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Transferrin Receptor Cluster of Differentiation 71: A Potential Novel Target for Bladder Cancer

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Abstract: Cluster of differentiation 71 (CD71) is important to the proliferation of many tumors and could be a potential target for tumor diagnosis. This study aimed to investigate the level of CD71 expression in bladder cancer (BC) tissues and urine samples for initial detection of BC. Eighty-seven bladder tumor tissues and 219 urine samples were collected from patients diagnosed with BC in the Urology Department of the Second Affiliated Hospital of Kunming Medical University. Quantitative real-time reverse transcription PCR (qRT-PCR), immunohistochemistry (IHC), and enzyme-linked immunosorbent assay were used to detect the gene and protein expression levels of CD71 in BC tissues and urine samples. Chi-square test was then carried out for analyses. Compared with non-BC samples, the expression levels of CD71 in BC tissues and urine increased significantly ($P < 0.050$). In conclusion, CD71 was highly expressed in BC tissues and urine. Thus, the concentrations of urine CD71 could be a new target for clinical detection of BC.

Keywords: Bladder cancer, Cluster of differentiation 71, Urine diagnosis

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1. Introduction

Bladder cancer (BC) is the ninth most common cancer in the world, with an estimated 430,000 newly diagnosed cases, leading to 165,000 deaths each year[1]. Furthermore, BC is 4 times more likely to occur in men than in women, and the incidence and mortality rate of men were about 9.6 and 3.2/100,000 people, respectively, in 2018[2]. Approximately 70% of BC patients were diagnosed with non-muscular invasive BC characterized by a high recurrence rate, while the remaining 30% were diagnosed with MIBC characterized by poor prognosis[3]. The standard diagnostic methods for BC, including cystoscopy and urine cytology, may cause complications and are highly subjective, expensive, and invasive, not to mention the poor sensitivity and specificity of cystoscopy for flat carcinoma and the relatively low sensitivity of urinary cytology for diagnosis of low-grade tumors[4,5]. Thus, novel methods with high reliability, sensitivity, and specificity for diagnosis of BC are warranted.

Cluster of differentiation 71 (CD71), also known as transferrin receptor 1 (TfR1), belongs to type II transmembrane dimer glycoprotein, which consists of a small cytoplasmic domain, a single-channel transmembrane domain and a complex extracellular domain[6]. TfR1 is a major receptor responsible for iron supply in cells through extracellular domain binding to serum Fe^{3+}-loaded transferrin and mediating endocytosis[7,8]. There are five highly correlated palindrome sequence elements in the 3'-untranslated region of TfR mRNA, called iron response elements (IREs). IREs can bind to iron regulatory proteins in cytoplasm, thereby regulating the stability of TfR mRNA and controlling its expression[9]. CD71 exists in various kinds of nucleated cells, with normal cells has a low level of CD71 expression, while proliferating cells have a high level of CD71 expression[10]. Accumulating evidence show that CD71 expression is higher in inflammation and tumors than that in normal tissues, and studies have found that TfR1 plays a key role in the growth and survival of pancreatic cancer, glioma, and other tumors[11,12]. However, the expression and role of CD71 in BC cells remain obscure.

The purpose of this study is to investigate the significance of CD71 in detecting BC by collecting tissue and urine samples from patients with bladder cancer and then detecting the expression of CD71 mRNA in BC tissue and the content of CD71 protein in urine.

2. Materials and methods

2.1. Materials

Primary BC and normal bladder tissues were available from patients undergoing surgery. Under the guidance of a skilled pathologist, we collected specimens of normal bladder urothelium with the length of more than 3 cm from the margin of BC tissue. After surgical removal, all specimens were then frozen in liquid nitrogen. Urine samples from patients with BC or healthy controls were collected before treatment. All human studies had been investigated and approved by IRB, Institute of Biophysics, Chinese Academy of Sciences, and written informed consent had been obtained from human subjects in accordance with the Declaration of Helsinki. Clinicopathological classification and staging were ascertained on the basis of the criteria set by the American Joint Committee on Cancer.

2.2. Detection of CD71 gene expression by qRT-PCR

About 5 mg of bladder tissue was taken and 1 ml of Trizol reagent was added. Total RNA was extracted and dissolved in RNase-free water. The concentration and purity of total RNA were determined. Using 100 ng of total RNA as template, a 20-ml reaction system was prepared according to the instructions of the reverse transcription kit, and the reverse transcription was carried out for converting RNA into cDNA. A 50-ml reaction system was prepared using 4 μl of cDNA as template. The reaction system was pre-denatured at 95 °C for 30 s, then at 95 °C for 5 s, followed by 60 °C for 30 s, with 40 cycles of reaction in total. The glyceraldehyde-3-phosphate dehydrogenase housekeeping gene was used as internal reference, and three replicates were used for each specimen. The delta Ct value for CD71 gene expression was calculated using the formula as shown below:

ΔCt = Ct value of target gene – Ct value of internal reference gene

2.3. Immunohistochemistry (IHC) staining of CD71

CD71 IHC staining of human BC tissues and normal bladder tissues was performed. Briefly, the sections of human BC tissues or normal bladder tissues were subject to degreasing, rehydration, incubation with 3% hydrogen peroxide (H_2O_2) for 30 min, washing, and sealing with 5% goat serum (Millipore, USA) at room temperature for 30 min. Afterward, the sections were incubated with CD71 antibody (1:400) overnight at 4 °C. The sections were incubated with biotin-binding goat anti-mouse IgG (H+L) secondary antibody (1:50) at 37 °C for 30 min and then washed with phosphate-buffered saline for 3 times. According to the instructions of the manufacturer, brown precipitates were produced using 3,3'-diaminobenzidine (DAB) horseradish peroxidase color development kit. The slides were observed under an optical microscope.

2.4. Detection of CD71 protein expression by enzyme-linked immunosorbent assay (ELISA)

A specific monoclonal antibody (ABC71) against CD71 which was used for horseradish enzyme labeling was screened using the hybridoma technique. Another anti-TfR antibody (MEM-189) was used for coating. A double antibody sandwich ELISA kit was prepared using ABC71 and MEM-189 to detect free CD71 in the urine samples.

2.5. Statistical analysis

Statistical software SPSS 18.0 was used to analyze all the data. The count data were expressed as percentage (%) and case (n) and were analyzed using Chi-square test. The difference was statistically significant at $P < 0.05$.

3. Results

The expression of CD71 was detected in the specimens of BC and normal bladder tissues by qRT-PCR. It was noted that CD71 expression was significantly increased in BC tissues (**Table 1**). IHC staining showed that the expression of CD71 protein was significantly different between BC and normal bladder tissues (**Table 2**). The expression of CD71 protein was also detected in the urine samples of BC patients and normal individuals. Similarly, the expression of CD71 protein in urine of BC patients was

evidently elevated (**Table 3**). The differences in the above are statistically significant ($P < 0.05$).

4. Discussion

BC is one of the most prevalent cancers worldwide, and about 80,500 new cases and 32,900 BC-related deaths occurred in China, in 2015[13]. With the growing understanding of BC biology, different molecular characteristics of BC may provide evidence for the diagnosis, prognosis, and progression of BC. Many unique, non-invasive urine diagnostic methods for BC are being studied with the goal to improve the diagnostic methods that are currently in use. Among them, NMP-22 BC test and BTA stat® test were FDA-approved commercial detection reagents, while other tests that were not FDA-approved include BLCA-1, Survivin, lactate, FGFR3, miR-26a, TWIST1 methylation, and other urinary protein, metabolite, and genetic biomarkers[14,15]. Recently, extracellular vesicles in body fluids have attracted increasing interests. Studies have found that proteins, lncRNAs, miRNAs, and mRNAs in extracellular vesicles could be used as potential biomarkers for the diagnosis of BC, including HEXB, LASS2, LncRNA-HOTAIR, and LncRNA-PCAT1[3,14]. Furthermore, Li *et al*.[4] found that telomerase activity of cells in urine could serve as a biomarker for non-invasive diagnosis of BC. However, the sensitivity or specificity of existing biomarkers for the diagnosis of BC, especially for the diagnosis of low-grade and early BC, still needs further research and validation.

CD71 is a promising source of biomarkers for the diagnosis and prognosis of diseases, including cancer. Dunsmore *et al*.[16] found that lower frequency and/or damaged function of $CD71^+$ red blood cell during pregnancy may make inflammatory bowel disease patients more likely to develop stronger proinflammatory environment in the gastrointestinal tract. Babu *et al*.[17] have demonstrated that decreased expression of miR-148a in hepatocellular carcinoma may increase the expression of TfR1, thereby increasing the iron level and cell proliferation of tumor cells. Basuli *et al*.[18] have determined that an increase in TfR1 was observed in the genetic model of ovarian cancer tumor-initiating cells, which resulted in the accumulation of excess iron in the cells and increased dependence on iron proliferation. These results are support that CD71 may also be involved in the occurrence and development of BC tumors, thereby providing the basis for further verification.

The present study is limited by the relatively small sample size as the study was a single-center study conducted in the Second Affiliated Hospital of Kunming Medical University. Furthermore, the latent mechanism of CD71 on the development and progression of BC remains to be elucidated in both *in vivo* and *in vitro* studies. Therefore, further studies that include larger number of clinical samples, multicenter studies, and functional analysis are needed to corroborate CD71 as a complementary non-invasive biomarker for BC.

By analyzing the expression of CD71 gene in BC tissues and normal bladder tissues, we found that the expression of CD71 gene in BC tissues was higher than that in normal bladder tissues. Subsequently, a specific monoclonal antibody (ABC71) against CD71 was screened using hybridoma technique, and an ELISA kit for detecting CD71 was prepared by combining another antibody against CD71. Using this kit to detect free CD71 in urine, it was found that the content of CD71 protein in urine of patients with BC was significantly higher than that of healthy individuals. In conclusion, CD71 may be an optimal biomarker of BC, providing pivotal value for the diagnosis, prognosis, and treatment of BC. The exploitation of CD71 will enable us to detect BC using fewer amount of samples and thus reduce the misdiagnosis rate of BC.

Table 1. Comparative analysis of CD71 gene expression in BC and normal bladder tissues

Groups	N	ΔCt value of CD71 gene expression
BC tissues	87	7.30 ± 0.05*
Normal bladder tissues	35	0.54 ± 0.03

*$P < 0.05$. BC: Bladder cancer, CD71: Cluster of differentiation 71

Table 2. Comparative analysis of CD71 protein expression in BC and normal bladder tissues

Groups	N	CD71 protein expression
BC tissues	87	5.33 ± 0.04*
Normal bladder tissues	35	0.29 ± 0.04

*$P < 0.05$. BC: Bladder cancer, CD71: Cluster of differentiation 71

Table 3. Comparative analysis of CD71 protein expression in urine of BC and normal urine

Groups	N	CD71 protein expression (ng/ml)
BC urine	219	328.26 ± 28.72*
Normal urine	134	45.35 ± 8.36

*$P < 0.05$. BC: Bladder cancer, CD71: Cluster of differentiation 71

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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