recurrence rate of patients in T1-T2 stage was lower than that in T3-T4 stage (Figure 6A). A statistically significant correlation was shown between the recurrence and stage (log-rank test, $p = 0.0002$). In a similar fashion, based on WHO stage, no patients in G1 stage appeared relapse, while 2 patients in G2 stage and 3 patients in G3 stage presented relapse. The 5-year recurrence rate was statistically correlated with the WHO stage (log-rank test, $p = 0.0053$) (Figure 6B).

Discussion

In 1973, the existence of HMG was reported in bovine thymus cells, and this type of protein has emerged as relatively high mobility in polyacrylamide gel[11-13]. Later, HMG proteins were categorized into HMGB, HMGA, and HMGN. Though different kinds of HMG proteins were characterized with diverse structures, they shared similar function[14,15]. HMG protein family participates in DNA replication, transcription, recombination, and repair, while the most important function is to regulate gene transcription and expression[16-18].

HMGN is a set of nucleoprotein combining with nucleosome. It contains unique nucleosomal binding domain (NBD) that can identify the

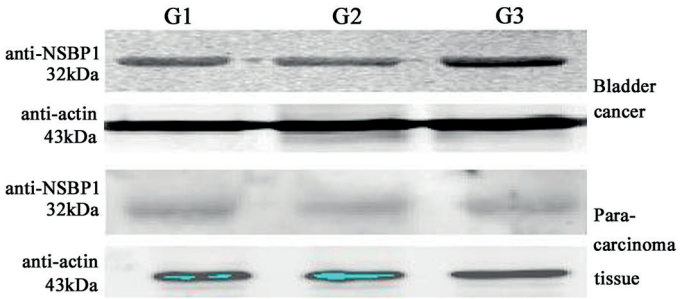

Figure 5. HMGN5 protein expression in bladder cancer tissue and para-carcinoma tissue according to WHO stage.

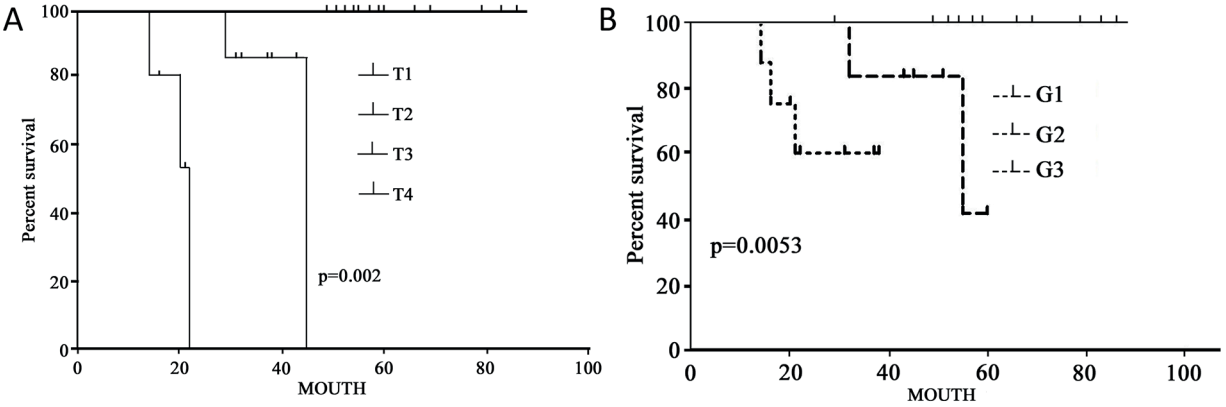

Figure 6. Patient relapse analysis according to UICC-TNM stage.

nucleoprotein of nucleosome core particle at 146 bp. HMGN reduces the density of highly ordered chromatin structure and increases the efficiency of transcription and translation through interacting with nucleosome[19,20]. HMGN5, also known as NSBP1, possesses the total length of 8600 bp. It contains 6 exons, 5 intron and non-coding regions, with the total cDNA length at 1865 bp. HMGN5 competes with histone connection body H1 to bind with nucleosome, and weakens the stability effect of histone H1 on nucleosome, leading to the depolymerization of chromosome structure[21].

Recent evidence has discovered novel marker correlated with poor clinical outcome in bladder cancer[22]. Our study detected HMGN5 mRNA and protein expression in bladder cancer and para-carcinoma tissue in different stage and further analyzed the relationship between HMGN5 expression and bladder cancer recurrence by follow-up. The result indicated that the level of HMGN5 mRNA/protein was significantly elevated in bladder cancer tissue compared with that in para-carcinoma tissue, which was gradually upregulated following bladder cancer upstage according to UICC-TNM stage and WHO stage. Follow-up results and Kaplan-Meier survival curve analysis demonstrated that patients with high HMGN5 level have evident inclination of recurrence.

Conclusions

We analyzed HMGN5 impact on bladder cancer clinicopathological staging and prognosis, providing theoretical basis for the diagnosis and treatment of bladder cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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